

Chondrogenesis of mesenchymal stem cells in gel-like biomaterials *in vitro* and *in vivo*

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1. ABSTRACT

Gel-like carrier materials were introduced into cell therapy of cartilage lesions to improve chondrocyte retention and distribution in the defect. Mesenchymal stem cells (MSC) are now discussed as an alternative cell source for repair. We here asked whether distinct gel-like carriers can support chondrogenesis of MSC *in vitro* and lead to stable cartilage-like transplants *in vivo*. Chondrogenesis of MSC embedded in collagen type I gel, fibrin glue, MatrigelTM and PuraMatrixTM peptide hydrogel was assessed and gene expression analysis, proteoglycan content, and collagen synthesis were quantified. Differentiated constructs were transplanted subcutaneously into SCID mice. All carriers supported chondrogenesis *in vitro*, but displayed material-dependent differences on COL2A1 gene expression, total collagen synthesis and proteoglycan deposition. The undesired calcification and microossicle formation in ectopic transplants *in vivo* was consistently suppressed by MatrigelTM. In sum, gel-like biomaterials were suitable carriers for MSC and promoted chondrogenesis. Suppression of calcification by particular gel-like materials makes their use even more attractive for MSC-based tissue engineering approaches in cartilage repair.

2. INTRODUCTION

Cartilage shows a very limited capacity for tissue regeneration and in order to achieve repair of cartilage defects a variety of cells or tissues have been used for transplantation procedures in animal models as well as in humans (1). In tissue engineering, cells, biomaterials, and bioactive factors are combined to create transplantable tissues and organs for research and clinical applications. Successful treatment of articular cartilage defects by transplantation of autologous chondrocytes has been reported (2). First generation autologous chondrocyte transplantation (ACT) procedures used cell suspensions without carrier materials which were injected beyond a sutured periosteal flap. Due to the risk of cell leakage, uneven distribution of the cells within the defect and hypertrophy of the repair tissue, new matrix-induced techniques were developed within the last years. The second generation ACT procedures mainly aimed at replacing the periosteal flap with membranes composed of bioresorbable matrices to simplify the surgical procedure and to minimize complications resulting from periosteal hypertrophy (3-4). Further, cells were embedded in three dimensional scaffolds to facilitate their transplantation (5-7). The major advantage of hydrogels in comparison to solid porous scaffolds is the opportunity to achieve a more

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homogenous cell distribution within the material as desired for tissue regeneration, their improved adaptation to shape and their higher fixation capacity in the defect.

Autologous mesenchymal stem cells (MSC) are discussed as an attractive alternative cell source to obtain chondrocytes since they can be isolated from diverse tissues without irreversible tissue damage (8-12). Bone marrow comprises a population of mesenchymal stem cells with the capacity to differentiate towards the chondrogenic lineage *in vitro* which could be used for cartilage regeneration (13-15). Subcutaneous adipose tissue must be regarded as a particularly attractive cell source because it is easily accessible, rather abundant, and self-replenishing. Adipose tissue-derived MSC have a multilineage differentiation capacity like bone marrow-derived MSC (11-12, 16-17), although their chondrogenic differentiation capacity was reduced under standard chondrogenic culture conditions driven by transforming growth factor beta (TGF-beta) (18). We previously optimized *in vitro* chondrogenesis of adipose tissue-derived MSC and eliminated their reduced chondrogenesis by combined application of TGF-beta 3 and bone morphogenetic protein 6 (BMP-6) (19). Chondrogenesis of MSC from both sources was associated with hypertrophy according to premature collagen type X expression, up-regulation of alkaline phosphatase activity and *in vivo* calcification and vascularisation of spheroids after ectopic transplantation in SCID mice (19-20).

Due to their multilineage capacity, MSC react sensitively to environmental conditions and it is conceivable, that carrier materials used as vehicles for transplantation could affect the chondrogenic differentiation capacity of MSC. Hydrogels like alginate and agarose were reported to allow *in vitro* chondrogenesis of human MSC (21), but these materials are uncommon in transplantation protocols to repair cartilage defects. Collagen type I and fibrin are naturally occurring molecules which are frequently used for transplantation procedures of autologous chondrocytes and tissue engineering approaches (5, 22-24).

In extension to previous reports using only one carrier material (25-26) the aim of this work was to analyze the influence of different gel-like carrier materials on chondrogenesis of MSC. The commonly used collagen and fibrin-based gel-like biomaterials consisting of one single predominant molecule were compared with Matrigel as a complex mixture of a variety of naturally occurring extracellular matrix molecules and growth factors, and PuraMatrix as a synthetic peptide hydrogel with a clearly defined composition. Besides effects on cell viability, construct size, collagen and proteoglycan deposition, the influence of these biomaterials on the *in vivo* stability of MSC-derived ectopic transplants in regard to undesired calcification was studied.

3. MATERIALS AND METHODS

3.1. Isolation and cultivation of mesenchymal stem cells

Lipoaspirates generated during liposuction procedures were obtained from Proaesthetic Private Clinic, Heidelberg. Human adipose tissue-derived MSC were

isolated from lipoaspirates of 12 donors (mean age and standard deviation 31.8 ± 7 years). Isolation was carried out according to the method described by Hauner *et al.* (27). Briefly, lipoaspirates were digested with Krebs-Ringer solution buffered with 25 mM HEPES, 20 mg/ml bovine serum albumin (BSA), and 1.5 mg/ml collagenase B (Roche Diagnostics, Mannheim, Germany) and filtered with a 250 μm and 150 μm nylon mesh (neoLab). Erythrocytes were removed using erythrocyte lysis buffer (0.154 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA). The remaining cells were washed with PBS and seeded into culture flasks. One day after isolation, cultures were washed with PBS to remove non-adherent material. For expansion, adipose tissue-derived MSC were plated at a cell density of $1-6 \times 10^3$ cells/ cm^2 in monolayer cultures and medium was replaced once a week. Expansion culture was carried out for two to three passages with medium consisting of DMEM high glucose (Gibco), 40 % MCDB201, supplemented with 2 % fetal calf serum (FCS), 2×10^{-8} M dexamethasone, 10^{-7} M ascorbic acid 2-phosphate, 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, 5 $\mu\text{g}/\text{ml}$ selenous acid (all Sigma-Aldrich, Deisenhofen, Germany), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco, Invitrogen, Karlsruhe, Germany), 10 ng/ml recombinant human epidermal growth factor (Strathmann Biotech, Hamburg, Germany) and recombinant human platelet-derived growth factor BB (Strathmann Biotech, Hamburg, Germany) (18). The studies were approved by the local ethics committee. Informed consent was obtained from all individuals included in the study.

3.2. Chondrogenic differentiation of MSC

After expansion in monolayer, MSC were harvested using trypsin/EDTA, and carrier-free pellets consisting of 0.5×10^6 cells were formed by centrifugation. Chondrogenic medium consisted of DMEM high glucose supplemented with 0.1 μM dexamethasone, 0.17 mM ascorbic acid 2-phosphate, 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, 5 $\mu\text{g}/\text{ml}$ selenous acid, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/ml BSA, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Standard culture was carried out with 10 ng/ml recombinant human transforming growth factor beta 3 (TGF-beta 3) and 10 ng/ml recombinant human bone morphogenetic protein 6 (BMP-6, R & D Systems, Wiesbaden, Germany). For limiting chondrogenic conditions chondrogenic medium without BMP-6 was applied.

3.3. Tissue engineering constructs

Micromasses were formed by inclusion of a cell suspension into collagen type I gel (IBFB, Leipzig, Germany), fibrin (Tissucol Duo STM, Baxter, Unterschleissheim, Germany), the basement membrane extract MatrigelTM (High Concentration, BD Biosciences, Heidelberg, Germany), or PuraMatrixTM Peptide Hydrogel (BD Biosciences, Heidelberg, Germany) with a final volume of 50 μl . A comparison of different cell counts in a pilot experiment revealed an optimal cell number of 0.5×10^6 cells per construct which was used for all experiments. Collagen constructs were prepared as recommended by the manufacturer: lyophilized collagen was resolved in 0.1 M acetic acid to a concentration of 4 mg/ml. 15 μl of resolved

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collagen were mixed with 25 μ l of cell suspension in chondrogenic medium without growth factors and 10 μ l of 0.2 M NaOH to achieve a pH of approximately 7. For fibrin constructs a fibrinogen solution containing 70-110 mg/ml of fibrinogen was diluted 1:15 in PBS and a solution containing 500 I.U./ml of thrombin was diluted 1:50 in PBS. Cells were suspended in 25 μ l of diluted fibrinogen solution and mixed with 20 μ l of diluted thrombin solution. Matrigel constructs were prepared by merging 25 μ l of chilled Matrigel with 25 μ l of chilled cell suspension in chondrogenic medium without growth factors. To generate PuraMatrix constructs 25 μ l of a cell suspension in 10 % sucrose were mixed with 25 μ l of PuraMatrix. Medium was changed three times within the next 30 minutes to adjust pH. The materials were allowed to gel at 37°C before chondrogenic medium was added. Matrix constructs and carrier-free pellets were cultured with chondrogenic medium for up to 6 weeks.

3.4. Cell viability assay

Cell viability was analyzed after four weeks of culture under chondrogenic conditions. Staining with fluorescein diacetate (0.1 mM FDA, Sigma Aldrich, Deisenhofen, Germany) was used to visualize living cells and propidium iodide (5 μ g/ml PI, Molecular Probes, Invitrogen, Karlsruhe, Germany) to stain dead cells (28). Optical sections were generated by the method of structured illumination (ApoTome Microscope Technique, Carl Zeiss, Jena, Germany) and red and green cells were counted by two independent observers.

3.5. RNA isolation and quantitative real time PCR

Quantitative gene expression analysis was performed for three MSC populations after differentiation for two and four weeks. Stem cell micromasses (pellets and matrix constructs) were minced with a polytron (Kinematica, Littau-Luzern, Switzerland). Total RNA was then isolated using a standard guanidinium thiocyanate/phenol extraction (peqGOLD Trifast; Peqlab, Erlangen, Germany). Polyadenylated mRNA was isolated from total RNA using oligo d(T) coupled to magnetic beads (Dynabeads; Dynal, Oslo, Norway) according to the manufacturer's instruction. Three micromasses were used for RNA isolation after two weeks of culture, six were used for RNA isolation after four weeks of culture.

20 ng of mRNA were subjected to first strand cDNA synthesis using reverse transcriptase (Omniscript®, Qiagen, Hilden, Germany) and oligo-d(T) primers. Expression levels of individual genes were analyzed by quantitative PCR using LightCycler™ technology (Roche diagnostics) according to the manufacturer's instructions. First strand cDNA was diluted 1:5 and 2 μ l were subjected to quantitative real time PCR using the following gene-specific 5' and 3' PCR primers, respectively: beta-actin: CTC TTC CAG CCT TCC TTC CT, CGA TCC ACA CGG AGT ACT TG; COL2A1: TGG CCT GAG ACA GCA TGA C, AGT GTT GGG AGC CAG ATT GT; COL10A1: TTT ACG CTG AAC GAT ACC AAA, TTG CTC TCC TCT TAC TGC TAT, ALP: CAC CAA CGT GGC TAA GAA TG, ATC TCC AGC CCT GGT CTC CTC. PCR amplification conditions were: 10 min denaturation at 95°C followed by 40 cycles of denaturation

at 95° C, annealing at 58° C for 7 s and extension at 72° C for 7 to 17 s. Specificity of the PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. The expression of the gene of interest in each sample is given as the percentage of beta-actin expression. The number of cDNA copies is correlated with the apparent threshold cycle (CT). Building the difference between CT of gene of interest and CT of beta-actin from one sample gives Δ CT values which can be expressed as percentage of beta-actin.

3.6. Total collagen synthesis

[³H]-proline incorporation was used as a measure for collagen synthesis. Matrices and pellets were incubated in 0.2 ml chondrogenic medium supplemented with 10 μ Ci/ml [³H]-proline (Amersham, UK) for 24 h at day 7, 14, and 28. Afterwards, the matrices were washed 3 times with 1 ml PBS for at least 1 h and digested in 0.5 ml 10 mM Tris, 0.1 mM EDTA, 2 % SDS, pH 8.0 with 100 μ g/ml Proteinase K (Roche, Mannheim, Germany) at 60°C overnight. 0.25 ml of the digested sample were used to measure incorporated radioactivity using a liquid scintillation counter (Win-Spectral, Wallac) and 2 ml OptiPhase Hi safe 3 scintillation fluid (Wallac). Adipose tissue-derived MSC from two different donors were used and six samples were prepared per donor and carrier material. Data were evaluated by Mann-Whitney-U test.

3.7. Quantification of proteoglycan content

Alcian blue staining was performed according to the method described by De Bari *et al.* (29). Matrices and pellets were washed with PBS, fixed with methanol for 30 minutes at -20°C, washed with Milli-Q water, and covered with 0.5 % Alcian blue 8 GS (Chroma) in 1M HCl. After overnight staining, cultures were washed extensively with Milli Q-water and extracted with 0.2 ml of 6 M guanidine HCl in Milli-Q water for 6 h at room temperature. The optical density of the extracted dye was measured at 650 nm using an ELISA reader MRX (Dynatech Laboratories). Adipose tissue-derived MSC from four different donors were used and four samples were prepared per donor and carrier material. Data were evaluated by Mann-Whitney-U test.

3.8. DNA quantification

Micromasses were homogenized in 500 μ l of 0.01 % Triton X-100. 25 μ l of the cell extract were mixed with 150 μ l of cold EDTA, pH 12.3, incubated at 37°C for 20 minutes and neutralized with 10 μ l of KH₂PO₄. After addition of 50 μ l of Hoechst 33258 solution (200 μ g/ml in water) fluorescence was measured at 355 nm. Data were referred to a DNA standard curve prepared from sheared salmon sperm DNA.

3.9. Histology

Micromasses were fixed in PBS containing 4 % paraformaldehyde for 2 h at room temperature, dehydrated in alcohol, washed in acetone, and infiltrated with paraffin. Paraffin sections of 5 μ m were dried, deparaffinized using XEM-200 (Vogel, Giessen, Germany), and rehydrated in alcohol. Staining procedures were performed using standard protocols. Sections were stained with 1 % alcian

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Table 1. Experimental design: *in vitro* characterization of stem cell-derived cartilage tissue

Days after chondrogenic induction	Analysis	Number of donors (i.e. Experiments)	Total number of micromasses/material
7-28	[³ H]-proline incorporation	2	12
14	gene expression	3	9
28	gene expression	3	18
28	alkaline phosphatase	3	18
42	proteoglycan deposition	4	16
35-42	histology	4	8

Table 2. Experimental design: *in vivo* analysis

Transplanted micromasses	Explantation at 4 weeks: Number of donors (i.e. Experiments)	Total number of micromasses/material (4 weeks)	Explantation at 8 weeks: Number of donors (i.e. Experiments)	Total number of micromasses/material (8 weeks)
collagen	4	8	-	-
fibrin	4	8	-	-
PuraMatrix	4	8	3	6
Matrigel	7	14	3	6
carrier-free pellets	7	14	3	6

blue (pH 2.5) (Chroma, Köngen, Germany) for 30 minutes to detect proteoglycan synthesis as an indication of cartilage matrix production. Counterstaining was performed for 3-5 minutes with fast red before permanently mounting with Eukit (HICO-MIC, Hirtz&Co, Cologne, Germany). To detect calcification, sections were stained with alizarin red for 5 minutes at room temperature (0.5 %, Chroma, Münster, Germany) and counterstained with fast green FCF (0.04 % in 0.1 % acetic acid, Certistan, Darmstadt, Germany) for 20 seconds.

Immunohistological staining was performed as described previously (18). Sections were pretreated with 2 mg/ml of hyaluronidase (Merck, Darmstadt, Germany) for 15 minutes at 37°C and subsequently with 1 mg/ml of pronase (Roche Diagnostics; Mannheim Germany) for 30 minutes at 37°C. PBS containing 5 % BSA was used to block non-specific background for 30 minutes. Sections were incubated overnight at 4°C with a monoclonal mouse anti-human collagen type II (clone II-4C11, ICN Biomedicals, Aurora, Ohio, USA) antibody in PBS containing 1 % BSA. After washing with Tris buffered saline, reactivity was detected using biotinylated goat anti-mouse secondary antibody (1:500; Dianova, Hamburg, Germany), streptavidin-alkaline phosphatase (Dako, Glostrup, Denmark) for 30 minutes at room temperature, and fast red (Sigma-Aldrich, Deisenhofen, Germany) for 20 minutes at room temperature. Sections were permanently mounted with Aquatex (Merck, Darmstadt, Germany) and examined by light microscopy. To distinguish between cells of human and murine origin, untreated sections or collagen type II-immunostained sections were further processed for *in situ* hybridization for human Alu genomic repeats as described previously (30).

A summary of the experimental design for the *in vitro* characterization of the stem cell-derived cartilage tissues is given in Table 1.

3.10. Alkaline phosphatase enzyme activity assay

Culture supernatants of six micromasses were collected at day 28 after chondrogenic induction and stored at -20°C until further use. For measurement of alkaline phosphatase (ALP) activity 100 µl of each supernatant

were incubated with 100 µl of substrate solution (10 mg/ml p-nitrophenylphosphate, Sigma-Aldrich, Deisenhofen, Germany) in 0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂ (pH 9.6). After incubation for 150 minutes, measurement was carried out at 405 nm in an ELISA reader.

3.11. Subcutaneous transplantation into SCID mice

Five groups of transplants (collagen, fibrin, Matrigel, PuraMatrix and carrier-free pellets) were prepared with cells derived from 4 or 7 donors. The micromasses were cultured for 5-6 weeks under chondrogenic conditions. A total of 8 to 14 micromasses was analyzed per group. For transplantation, pellets and matrices were attached to a non-resorbable surgical suture with a small amount of fibrin glue to facilitate transplantation and harvest. Subcutaneous pockets were prepared in the upper dorsal area of 10 anesthetized SCID mice. Up to four pouches were prepared per mouse and two constructs of each material and carrier-free pellets were transplanted into the pouches. Seven mice were killed at 4 weeks and 3 mice were killed at 8 weeks after transplantation. Explants were analysed by histological and immunohistological procedures. The animal experiments were approved by the Local Animal Experimentation Committee Karlsruhe.

The experimental design for the *in vivo* characterization of the stem cell-derived cartilage tissues is summarized in Table 2.

4. RESULTS

4.1. Enhanced construct volume in Matrigel and PuraMatrix

Compared to matrix-free high density pellets all carrier-augmented cell constructs displayed a larger volume of 50 µl at the time of preparation. During the first days of chondrogenic culture, significant contraction occurred in the collagen and fibrin constructs, whereas Matrigel and PuraMatrix supported the initial size and showed only a slight shrinkage. After 6 weeks of *in vitro* culture, collagen and fibrin-augmented adipose tissue-derived MSC constructs were only slightly larger than the carrier-free pellets. Matrigel and PuraMatrix nearly retained their initial

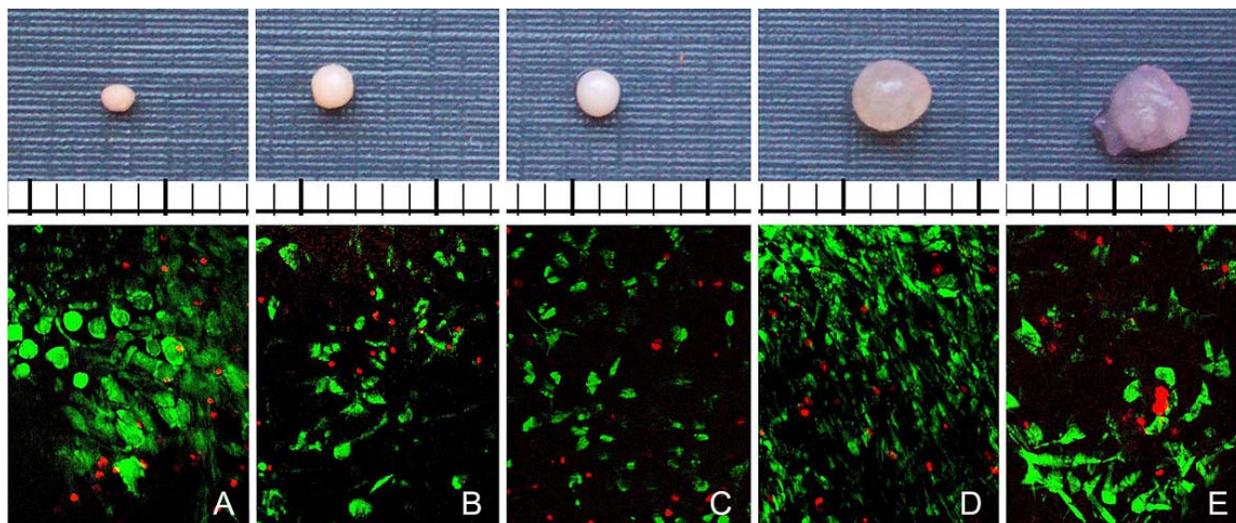


Figure 1. Carrier-augmented MSC constructs after culture under chondrogenic conditions. The size of the constructs at 6 weeks can be estimated from the mm scale (top) and cell viability is visualized after FDA/PI staining of optical sections (bottom). Dead cells (red) are clearly outnumbered in all materials after 4 weeks of culture (A) Carrier-free pellet; (B) collagen type I gel; (C) fibrin; (D) Matrigel; (E) PuraMatrix.

volume indicating improved defect-filling capacity over time (Figure 1).

4.2. No influence of carrier-materials on cell viability

Cell viability was assessed after 4 weeks of culture under chondrogenic conditions by staining with FDA/PI. Irrespective of the carrier materials, dead cells (< 30 %) were outnumbered by living cells and no differences in cell viability were evident between the different gel-like carriers or to the carrier-free pellet (Figure 1).

4.3. Induction of COL2A1 mRNA in all carrier materials

Quantitative gene expression analysis of adipose tissue-derived MSC after two and four weeks of chondrogenic induction (n=3) revealed an up-regulation of the cartilage specific marker COL2A1 as well as the hypertrophic marker COL10A1 (Figure 2). Two weeks after induction COL2A1 signals appeared only for carrier-free pellets and Matrigel constructs indicating that chondrogenesis was somehow delayed within the collagen, fibrin and PuraMatrix constructs (Figure 2A). At four weeks after induction, the carrier-free pellets and Matrigel constructs showed higher expression levels of COL2A1 than the collagen and fibrin constructs indicating that differentiation was induced in all materials, but to a lower extent in collagen and fibrin (Figure 2B). For PuraMatrix, gene expression analysis was not possible after 4 weeks of culture due to extremely low RNA yields. COL10A1 mRNA expression usually precedes induction of COL2A1 by several days (19-20) and COL10A1 was indeed detected within the carrier-free pellets and all materials at 2 weeks (Figure 2A) and 4 weeks (Figure 2B) indicating no influence of the carriers on the expression of this hypertrophic marker gene.

4.4. Enhanced collagen synthesis in Matrigel and fibrin

[³H]-proline incorporation was used as a measure for collagen synthesis of MSC biocomposites. [³H]-proline

incorporation was significantly enhanced by some of the carrier materials compared to the carrier-free pellets (Figure 3A). Collagen constructs showed an enhanced [³H]-proline incorporation only at day 14 (1.9-fold, $p \leq 0.05$), whereas fibrin (mean 2-fold, $p \leq 0.05$) and Matrigel constructs (mean 2.8-fold, $p \leq 0.05$) displayed an improved [³H]-proline incorporation at each time point. PuraMatrix had no influence on [³H]-proline incorporation except for day 28 when collagen synthesis was slightly reduced (0.7-fold $p \leq 0.05$). Total collagen synthesis, thus, was promoted especially by Matrigel and fibrin.

4.5. Elevated proteoglycan deposition in PuraMatrix

Proteoglycan deposition in MSC biocomposites was assessed at termination of chondrogenic culture at six weeks (Figure 3B). While collagen constructs contained similar amounts of proteoglycan like the carrier-free control pellets, proteoglycan deposition was significantly reduced in fibrin (0.6-fold, $p < 0.01$) and Matrigel constructs (0.8-fold, $p < 0.05$), whereas in PuraMatrix accumulation of proteoglycan was significantly enhanced (1.5-fold, $p < 0.05$). Proteoglycan contents were not referred to DNA content due to background problems with the Matrigel material which obviously contained DNA. Since the PuraMatrix constructs showed a slightly higher DNA content ($5.9 \pm 0.75 \mu\text{g}/\text{construct}$, mean and standard deviation) than the carrier-free pellets ($3 \pm 0.91 \mu\text{g}/\text{pellet}$, mean and standard deviation) enhanced proteoglycan deposition in these constructs may be a consequence of moderately higher cell counts.

4.6. Deposition of cartilaginous matrix in all materials

Histological analysis of matrix constructs seeded with MSC (n=4 donor cell populations) and cultured under chondrogenic conditions for 6 weeks revealed that differentiation was supported by all materials and a cartilaginous matrix was produced throughout the constructs. There were only slight differences in the

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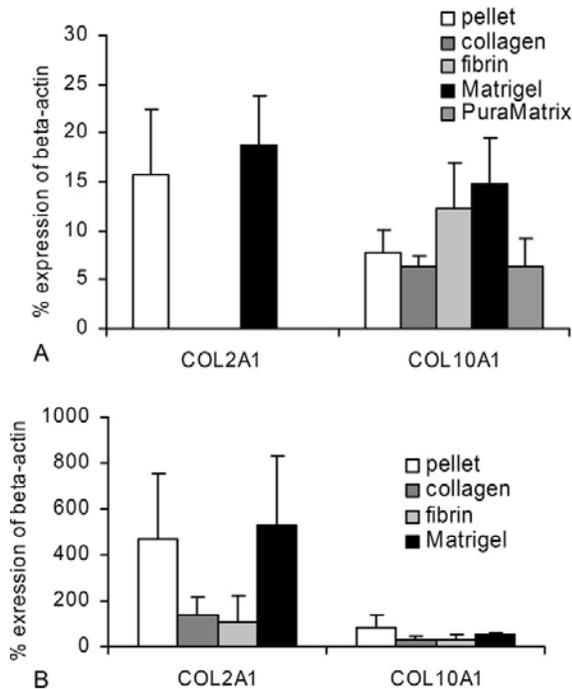


Figure 2. Col2A1 and Col10A1 mRNA expression detected after two (A) and four (B) weeks of culture under chondrogenic conditions. Gene expression was analysed by quantitative real time PCR and normalized to the expression of the house keeping gene beta-actin. Mean and standard deviation of three donor cell populations of MSC are given.

intensity of the alcian blue (Figure 4A-E) and collagen type II staining (data not shown). We challenged MSC differentiation of 4 donor cell populations under suboptimal conditions i.e. in the absence of BMP-6, where only approximately 10 % of carrier-free adipose tissue-derived stem cell cultures are able to differentiate (19). Under these conditions all carrier-free pellets were negative for proteoglycans and collagen type II (Figure 4F, K), while PuraMatrix constructs of all four donor cell populations showed positive collagen type II and alcian blue staining (Figure 4J, O). Within the other carrier-materials, two donor cell populations were completely alcian blue (not shown) and collagen type II negative (Figure 4L-N) while some cells of the other two donor cell populations were able to differentiate (Figure 4G-I).

4.7. Determination of alkaline phosphatase expression and activity

The late phase of *in vitro* chondrogenesis is characterized by up-regulation of ALP enzyme activity which is crucial for *in vivo* matrix calcification (19, 20) but undesired in cartilage repair. To quantify this aspect of hypertrophic differentiation, ALP mRNA expression and ALP enzyme activity of cultures (n=3 donors, Figure 5) were analyzed after 28 days of chondrogenic induction. Quantitative real time PCR revealed the strongest ALP expression in carrier-free pellets (0.8 % beta-actin) and collagen constructs (0.6 % beta-actin). Twelve-fold lower expression levels were found in the fibrin constructs, 30-

fold lower expression levels in the Matrigel constructs (Figure 5A).

Consistent with this, the ALP enzyme activity was high in medium supernatants of carrier-free pellets at day 28 (Figure 5B). Reduced mean enzyme activity was evident in the supernatant of collagen constructs (3-fold lower compared to carrier-free pellets) while low (fibrin: 14-fold lower, and Matrigel: 20-fold lower) or not detectable ALP activity (PuraMatrix) was observed for the other carrier-augmented cultures. Undesired ALP activity was, thus, reduced by all carrier materials and completely prevented by PuraMatrix.

4.8. Stabilization of ectopic cartilage formation by PuraMatrix and especially by Matrigel

In order to investigate, whether carrier materials may positively influence the ectopic cartilage formation capacity, differentiated matrix constructs and carrier-free pellets were pre-induced for 5-6 weeks with 10 ng/ml TGF-beta 3 and 10 ng/ml BMP-6 and transplanted into SCID mice. Explants analyzed at 4 weeks after transplantation were still positive for collagen type II (Figure 6A-E) and proteoglycans (alcian blue staining, Figure 6F-J). Alizarin Red staining, however, revealed a calcification of the carrier-free pellets and the collagen and fibrin constructs of all donors (Figure 6K, L, M). In PuraMatrix constructs calcification was found in two of four donor cell populations (Figure 6O), while all Matrigel constructs (n=7 donors) showed no calcification (Figure 6N). Collagen type II stained sections were used for *in situ* hybridization for human Alu genomic repeats indicating that the explanted constructs consisted of human cells (Figure 6P). To decide whether calcification was delayed or prevented in the Matrigel constructs, the *in vivo* period was prolonged to 8 weeks (n=3 donors). Matrigel transplants still showed no calcification for two donor cell populations and only a slight calcification for one donor cell population (data not shown) indicating an enhanced stabilization of the chondrocyte phenotype by Matrigel.

5. DISCUSSION

The importance of cellular condensation in chondrogenesis of mesenchymal stem cells was evident from both *in vivo* and *in vitro* observations. High cell densities were not only required for chondrogenesis to occur (31, 32) but the extent of cell condensation was also correlated with the level of chondrogenesis that could be achieved (33, 34) and obviously resulted in the initiation of gap-junction-mediated cell-cell communication in condensing mesenchyme (35, 36). Cell-cell and cell-matrix interactions as well as secreted factors seemed crucial for this process. Thus, chondrogenesis of adult mesenchymal stem cells was only successful in high density pellet culture but not in monolayer or cell suspension cultures. So far, only limited information was available on the effect of different scaffold materials on chondrogenesis of MSC, especially of biomaterials in which the cells are equally dispersed and kept apart from each other due to a gel-like composition of the matrix (37, 26). Since such materials become, however, more and more important in transplantation protocols for repair of articular cartilage

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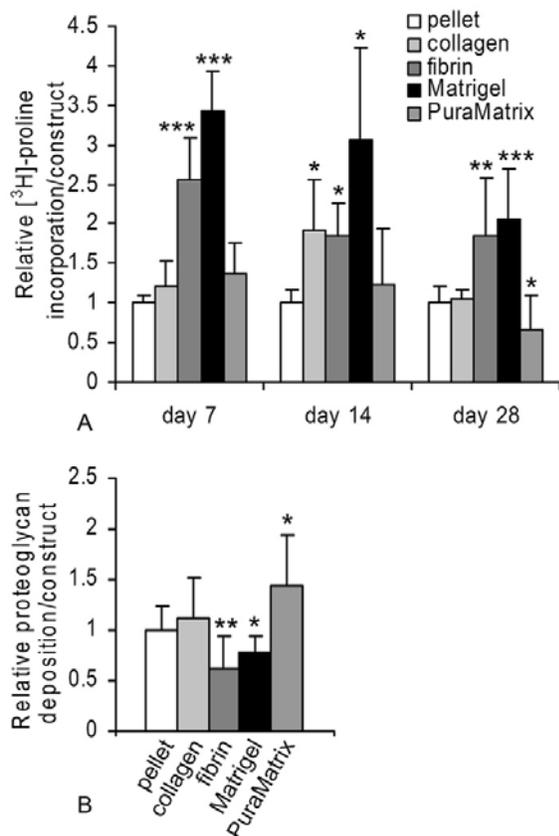


Figure 3. (A) Collagen synthesis of carrier-augmented constructs and carrier-free pellets quantified by [³H]-proline incorporation. After 7, 14 and 28 days of chondrogenic induction, constructs of 2 donor cell populations (6 samples for each donor and each material) were labelled with [³H]-proline for 24 hours and incorporated radioactivity was detected with a beta counter. [³H]-proline incorporation of carrier-free control pellets was set as 1. (B) Proteoglycan deposition of carrier-augmented constructs and carrier-free pellets quantified by alcian blue detection. After 6 weeks of *in vitro* chondrogenesis constructs were fixed, stained with alcian blue, washed with PBS, and eluted dye was measured at 650 nm. Data are presented as mean and standard deviation (4 samples each donor and each material). Proteoglycan deposition of carrier-free control pellets was set as 1. Data were evaluated by Mann-Whitney-U test: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to carrier-free pellets.

defects, we asked which gel-like materials support chondrogenesis, whether differential effects are seen on different aspects of *in vitro* chondrogenesis and whether there is a potential to influence and improve differentiation stability *in vivo*.

All gel-like biomaterials enhanced the volume of the constructs and separation of MSC by such biomaterials giving resistance to condensation induced by TGF-beta did not prevent the cells from differentiating *in vitro*. Obviously, at appropriate *in vitro* culture conditions, a cell density of 0.5×10^6 cells/construct (1×10^4 cells/mm³) was sufficient to support chondrogenesis in all gel-like

materials, and changes occurring later during shrinkage, like in collagen and fibrin matrices, seemed not to suppress chondrogenesis. Taken together this demonstrated that a high density packing of cells side by side of each other, with formation of intimate cell-cell interactions like in carrier-free pellet cultures, was not an absolute requirement to trigger chondrogenesis. Gel-like matrices like PuraMatrix provided an even more favourable environment to support differentiation since BMP-6 supplementation required for chondrogenic differentiation of adipose tissue-derived MSC could be omitted without losing chondrogenic induction.

Not all tested materials were similarly suited to support chondrogenesis *in vitro* and differential effects were seen on distinct phases of cell development. The collagen, fibrin and PuraMatrix constructs displayed a reduced level of induction of COL2A1 gene expression indicative of suboptimal conditions for the early phase of chondrogenesis at two weeks. According to [³H]-proline incorporation, total collagen synthesis was, however enhanced compared to pellets in the fibrin constructs, suggesting that other collagen types beside collagen type II may have been stimulated besides. Proteoglycan deposition at termination of culture indicated that this mid phase aspect of chondrogenesis was reciprocal to collagen synthesis since it was reduced in fibrin and Matrigel, those materials supporting higher collagen synthesis. Vice versa PuraMatrix constructs were characterized by lower collagen synthesis but accumulated slightly higher proteoglycan amounts in the matrix. It remains to be determined, whether higher synthesis of proteoglycans or their improved retention in the constructs are the reason for this result, since the extremely high content of charged amino acids in PuraMatrix may favour proteoglycan binding.

Hypertrophic and, thus, undesired late aspects of chondrogenesis like ALP enzyme activity were most pronounced in pellet cultures followed by collagen gel. Consistent with the role of ALP in matrix calcification, these composites underwent the strongest calcification *in vivo* together with fibrin which, however, had a lower ALP enzyme activity *in vitro*. PuraMatrix constructs consistently did not display any ALP activity *in vitro* although calcification of constructs was observed with some donor cells. Together this demonstrated that ALP enzyme activity detected *in vitro*, was no secure means to predict the extent of *in vivo* calcification. Since constructs are *in vivo* no longer under the influence of medium components like TGF-beta, a known suppressor of chondrocyte maturation and calcification (38), ALP activity may have developed differently during the 4 weeks *in vivo* period. Matrigel as a complex biomaterial contains growth factors including small amounts of TGF-beta and is mainly composed of collagen type IV, a matrix molecule with TGF-beta binding capacity (39). It may further contain a variety of factors capable to suppress *in vivo* calcification. Thus, the complex composition of Matrigel and a potential TGF-beta storage capacity during culture may have mediated its enhanced capability to endure stabilization of ectopic transplants in the absence of calcification.

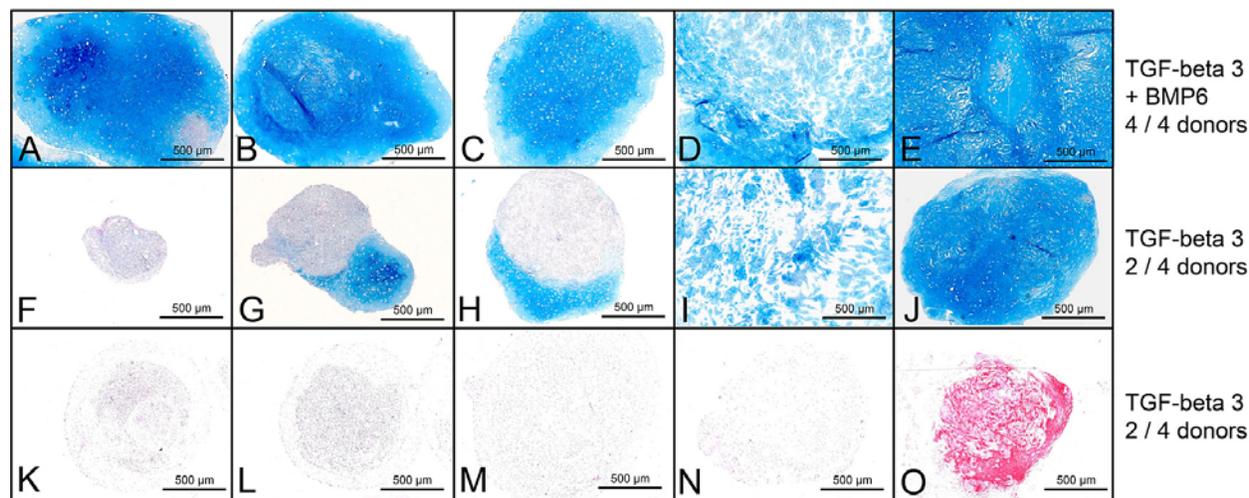


Figure 4. Alcian blue staining (A-J) and immunohistochemical staining for type II collagen (K-O) in paraffin sections after chondrogenic differentiation of MSC constructs for 6 weeks. MSC constructs of two out of four different donor cell populations (donor 1: A-J, donor 2: K-O) are shown. Cultures were induced in the presence of TGF-beta 3 and BMP-6 (A-E) or TGF-beta 3 only (F-O). (A, F, K) Carrier-free pellets; (B, G, L) collagen type I gel; (C, H, M) fibrin; (D, I, N) Matrigel; (E, J, O) PuraMatrix. At optimal conditions, four of four donor cell populations displayed a deposition of cartilaginous matrix in all carrier materials (A-E), while at suboptimal conditions carrier-free pellets were always negative (F, K). At suboptimal conditions, two of four cell populations showed a deposition of cartilaginous matrix in all carrier-materials (G-J), but two other donor cell populations only deposited detectable amounts of cartilaginous matrix in PuraMatrix (O).

The differential effects of gel-like materials elucidated in this study may be due to differences in the biochemical composition and the ultrastructure of the different materials. Both may mediate a distinct capacity to support cell metabolism and to bind and retain growth factors or newly synthesized extracellular matrix molecules. Fibrin glue contains TGF-beta 1, bFGF, EGF and VEGF. Matrigel consists of a complex mixture of basement membrane-derived extracellular matrix proteins, mainly laminin, collagen type IV and entactin as well as multiple growth factors, mainly IGF-1, TGF-beta and EGF. Matrigel is derived from EHS (Engelbreth-Holm-Swarm) mouse sarcoma and is frequently used as a material for *in vitro* and *in vivo* studies. As it is derived from tumour tissue, it must be regarded as potentially cancerogenic and is therefore, in its current formulation, not suitable for application in humans. Since it is composed of mainly laminin and collagen type IV, laminin or collagen type IV-based gel-materials may be considered as attractive for future material developments. As growth factor depleted preparations of Matrigel are available, it is appealing to study which growth factors may have improved chondrogenesis. This could allow to upgrade safe materials with favourable features of Matrigel by enhancing them with the active compounds.

Synthetic biodegradable materials have the advantage to be available in large amounts, can be designed with great flexibility to meet desired properties and are devoid of animal-derived material and pathogens. Peptide hydrogels are a new class of biomaterials consisting of defined amino acids and water. They are biocompatible and resorbable and were shown to support cell attachment and differentiation of a variety of mammalian primary cell

types. The self-assembling peptide hydrogel RAD16-I, also called PuraMatrixTM, was reported to enhance osteoblast differentiation by providing a more permissive environment for osteoblast growth (40). It should be safe for human application, but a further improvement of initial handling characteristics is desired, since in the beginning of the culture the constructs were fragile. Although they gained in stiffness during the culture period due to extracellular matrix synthesis of the encapsulated cells constructs were still too soft to allow fixation in a cartilage defect without a sealing cover. Another self-assembling peptide hydrogel, KLD12, was described to foster chondrocyte extracellular matrix production and cell division (41). Synthetic peptide hydrogels have the potential to be used for applications in humans and PuraMatrix seemed to be beneficial for chondrogenic differentiation of MSC. A reduced *in vivo* calcification of the MSC transplants like with Matrigel was not consistently achieved with PuraMatrix. If hypertrophy and calcification of constructs remain issues after transplantation into cartilage defects, PuraMatrix as a synthetic material may be modified with functional peptide sequences or upgraded through the inclusion of specific growth factors to achieve similar effects. Functionalization of PuraMatrix (RAD16-I) was demonstrated by Genové *et al.* who modified this peptide scaffold by extension with three short sequence motifs present in laminin 1 and collagen type IV, the two major protein components of the basement membrane (42). According to our results, this material may be attractive for future studies since it could combine effects of Matrigel and PuraMatrix.

Bone marrow is still the best characterized and most often applied source of stem cells which is also of great interest particularly with regard to microfracture

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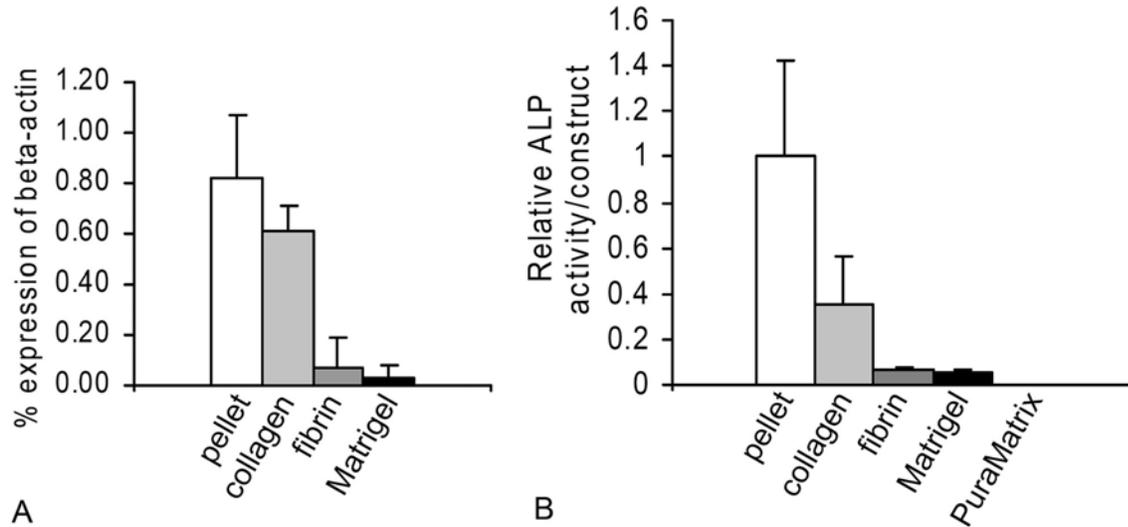


Figure 5. (A) ALP mRNA expression detected after 4 weeks of culture under chondrogenic conditions. Gene expression was analyzed by quantitative real time PCR and normalized to the expression of the house keeping gene beta-actin. Data are mean and standard deviation of three donor cell populations of MSC. (B) Alkaline phosphatase enzyme activity of culture supernatants at 28 days of culture under chondrogenic conditions. ALP-activity was determined by the hydrolysis of p-nitrophenylphosphate. Data are mean and standard deviation of MSC cultures of three different donors. ALP-activity of carrier-free control pellets was set as 1.

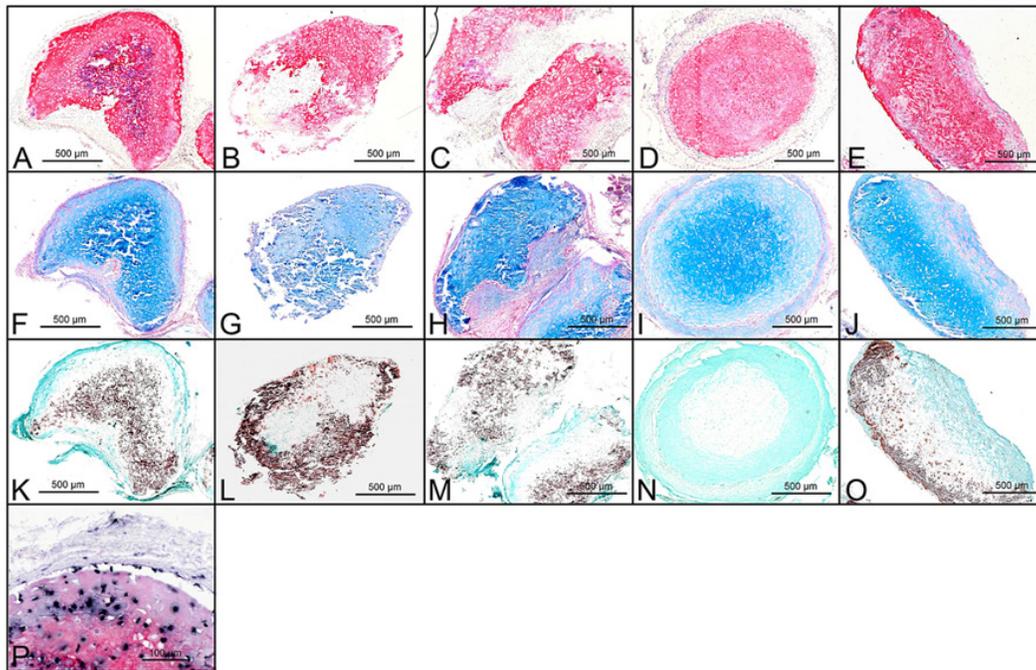


Figure 6. Collagen type II (A-E, P), alcian blue (F-J) and alizarin red (K-O) staining of paraffin sections of MSC constructs 4 weeks after transplantation into SCID mice. Before transplantation, constructs were cultured for 5 weeks under chondrogenic conditions with TGF-beta 3 and BMP-6. (A, F, K) Carrier-free pellet; (B, G, L, P) collagen type I gel; (C, H, M) fibrin; (D, I, N) Matrigel; (E, J, O) PuraMatrix. Transplants were positive for collagen type II (A-E, P) and rich in proteoglycans (F-J) but calcification was found in carrier-free pellets as well as in collagen and fibrin (K-M). In PuraMatrix constructs calcification was only observed for two of four donor cell populations (O) while all Matrigel constructs showed no calcification (N). *In situ* hybridization for human Alu genomic repeats of collagen type II-immunostained sections confirmed that the cells in the explanted constructs were of human origin (P).

techniques as an alternative treatment for cartilage defects. For this reason, we extended our analysis on the influence of carrier materials on chondrogenesis and ectopic cartilage formation capacity of adipose tissue-derived MSC to bone marrow-derived MSC from four donors (data not shown). Histology revealed *in vitro* results similar to adipose tissue-derived MSC, but a somehow higher tendency for calcification in Matrigel after ectopic transplantation (weak calcification with cells from two out of four donors). MSC from bone marrow may, thus be more prone to differentiation towards osteogenesis than adipose tissue derived stem cells due to their localization *in vivo*. This indicates that the choice of the right carrier material to prevent calcification may be even more important when bone marrow derived MSC are used for cartilage repair.

Two important points in tissue engineering of cartilage are the integration of transplants into the cartilage defect and the functionalization of the transplant in the joint. Our study provides no clue on these two issues and further experiments using an animal model with transplantation of MSC constructs into cartilage defects will have to address such transplantation-related questions of biogel-based MSC constructs.

6. CONCLUSIONS AND PERSPECTIVES

In summary, our study demonstrated that diverse gel-like carrier materials support the chondrogenic differentiation of MSC and can display differential effects on gene expression and biochemical composition of the regenerate. While collagen-type I gel and fibrin glue displayed their strength mainly in a good handling and potentially enhanced fixation capacity, but showed no further favorable cellular or molecular effects on MSC beyond pellet culture, PuraMatrix supported *in vitro* chondrogenesis to an extent that growth factor supplementation could be reduced. It further suppressed undesired ALP enzyme activity which is crucial for matrix calcification, however could not prevent as effectively as Matrigel the *in vivo* calcification of constructs at ectopic sites. Combining the positive effects of the synthetic scaffold and the basement membrane-derived factors of Matrigel with the features of a collagen gel or fibrin glue is an appealing challenge for upcoming studies.

In conclusion, our study guides the way to use gel-like biomaterials not only to improve transplantation and fixation techniques in chondrocyte-based cartilage repair, but also to influence early, enduring, and late aspects of chondrogenesis and to improve functionalization of MSC derived chondrocytes as an attractive cell source for tissue engineering.

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Abbreviations: ACT: autologous chondrocyte transplantation, MSC: mesenchymal stem cells, TGF-beta: transforming growth factor beta, BMP: bone morphogenetic protein, FDA: fluorescein diacetate, PI: propidium iodide, ALP: alkaline phosphatase, SCID: severe combined immunodeficiency, EHS: Engelbreth-Holm-Swarm

Key Words: Carrier Materials, Proteoglycan, Collagen Type II, Calcification, Ectopic Cartilage Formation

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