

Chondrogenic Differentiation of Human Chondrocytes and Stem Cells in Different Cell Culture Systems Using IGF-1-Coupled Particles

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Abstract

Various cell-based therapies use the transplantation of *ex vivo* cultured chondrocytes or stem cells to support repair of cartilage defects. Cell expansion *in vitro* is required prior to transplantation accompanied by cell dedifferentiation, resulting in unwanted fibrocartilage formation *in vivo*. Targeted application of growth factors during *in vitro* cultivation is intended to enhance chondrogenic differentiation of cells. In previous studies, collagen-based scaffolds enriched with silica particles coupled with the insulin-like growth factor (IGF) 1 were tested, concerning their suitability to increase the *in vitro* redifferentiation of human chondrocytes. Accordingly, in the present study chondrogenic differentiation potential of IGF-1-coupled particles was investigated using human chondrocytes cultured in scaffold-free spheroid pellet culture. Further, influence of IGF-1-coupled particles on mesenchymal stem cells derived from bone marrow (BM-MSCs) cultured onto collagen-based scaffold or in pellet culture was examined as well pellet culture was examined.

Chondrogenic differentiation was induced by the growth factor IGF-1 applied as I) soluble IGF-1 or II) conjugated to red fluorescent silica particles. In addition, control silica particles conjugated with NH₂ were used to exclude adverse side effects. Besides cell proliferation, collagen type II and glycosaminoglycan synthesis was quantified and histological staining performed to investigate the chondrogenic differentiation.

In pellet culture, IGF-1-coupled particles were applied during the pellet formation only. Traceable red fluorescent particles showed homogenous distribution within the pellets. Adverse effects were not detected. Human chondrocyte pellets displayed significantly increased collagen type II synthesis using IGF-1-coupled particles, compared to soluble IGF-1. Independent of the application mode, induction of chondrogenic differentiation of BM-MSCs cultured in pellets was not suitable with the addition of IGF-1 only. However, BM-MSCs cultivation onto collagen-based scaffold enriched with IGF-1-coupled particle showed superior glycosaminoglycan synthesis, compared to soluble IGF-1 application.

Using IGF-1 coupled to particles within a three-dimensional matrix resulted in an increased stimulatory chondrogenic effect, indicating a promising tool for controlled growth factor delivery during treatment of cartilage lesion.

Keywords: Human chondrocytes; Human mesenchymal stem cells; Chondrogenic differentiation; Insulin-like growth factor (IGF); Cartilage regeneration

Background

Impaired cartilage regeneration after lesion is still a major challenge in orthopaedic surgery. Cartilage tissue has a limited intrinsic capacity for self-regeneration, because of its avascularity and low cellularity. Traumatic or degenerative injuries of cartilage tissue may cause the development and progression of osteoarthritis [1]. Various cell-based therapies utilised the transplantation of *ex vivo* cultured cells to reinforce repair of cartilage defects [2–4]. Autologous chondrocyte implantation (ACI) includes the isolation and subsequent *in vitro* expansion of autologous chondrocytes from a minor load-bearing area of patient's cartilage, followed by cell implantation in terms of spheroid pellet cultures into the defect side. Using matrix-associated autologous chondrocyte implantation (MACI) cells were incorporated in a three-dimensional scaffold before transplantation [4]. However, the application of human chondrocytes involved considerable limitations, namely that two operations are needed (cartilage harvest and cell transplantation), there is risk of donor site morbidity, restricted availability of healthy tissue and cells, loss of their chondrogenic phenotype during expansion in monolayer cultures and limited replicative capacity [5]. Accordingly, in recent years adult mesenchymal stem cells derived from bone marrow (BM-MSCs) have attracted much attention due to their high self-renewal capacity, excellent proliferation rates, ability to differentiate

into multiple stromal cell lineages as well as minimal harvest morbidity. The application of BM-MSCs for cartilage regeneration revealed promising clinical outcome for cartilage repair and, therefore, BM-MSCs are a promising alternative cell source for cartilage restoration [6–8].

These cell-based interventions, especially ACI, are widely used for functional restoration of cartilage lesions [1,9]. Although these techniques have proven their feasibility in refilling chondral and osteochondral defects, some limitations are apparent [1,5]. During *in vitro* expansion on plastic, isolated chondrocytes dedifferentiate, resulting in a fibroblast-like morphology and an increased expression of collagen type I, while collagen type II release is reduced [5,6].

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An insufficient differentiation of BM-MSCs *in vitro* causes the same limitations. The dedifferentiated state of cells could impair the formation of hyaline cartilage and result in hypertrophic differentiation of cells after implantation. Histological analyses show reconstruction of a cartilage defect *in vivo* by fibrocartilage tissue instead of hyaline cartilage, as a result of which the fibrous repair tissue is not capable of withstanding the high biomechanical loading in the knee joint [4,10].

As dedifferentiation of chondrocytes contributes inferior cartilage repair *in vivo*, the induction of cell redifferentiation by the application of various growth factors and the use of a three-dimensional (3D) cell culture system is of great interest and has been intensively studied. Exposing human chondrocytes and BM-MSCs to specific bioactive molecules is assumed to promote chondrogenic differentiation, enhance regeneration potential significantly and thus improve the clinical outcome [11,12]. The most investigated molecules that stimulate the anabolic activity in cartilage include transforming growth factor (TGF)- β 1, 2, 3 and insulin-like growth factor (IGF)-1 [13-15]. IGF-1 is an essential growth factor which interacts with its specific receptor and initiates an intracellular signaling resulting in enhanced proteoglycan and type II collagen synthesis as well as superior proliferation of pre-chondrocytes [16]. Moreover, this growth factor induces the differentiation of BM-MSCs towards the chondrogenic phenotype [17]. In addition, chondrogenic redifferentiation after cell expansion is promoted by 3D cell cultivation in either spheroid pellet culture or on scaffolds made of collagen and hyaluronic acid [18]. The cultivation in spheroid pellet cultures allows chondrocyte condensation with cell-cell contacts similar to those observed in the preliminary stage of cartilage development [5,19]. New approaches pursue the integration of bioactive molecules in the 3D constructs, because of the limited supply of growth factors and nutrients within the centre of these [20].

In our previous studies, we used collagen-based scaffolds enriched with IGF-1 coupled to silica particles (sPa) for the *in vitro* redifferentiation of human chondrocytes [21]. The application of IGF-1-coupled sPa increased the initial type II collagen synthesis in chondrocytes, indicating an accelerated chondrogenesis. Regarding these promising findings, in the present study, the IGF-1-coupled silica particles were used to investigate their stimulatory chondrogenic potential on human chondrocytes cultured in spheroid pellets. BM-MSCs are a promising alternative cell source for cartilage regeneration as well, and therefore, chondrogenic differentiation potential of IGF-1-coupled particles regarding BM-MSCs cultured either on a collagen-based scaffold or as pellets was examined. Cell viability and extracellular matrix synthesis were investigated and compared to soluble IGF-1 treated cells to identify the most suitable form of growth factor application.

Materials and Methods

Cell isolation and cultivation

The articular knee cartilage used for the isolation of human chondrocytes was obtained from patients undergoing primary knee replacement. Furthermore, during this surgical procedure the femoral medullary space was opened and bone marrow aspirated, from which mesenchymal stem cells were isolated. The samples were collected only with informed consent from the patient and according to procedures approved by the Local Ethical Committee of the University of Rostock (registration number: A200917 and A2014-0025). Human chondrocytes (n=6, female: 65 \pm 11 years) were isolated under sterile conditions according to the protocol previously described [22]. The knee cartilage was minced and enzymatically digested by trypsin and collagenase A. Chondrocytes were washed prior to cultivation in Dulbecco's Modified

Eagle Medium (DMEM) containing 10% foetal calf serum (FCS), 1% amphotericin B and 1% penicillin-streptomycin (all from Fisher Scientific, Darmstadt, Germany) and supplemented with ascorbic acid (50 μ g/mL, Sigma, Seelze, Germany) under standard cell culture conditions (5% CO₂ and 37°C, 21% O₂). The culture medium was changed every two to three days.

Human mesenchymal stem cells (n=8; male: 66 years; female: 73 \pm 7 years) were isolated from bone marrow (BM) aspirates. Each aspirate sample (1-3 mL) was diluted in DMEM and layered over Histopaque⁻1077 (Sigma-Aldrich, Seelze, Germany). After density gradient centrifugation at 800 x g for 20 min, the mononuclear cell layer was removed from the interface and washed with phosphate buffered saline (PBS; PAA, Coelbe, Germany). Cells were counted using trypan blue dye (Serva, Heidelberg, Germany) and a hemocytometer. Nucleated cells were seeded at a density of 1600-4000 cells/cm² in DMEM containing the supplements FCS, amphotericin B and penicillin-streptomycin as mentioned above, and cultured under standard cell culture conditions [13]. After 72 h, non-adherent cells were removed and afterwards the medium was changed every three to four days.

Characterisation of Mesenchymal Stem Cells (BM-MSCs)

Flow cytometry

In order to analyse the surface marker expression profile of isolated mononuclear cells, flow cytometric analysis was performed using mouse anti-human fluorochrome-conjugated monoclonal antibodies. After washing and blocking using PBS/1% BSA, cells were incubated in the dark for 60 min on ice with the following antibodies against several CD (cluster of differentiation) antigens: CD90-FITC (IgG1), CD105-FITC (IgG1), CD44-PE (IgG1), CD14-PE (IgG1), CD29-APC (IgG1) (all from Biolegend, London, UK), CD45-APC (IgG1, AbD Serotec, Puchheim, Germany) and mouse isotype control for IgG1 as a negative control labelled with APC (Biolegend, London, UK). Afterwards, cells were washed and kept in PBS/1% BSA. Fluorescence intensity was assessed immediately after antibody staining using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, USA). Data analysis was performed using CellQuest software (Becton Dickinson) and evaluated using the software FlowJo (Tree Star, Inc., Ashland, Oregon, USA). For each sample the fluorescence intensity of each CD marker was analysed in comparison to the isotype control.

Isolated cells were found to express the markers CD90, CD105, CD44 and CD29 on the surface of 99.6%, 91.4%, 99.9% and 99.9% of the cells (Figure 1A). Whereas, cells show strongly reduced expression of the macrophage-specific surface marker CD14 (49.7%) and the hematopoietic lineage marker CD45 (0.38%).

Verification of the multipotent character of isolated BM-MSCs

The differentiation towards osteogenic, chondrogenic and adipogenic lineage was induced to examine the multilineage differentiation capacity of isolated cells. For osteogenic differentiation, isolated cells (4000 cells/cm²) were incubated in Minimum Essential Media (MEM; Biochrom, Berlin, Germany) containing 10% FCS, 1% amphotericin B, 1% penicillin-streptomycin, 1% HEPES) and supplemented with osteogenic differentiation factors (all from Sigma-Aldrich, Seelze, Germany) ascorbic acid (50 μ g/mL), β -glycerophosphate (10 mM) and dexamethasone (100 nM) at 37°C, 5% CO₂ for 21 days. Afterwards, immunohistochemical detection of the enzyme alkaline phosphatase (ALP) was performed using the Fuchsin+Substrate Chromogen System (Dako, Hamburg, Germany)

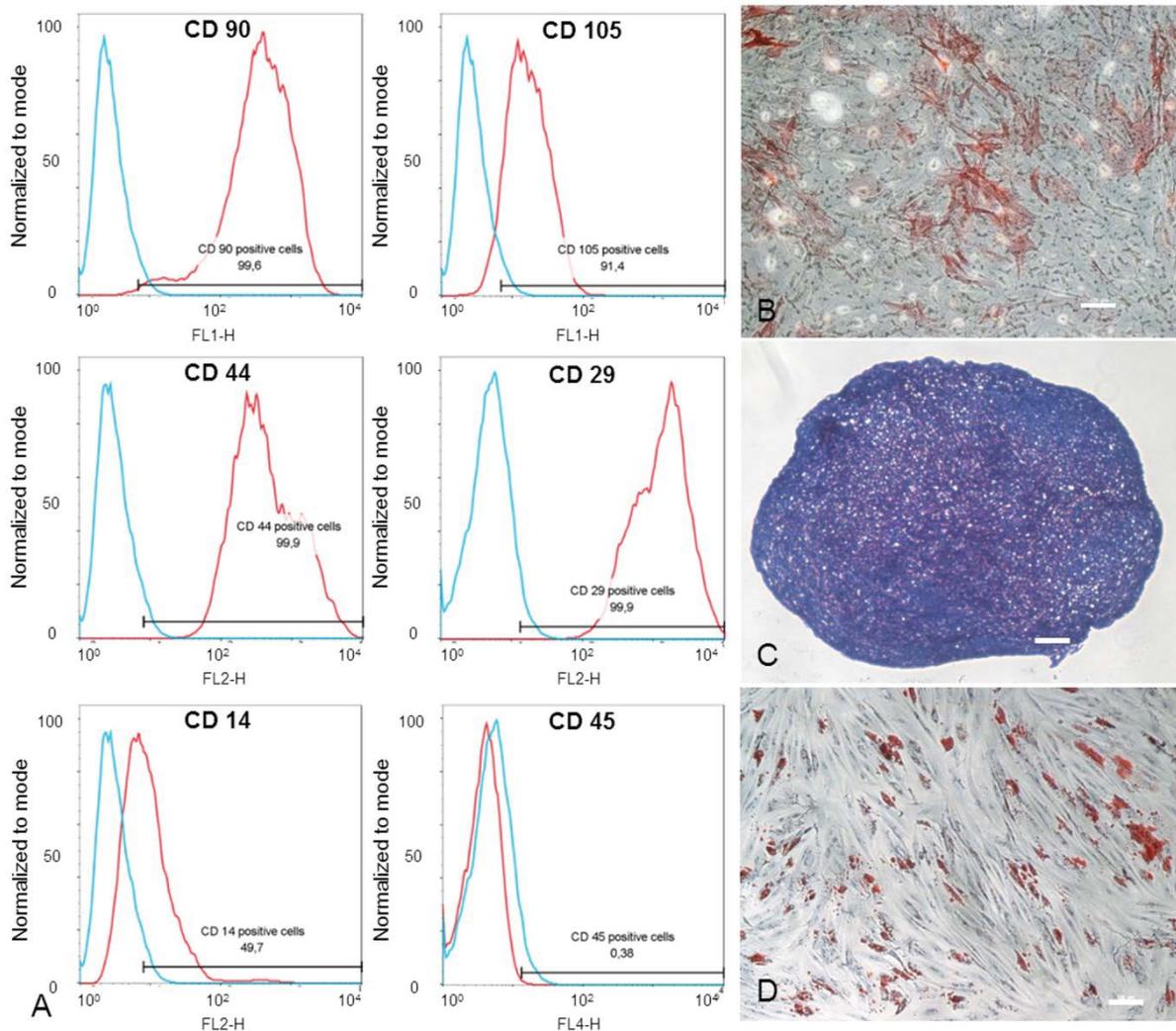


Figure 1: Characterisation of bone marrow-derived mesenchymal stem cells (BM-MSCs). Cells were isolated from human bone marrow and expanded *in vitro*. **(A)** Flow cytometric analysis of cell surface antigens was performed using BM-MSCs in the first passage and the positive expression of CD90, CD105, CD44 and CD29 was detected, while lacking expression of negative markers CD14 and CD45. The histograms display the fluorescence profiles of BM-MSC stained with the particular surface antibody (red lines) and the isotype control (blue lines) of a representative sample. **(B-D)** Differentiation of BM-MSCs was induced in the fourth passage. Achievement of *in vitro* differentiation in osteogenic, chondrogenic and adipogenic lineage was examined after 21 days (white bar indicates 100 μm). **(B)** Osteogenic differentiated cells were positively probed for the expression of alkaline phosphatase (red color). **(C)** Chondrogenic differentiation of BM-MSCs in pellet culture was visualised by dark violet staining of proteoglycans using toluidine blue. **(D)** Adipogenesis was detected by specific lipid vesicles being stained with oil red.

following the manufacturer's protocol. Positive staining of ALP, an enzyme involved in bone matrix mineralisation, was conducted to confirm osteogenic differentiation of isolated cells after 21 days (Figure 1B).

For chondrogenic differentiation, aliquots of 5×10^5 BM-MSCs were centrifuged and cultured in a conical tube to form a pellet prior to transfer to 96-well plate after 72 h. Cell pellets were incubated for 21 days in serum-free DMEM (containing amphotericin B and penicillin-streptomycin) supplemented with 1% ITS™ (Becton Dickinson, Heidelberg, Germany), ascorbic acid (50 μg/mL), dexamethasone (100 nM), insulin-like growth factor 1 (IGF-1; 50 ng/mL, RD Systems, Wiesbaden, Germany) and transforming growth factor beta 1 (TGF-β1; 50 ng/mL, tebu-bio, Offenbach, Germany) under standard cell culture conditions. After 21 days, chondrogenic phenotype was indicated by the proteoglycan deposition being stained violet, using toluidine blue (Figure 1C).

For adipogenic differentiation, BM-MSCs (3×10^4 cells/cm²) were incubated in DMEM (containing FCS, amphotericin B and penicillin-streptomycin) supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 10 μM insulin, 1 μM dexamethasone and 200 μM indomethacin for 21 days. In order to investigate the adipogenic differentiation potential, cells were analysed for the deposition of triglyceride droplets, which were detected by oil red staining (Figure 1D). The results show that the isolated bone marrow cells had the ability of stem cells for multilineage differentiation toward the osteogenic, chondrogenic and adipogenic lineages.

Conjugation of IGF-1 on the surface of fluorescent silica particles

Red fluorescent silica particles (sicastar®-redF, micromod

Partikeltechnologie GmbH, Rostock, Germany) with a diameter of 1 μm and amino groups on the surface were applied for the conjugation with recombinant human growth factor IGF-1. The detailed procedure was previously described [21]. In brief, the silica particles were prepared by a modified Stöber process including the covalent binding of fluorescent dye. The conjugation strategy of IGF-1 on the surface of the fluorescent silica particles (Excitation: 569 nm, Emission: 585 nm) is based on the insertion of maleimide groups on the surface of amino-functionalised particles via a polyethylene glycol (PEG) spacer. In the second step, the maleimide-functionalised particles react with the thiolated IGF-1 to form a stable chemical bond between IGF-1 and particles. The IGF-1 coating on the particle surface was analysed by the protein quantification assay micro-BCA assay (Thermo Fisher Scientific, Darmstadt, Germany). The final suspension contained 12.5 μg particle per mL suspension corresponding to 50 ng/mL of the growth factor IGF-1. Additionally, control silica particles with NH_2 -groups on the surface were used with the same concentration (12.5 μg particles per mL suspension) to exclude adverse effects of the red-fluorescent silica particles sicastar[®]-redF.

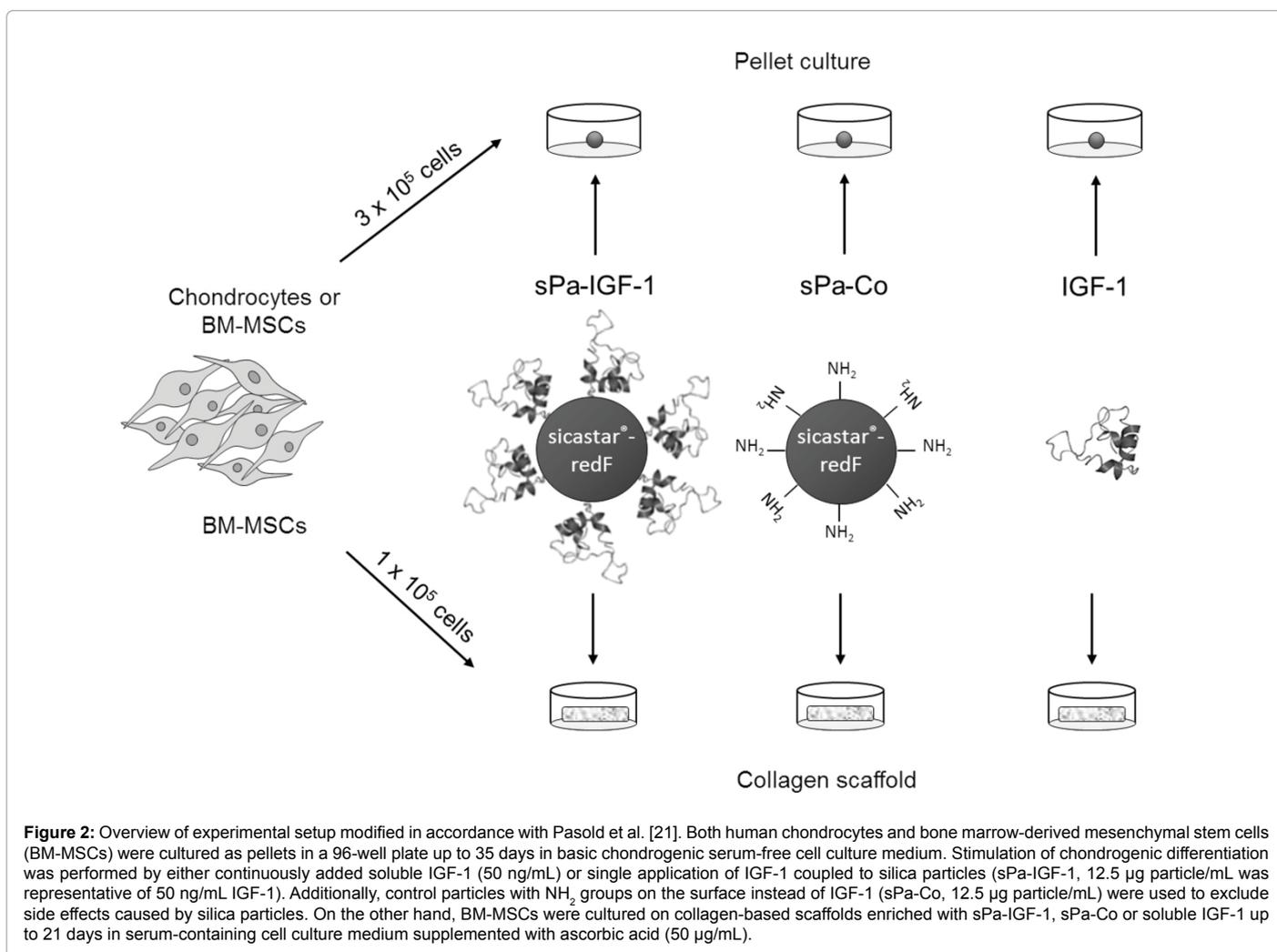
Chondrogenic differentiation promoted by growth factor IGF-1

Both human chondrocytes and human BM-MSCs were washed

with PBS and detached using 0.25% trypsin/EDTA solution (Fisher Scientific, Darmstadt, Germany). Afterwards, chondrocytes were cultured as spheroid pellets, a scaffold-free high-density culture system. BM-MSCs were either cultured as spheroid pellets or cultured onto a collagen-based scaffold. Independent of the culture system, the following three treatment groups was distinguished to investigate the chondrogenic differentiation potential of the IGF-1-coupled particles (Figure 2):

- I) IGF-1-coupled particles sPa-IGF-1 (12.5 μg particle/mL, 50 ng/mL IGF-1),
- II) Control silica particles sPa-Co (12.5 μg particle/mL, 50 ng/mL NH_2),
- III) Soluble IGF-1 (50 ng/mL)

For pellet cultivation, aliquots of 3×10^5 cells were centrifuged at $800 \times g$ for 5 min in 15-mL conical tubes. Cells were suspended in 500 μL DMEM containing 1% ITS[™], ascorbic acid (50 $\mu\text{g}/\text{mL}$), dexamethasone (100 nM) and the supplements I), II) or III). After 72 h of incubation in the tubes, the spherical cell aggregates were transferred into wells of a 96-well plate and cultured under standard cell culture conditions. The medium was changed every two to three days. Both sPa-IGF-1 (group I) and sPa-Co (group II) were added only at the time of pellet formation.



Afterwards, cells were cultured in DMEM containing ITS™, ascorbic acid and dexamethasone. However, cells of group III were cultured in the same basic medium continuously supplemented with soluble IGF-1. Pellets were cultured under standard cell culture conditions up to 35 days.

Besides the cultivation in pellets, BM-MSCs were also cultured onto three-dimensional, bioresorbable collagen-based scaffolds which are two-layered scaffolds made of type I collagen and hyaluronic acids (Medical Biomaterial Products GmbH, Neustadt-Glewe, Germany). BM-MSCs (1×10^5 cells/cm²) were seeded onto the scaffold and were incubated in DMEM (with 10% FCS, 1% amphotericin B, 1% penicillin-streptomycin) supplemented with ascorbic acid (50 µg/mL) and the additives I) sPa-IGF-1, II) sPa-Co or III) soluble IGF-1 only at the time of cell seeding. After three days of incubation under standard cell culture conditions, the first medium change was performed, so that the unbound particles were washed away. The cell culture medium (FCS-containing DMEM and ascorbic acid) was changed every two to three days and cells were incubated under standard cell culture conditions up to 21 days.

Cell viability testing

The CyQuant Proliferation assay is a colorimetric-based test quantifying the amount of DNA which correlates with the cell number. The pellets were lysed and CyQuant test was performed according to the manufacturer's protocol. After measurement using microplate reader Infinite[®] 200 PRO (TECAN, Maennedorf, Switzerland) the number of cells was calculated according to a standard curve.

For cells cultured on scaffolds, the metabolic activity was investigated using the WST-1 assay (Roche, Grenzach-Wyhlen, Germany) as recommended by the manufacturer and quantified using a spectrophotometer (Infinite[®] 200 PRO, TECAN, Maennedorf, Switzerland). Next, the BM-MSCs cultured onto collagen-based scaffolds were stained using calcein-acetoxymethyl in accordance with manufacturer's guidelines of the LIVE/DEAD[®] assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The green fluorescent (494-517 nm) calcein-acetoxymethyl indicated intracellular esterase activity and enabled an optical appraisal of the cells. The red fluorescent (528-617 nm) ethidium homodimer-1 was not used, because it interferes with the red fluorescent silica particle. Images of the cells were taken with a fluorescence microscope (Nikon Type 120, Nikon Instruments, Tokyo, Japan) and evaluated with the software NIS-Elements (Nikon Instruments, Tokyo, Japan). After 21 days of incubation, the distribution of particles within the collagen scaffold was investigated using confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena, Germany).

Pro-collagen type II quantification

The production of pro-collagen type II (CPII) on the protein level was examined via enzyme-linked immunosorbent assay (ELISA; QUDEL Corporation, San Diego, USA). The quantity of the C-terminal pro-peptide of CPII reflects the synthesis of mature collagen type II, because this pro-peptide is cleaved from the collagen molecule by specific proteases during incorporation into extracellular collagen fibrils. Therefore, supernatants of samples were collected after 7, 14 and 35 days and stored at -20°C. The CPII assay was conducted according to manufacturer's specifications. Absorbance was measured at a wavelength of 450 nm using a spectrophotometer (Infinite[®] 200 PRO, TECAN, Maennedorf, Switzerland). The CPII content was determined on the basis of a standard curve.

Glycosaminoglycan content

The Blyscan[™] glycosaminoglycan assay (Biocolor Pharmaceuticals,

Inc., Carrickfergus, UK) is a quantitative test for the analysis of sulphated proteoglycans and glycosaminoglycans (GAG) using 1,9-dimethylmethylene blue. After 3, 7, 14 and 21 days, the samples were collected and papain extraction (20 units/mg in 0.2 M sodium phosphate buffer, pH 6.4; Sigma-Aldrich, Seelze, Germany) was performed at 65°C overnight prior to GAG quantification following the manufacturer's instructions. Absorbance was measured at 656 nm using a spectrophotometer (Infinite[®] 200 PRO, TECAN, Maennedorf, Switzerland) and the GAG content was calculated using a reference standard curve. For determining the background signal of the collagen-based matrix, cell-free scaffolds which were also incubated in medium over respective time points were analysed and used as blank. The amount of GAG was then normalised to metabolic activity per sample.

Histology

Pellets were fixed in 4% buffered formalin for 24 h, embedded in paraffin and subsequently cut into 5 µm thick sections. These sections were deparaffinised and stained with either Heidenhain's AZAN trichrome or toluidine blue [23,24].

Statistical analysis

A minimum of five independent experiments was performed for statistical analysis. Statistical significance between groups was calculated either by one-way ANOVA posthoc or by Wilcoxon matched pair tests (two-tailed) using Graphpad Prism Software (La Jolla, CA, USA). The level of significance was set at $p < 0.05$. Unless otherwise indicated, data are represented by the mean value \pm standard deviation.

Results

Redifferentiation of chondrocytes cultured in pellets using IGF-1-coupled particles

During chondrocyte pellet formation, IGF-1-coupled particles were added for reinforcing the availability of the growth factor IGF-1 even in the centre of the pellets. Red fluorescent silica particles (sPa) were used for IGF-1 coupling to ensure particle tracking. These particles could be detected in the pellet sections prior to histological processing after both 14 and 35 days of cultivation by means of fluorescent microscopy. They were homogeneously dispensed within the cell pellet, indicating consistent distribution of the growth factor (Figure 3A). In addition, it has been shown that the application of IGF-1-coupled sPa had no influence on pellet formation.

Besides using IGF-1-coupled sPa, the influence of continuously added exogenous IGF-1 as well as the application of NH₂-functionalized particles (sPa-Co) on chondrocytes was determined. Cell number was investigated to examine cell proliferation (Figure 3B). An initial increased cell proliferation up to seven days was measured in all treatment groups. During further cultivation, cell numbers were slightly reduced. After 35 days, the highest number of cells was found for continuously added IGF-1 ($1.51 \times 10^6 \pm 2.55 \times 10^5$ cells/pellet) compared to pellets enriched with either IGF-1-coupled particles ($1.17 \times 10^6 \pm 3.59 \times 10^5$ cells/pellet) or control particles ($1.12 \times 10^6 \pm 3.76 \times 10^5$ cells/pellet). However, the differences did not reach a level of significance, indicating that using sPa neither had any influence on cell proliferation nor did it have any cytotoxic adverse effects.

Collagen type II synthesis was quantified and related to the number of cells. We found that after seven days of cultivation the chondrocyte pellets treated with sPa-IGF-1 produced an increased amount of collagen type II compared to cells cultivated with the continuous addition of IGF-

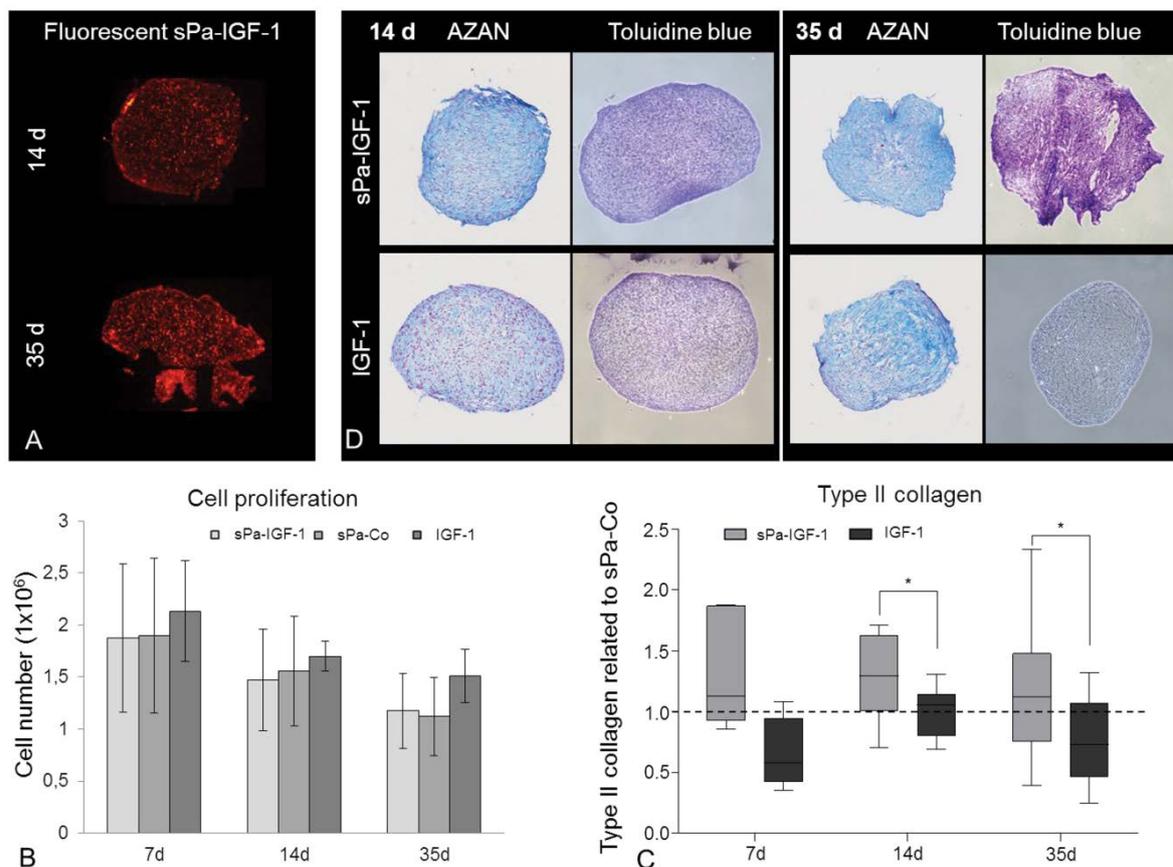


Figure 3: Effect of IGF-1-coupled particles on redifferentiation of human chondrocytes. Human chondrocytes were cultured in spheroid pellets enriched with I) IGF-1-coupled particles (sPa-IGF-1, 12.5 µg particle/mL corresponding to 50 ng/mL IGF-1) or II) NH₂-functionalized control particles (sPa-Co, 12.5 µg particle/mL) or III) IGF-1 (50 ng/mL) was continuously added to pellets. **A)** Fluorescence images of deparaffinised pellet sections showed the distribution of red fluorescent silica particles conjugated with IGF-1 after 14 and 35 days of cultivation (magnification 100x, n=4). **B)** The influence of IGF-1-coupled particles on cell proliferation was examined by cell number calculation at different time points (7, 14 and 35 days) (n=6). **C)** Type II collagen content in medium supernatant was quantified and related to respective number of cells. Data are normalised to sPa-Co and presented in box plots, whereby boxes identify interquartile ranges, horizontal lines within the boxes indicate the median, and whiskers denote minimum and maximum values (n=6). **D)** Representative images of histological staining of pellets performed at day 14 and 35 are shown. Pellets were stained using Heidenhain's AZAN trichrome which marks collagen deposition (blue) and toluidine blue which indicates proteoglycan deposition in purple (magnification 100x, n=6).

1 (p=0.06) (Figure 3C). After 14 days, we could detect a significantly increased collagen type II production (p=0.031) for pellets cultured with sPa-IGF-1, compared to cells treated with soluble IGF-1. Even after 35 days the same development was observed. The enhanced collagen type II synthesis demonstrated a stimulating impact on the redifferentiation of IGF-1-coupled sPa treated chondrocytes. Histological staining using AZAN could complete the results of collagen quantification (Figure 3D). After 14 days, pellets enriched with IGF-1-coupled sPa revealed stronger blue staining, indicating higher collagen deposition compared to pellets cultured with soluble IGF-1. By means of toluidine blue staining the accumulation of proteoglycans was detected after 14 days in both pellets enriched with IGF-1 coupled sPa and those cultured with soluble IGF-1 (Figure 3D left panels). But only pellets augmented with IGF-1-coupled particles were able to maintain the proteoglycan synthesis during cultivation up to 35 days (Figure 3D right panels).

Human BM-MSCs cultured in pellets enriched with IGF-1-coupled particles

During BM-MSC pellet formation, IGF-1-coupled particles were

applied to induce chondrogenic differentiation, as it has already been done for the human chondrocytes. Next to the addition of sPa-IGF-1, NH₂-functionalized control particles were added during pellet formation or soluble IGF-1 was applied continuously over the whole cultivation period. The red fluorescent sPa was distributed well within the pellet as has been observed for the chondrocytes (Figure 4A). Histological investigation showed that although the initial formation of BM-MSC pellets was not affected by sPa-IGF-1 application, the maintenance of the solid pellet shape up to 35 days was not possible (Figure 4B). After 35 days of cultivation, the pellet's original shape was marginally preserved, impairing histological analysis (data not shown). Additionally, both AZAN and toluidine blue could perform only a poor matrix staining, indicating low deposition of collagen and proteoglycans.

Human BM-MSCs cultured on collagen-based scaffolds enriched with IGF-1-coupled particles

Besides the pellet cultivation, BM-MSCs were seeded on collagen-based scaffolds and chondrogenically stimulated by the addition of the growth factor IGF-1 as I) soluble IGF-1 or II) coupled to particles (sPa-

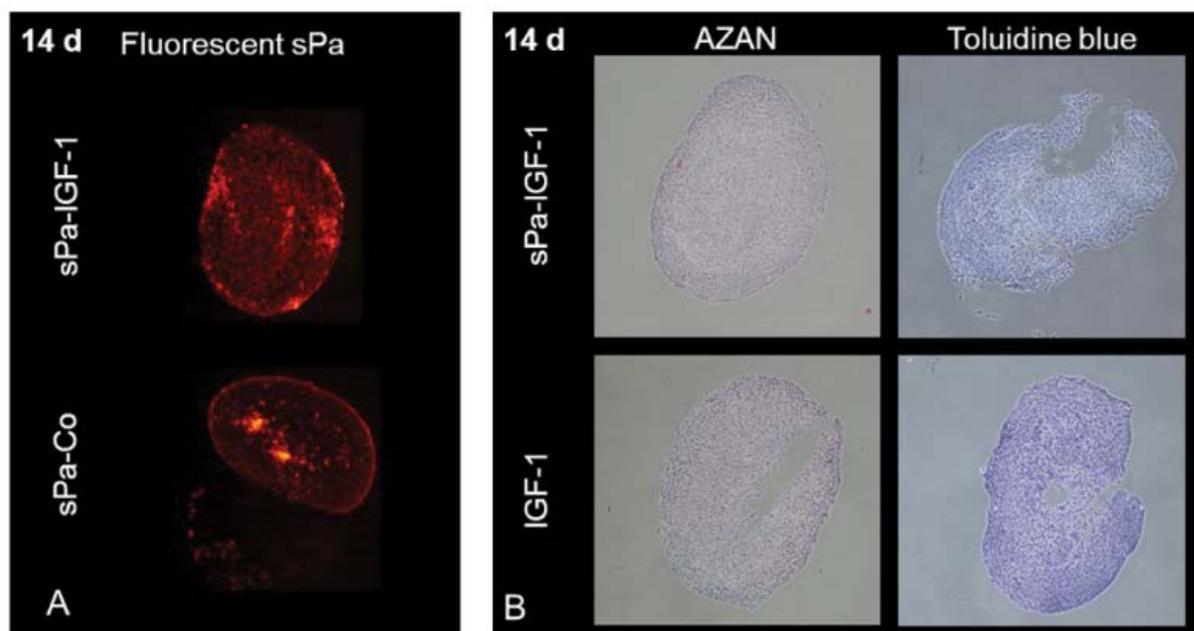


Figure 4: Effect of IGF-1-coupled particles on chondrogenic differentiation of human BM-MSCs. Human mesenchymal stem cells derived from the bone marrow (BM-MSCs) were cultured in spheroid pellets enriched with I) IGF-1-coupled particles (sPa-IGF-1, 12.5 μg particle/mL corresponding to 50 ng/mL IGF-1) or II) NH_2 -functionalized control particles (sPa-Co, 12.5 μg particle/mL) or III) IGF-1 (50 ng/mL) was continuously added to pellets. **A)** Fluorescence images of deparaffinised pellet sections showed the distribution of red fluorescent silica particles conjugated with IGF-1 and NH_2 , respectively, after 14 days of cultivation (magnification 100x, n=3). **B)** Representative images of histological staining of pellets performed at day 14. Pellets were stained using Heidenhain's AZAN trichrome, which marked collagen deposition blue and toluidine blue which indicated proteoglycan deposition in purple (magnification 100x, n=3).

IGF-1). Additionally, control particles (sPa-Co) were used to exclude any side effects triggered by the fluorescent silica particles. The particles sPa-IGF-1 and sPa-Co were added only at the time of cell seeding and unattached particles were removed during first medium exchange after 72 h. Using a confocal laser scanning microscopy, the distribution of particles was investigated after 21 days of cultivation. Red fluorescent particles could be detected up to a depth of 55 μm , which corresponded to 10% of the thickness (Figure 5A). After 14 days, the metabolic activity of cells cultured on collagen-based scaffolds supplemented with sPa-IGF-1 was slightly decreased compared to cells cultured with a single application of soluble IGF-1 and cultured with control particles, respectively. However, there were no significant differences between the treatment groups (data not shown). Furthermore, we found a strong increase of viable, green fluorescent cells from 7 up to 21 days in all three treatment groups, indicating cell proliferation (Figure 5B). There was no negative influence observed of the silica particles on cell viability. Investigations of collagen type II in the medium supernatant revealed no different expression rate between all treatment groups (data not shown). Because of the difficult histological processing of the used collagen-based scaffolds, the detection of proteoglycans (GAG) was realised by quantification of GAG deposition within the scaffold. Although the initial GAG synthesis was at the same level for all treatment groups, BM-MSCs cultured with IGF-1-coupled sPa displayed enhanced GAG synthesis after 7 days. After 14 days, GAG production was found to be significantly higher ($p=0.029$) compared to single application of soluble IGF-1, indicating enhanced chondrogenic differentiation (Figure 5C).

Discussion

Cell-based investigations for supporting cartilage regeneration

involve *in vitro* cultivation of chondrocytes and mesenchymal stem cells to increase the number of the cells required for subsequent cell implantation into the defect side. During *in vitro* expansion, human chondrocytes undergo rapid dedifferentiation enabling cell proliferation, which is why prior to *in vivo* transplantation chondrocytes have to be redifferentiated again. The induction of redifferentiation of chondrocytes and mesenchymal stem cells is initiated by the administration of growth factors and improved through three-dimensional cultivation [5,6].

In the present study, the influence of IGF-1 on both human chondrocytes and bone marrow-derived mesenchymal stem cells (BM-MSCs) was investigated. Cells were cultured in three-dimensional cell culture systems in terms of spheroid pellet culture. Additionally, BM-MSCs were cultured onto a collagen-based scaffold, as has been done with human primary chondrocytes in our previous study [21]. The growth factor was added either in the form of soluble IGF-1 or IGF-1-coupled particles.

Regarding pellet cultivation, soluble IGF-1 as well as IGF-1-coupled particles were added to the cell suspension and thus were present while cells formed cell aggregate. It was hypothesised that IGF-1-coupled particles accumulate within the cell pellet and form a construct of cells and growth factors similar to the pre-cartilage environment, which is also characterized by high-density condensation of undifferentiated cells and a mix of growth factors [19]. The use of fluorescent silica particles enabled tracing of particles within pellet. Pellet sections showed that the particles were integrated within the cell pellet without interfering with formation of cell aggregate. In addition, growth factor coupled particles were distributed homogeneously within the pellet, indicating consistent delivery of IGF-1. Moreover, investigations regarding cell

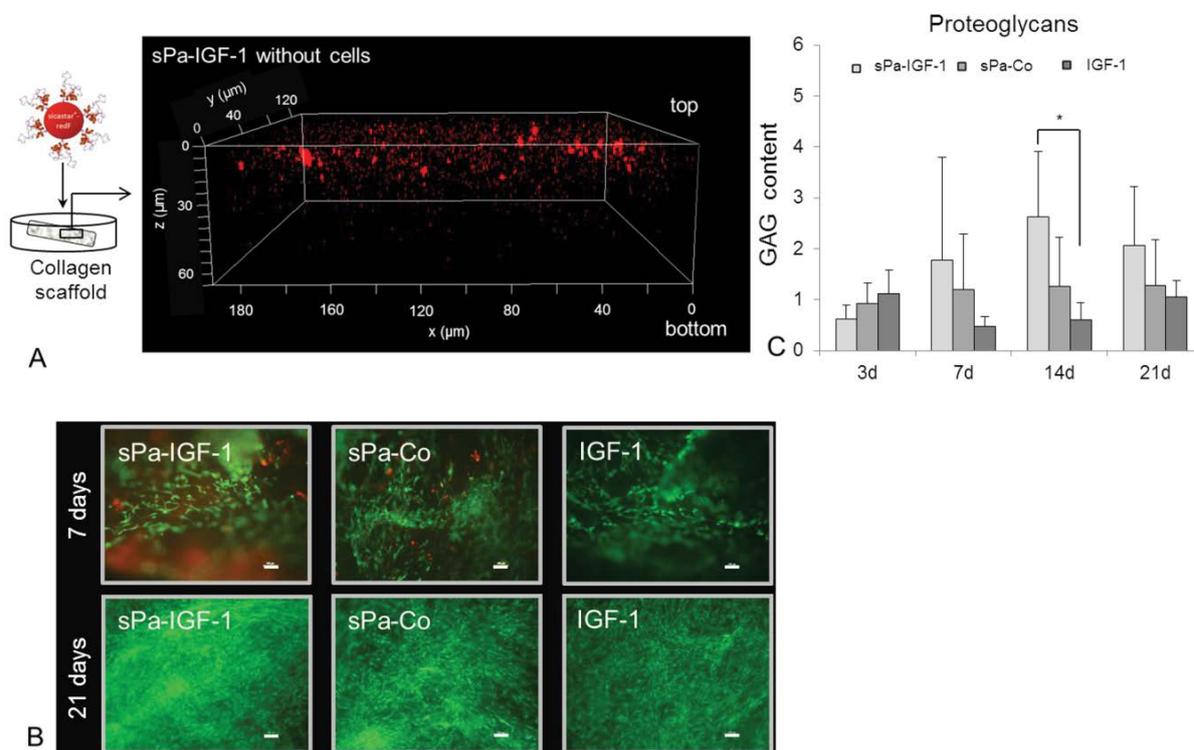


Figure 5: Cultivation of BM-MSCs onto collagen-based scaffold and induction of chondrogenic differentiation using IGF-1-coupled particles. Human BM-MSCs were seeded onto collagen-based scaffold with a single addition of either I) IGF-1-coupled particles (sPa-IGF-1, 12.5 μg particle/mL corresponding to 50 ng/mL IGF-1), II) NH_2 -functionalized control particles (sPa-Co, 12.5 μg particle/mL) or III) IGF-1 (50 ng/mL). All three cell/scaffold constructs were cultured up to 21 days in serum-containing medium supplemented with ascorbic acid only. **A)** Representative image of a segment of the collagen-based scaffold enriched with red fluorescent sPa-IGF-1 after 21 days of cultivation recorded by confocal laser scanning microscopy. **B)** Representative pictures of live staining after 7 and 21 days indicating a high number of green fluorescent, viable cells, and fluorescent particles (magnification 100x). **C)** Synthesis of proteoglycans (GAG) by BM-MSCs cultured on collagen-based scaffolds enriched with sPa-IGF-1, sPa-Co or soluble IGF-1. GAG content was normalised to metabolic cell activity to have an impression of the proteoglycan synthesis of each group which is independent of the cell number and metabolic activity (n=5).

proliferation give no hint of cytotoxic effects of the silica particles *in vitro* [25]. The slight decrease in cell numbers after initial proliferation emphasised the differentiation process in which cells focus rather on matrix synthesis [26].

The IGF-1-coupled particles were administrated only during pellet formation, whereas soluble IGF-1 was applied initially, and during further cultivation every two to three days. Using pellet cultivation chondrocytes were cultured in a three-dimensional environment allowing cell-cell-contact encouraging chondrogenic differentiation of the cells even without the addition of growth factors as it was observed for the cells of the particle control. The collagen type II synthesis was slightly, non-significantly increased compared to the use of soluble IGF-1. IGF-1 is a potent and well known inducer of chondrocytes redifferentiation [27]. However, our results show that the application of soluble IGF-1 has not promoted chondrogenic differentiation of the cells very well. Though using IGF-1-coupled particles for chondrocytes pellet cultivation, cells displayed significantly increased collagen type II expression after 14 and 35 days, compared to cells cultured with continuously added IGF-1. Histological staining also confirmed the increased chondrogenic differentiation potential of IGF-1-coupled particles. This indicated that bounding of IGF-1 on particles seems to result in prolonged stability and beneficial availability within pellets, reinforcing the chondrogenic differentiation potential of IGF-1, which indeed has a short lifetime [27].

Cultivation of chondrocytes in the form of spheroid pellets is widely used in regard to autologous chondrocyte implantation (ACI) to promote cartilage healing *in vivo* and to study the chondrocyte differentiation during formation of hyaline-like cartilage [19]. However, the limitations of this culture system are associated with the dense structure. Cells aggregate during pellet formation, develop cell-cell contacts and deposit synthesised extracellular matrix proteins, resulting in increased density of the pellet. Hence, the nutrient and growth factor delivery is restricted, and could cause apoptosis of cells within the pellet centre. The reinforcement of growth factor availability in the centre of the cell pellet by immobilising them on particles could solve this problem. Our results indicated that the conjugation of IGF-1 to particles is a tool for targeted application of growth factors and enhancing their supply within a dense cell aggregate [28].

Apart from chondrocytes, BM-MSCs were commonly used for supporting regeneration processes within the cartilage tissue after lesion [1,3,29]. To investigate the influence of IGF-1 on BM-MSCs, we performed three-dimensional cultivation of BM-MSCs in the form of a spheroid pellet culture system and also by using a collagen-based scaffold. Regarding pellet cultivation, the growth factor IGF-1 was added during pellet formation either coupled to particles or as soluble IGF-1, as has been done for chondrocytes before [21]. Our results showed a homogenous distribution of particles within the cell pellet, thus indicating a consistent dispersion of the growth factor IGF-1. Despite

that, our histological investigations demonstrated less proteoglycan and collagen deposition accompanied by a failed maintenance of the pellet stability over 14 days for all treatment groups. It is known that IGF-1 has anabolic effects on BM-MSCs and in combination with several growth factors like TGF- β 1-3 the applied concentration of IGF-1 (50 ng/mL) is proper for inducing adequate chondrogenic differentiation of BM-MSCs using pellet cultivation [30,31]. We found that the cultivation of BM-MSCs in the form of pellets with IGF-1-coupled particles, matching a final IGF-1 concentration of 50 ng/mL, was not suitable for chondrogenic stimulation. Considering that we only add the growth factor IGF-1 as a differentiation inducer, it might be possible that the concentration of IGF-1 and IGF-1-coupled particles has to be increased for initiation of the chondrogenic differentiation in BM-MSCs.

During matrix-associated autologous chondrocyte implantation (MACI), cells are transferred onto a scaffold imitating the cartilage environment [32]. Instead of chondrocytes, here we used BM-MSCs and added IGF-1-coupled particles at the time of cell seeding. We used a scaffold mostly made of type I collagen and containing hyaluronic acid, which is commonly used in tissue regeneration. In comparison to the scaffold-free pellet cultivation, the advantage is that the included collagen consists of functional groups that facilitate interactions with other molecules and cells. Additionally, the combination with hyaluronic acid supports the chondrogenesis of MSCs and the creation of hyaline-like cartilage even without the addition of growth factors [33,34]. We hypothesised that the IGF-1-coupled particles are incorporated into the scaffold and establish an environment mimicking hyaline cartilage, consisting of a collagen network enriched with IGF-1 for an enhanced induction of chondrogenic differentiation. Even after 21 days of cultivation, with a change of medium three times a week, red fluorescent particles were traceable within the scaffold, confirming the incorporation of IGF-1-coupled particles within the collagen-based scaffold. Due to small particle size, the IGF-1-coupled particles were able to infiltrate the porous collagen-based scaffold. For improving particle distribution and infiltration into deeper scaffold zones in further studies, particle addition could be performed under gentle rotation prior to cell seeding. No cytotoxic effect was observed using a collagen-based scaffold and silica particles investigated by WST-1 test quantifying metabolic activity (data not shown) and staining of living cells demonstrated a high number of viable BM-MSCs. These results agree with our previous study using human chondrocytes, where no adverse effects of IGF-1-coupled silica particles and collagen-based scaffold were detected either [13,21]. In addition, using IGF-1-coupled particles to induce chondrogenic differentiation of BM-MSCs, glycosaminoglycan synthesis was significantly enhanced, compared to BM-MSCs cultured with a single application of soluble IGF-1. The growth factor IGF-1 is known to increase the proliferative capacity of MSCs and also to enhance the synthesis of extracellular matrix proteins [7,15]. The use of particles as a carrier and delivery system seems to improve prolonged availability of IGF-1 within the scaffold, resulting in superior proteoglycan synthesis compared to single application of soluble IGF-1. However, the effect of the chondrogenic differentiation has to be investigated in further studies, because despite the increased glycosaminoglycan production, the staining of living cells displayed elongated fibroblastic morphology of BM-MSCs and not round chondrocyte-like phenotype. This problem could be overcome by the use of higher concentrations of IGF-1 or a combination of different growth factors conjugated to particles as well. Various factors, including bone morphogenetic proteins (BMP-2, -4 and -6) and TGF- β may be used in addition to IGF-1 to amplify chondrogenesis [35,36].

Particles function as a carrier system and support the homogenous

incorporation of IGF-1 in a collagen-based scaffold, and therefore could be a comprehensive tool to enhance chondrogenic differentiation of human chondrocytes and BM-MSCs. Even if the transplantation of these cells with or without a scaffold as part of ACI and MACI, respectively, already results in good cartilage repair, the addition of growth factors could increase the quality of the regenerating cartilage tissue, because the supply of growth factors and nutrients is restricted in cartilage due to limited vascularisation. Other growth factors could be conjugated to particles like TGF- β 1 or 3 and incorporated in the scaffolds or directly applied in pellet cultures for reinforcing chondrogenic differentiation. In the present study, fluorescent silica particles were used, but regarding clinical application the use of polysaccharide based particles are conceivable, because possible cytotoxic effects during degradation are more improbable.

Conclusion

Silica particles conjugated with IGF-1 are suitable to integrate this growth factor into cell aggregates and collagen-based scaffolds. This enables a direct chondroinductive effect on the cells. The homogenous distribution and immobilisation of the IGF-1 in the scaffold and in spheroid pellets represents an alternative therapeutic approach for an improved treatment of articular cartilage lesions.

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References

1. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, et al. (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* 331: 889-895.
2. Bernhard JC, Vunjak-Novakovic G (2016) Should we use cells, biomaterials or tissue engineering for cartilage regeneration? *Stem Cell Res Ther* 7: 56.
3. Cucchiari M, Madry H, Guilak F, Saris DB, Stoddart MJ, et al. (2014) A vision on the future of articular cartilage repair. *Eur Cell Mater* 27: 12-16.
4. Nukavarapu SP, Dorcenus DL (2013) Osteochondral tissue engineering: Current strategies and challenges. *Biotechnol Adv* 31: 706-721.
5. Steinert AF, Ghivizzani SC, Rethwilm A (2007) Major biological obstacles for persistent cell-based regeneration of articular cartilage. *Arthritis Res Ther* 9: 213.
6. Caldwell KL, Wang J (2015) Cell-based articular cartilage repair: The link between development and regeneration. *Osteoarthritis Cartilage* 23: 351-362.
7. Bornes TD, Adesida AB, Jomha NM (2014) Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: A comprehensive review. *Arthritis Res Ther* 16: 432.
8. Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH et al. (2010) Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. *Am J Sports Med* 38: 1110-1116.
9. Ko CK, Lee EY, Jang JD, Kim SJ, Suh DS, et al. (2012) Cartilage regeneration using a fibrin and autologous cultured chondrocytes mixture in a canine model. *J Tissue Sci Eng* 03: 1-3.
10. Umlauf D, Frank S, Pap T, Bertrand J (2010) Cartilage biology, pathology and repair. *Cell Mol Life Sci* 67: 4197-4211.
11. Lam J, Lu S, Kasper FK, Mikos AG (2015) Strategies for controlled delivery of biologics for cartilage repair. *Adv Drug Deliv Rev* 84: 123-134.
12. Kwon H, Paschos NK, Hu JC, Athanasiou K (2016) Articular cartilage tissue engineering: The role of signaling molecules. *Cell Mol Life Sci* 73: 1173-1194.
13. Vemuri MC, Chase LG, Rao MS (2011) Mesenchymal stem cell assays and applications. *Methods Mol Biol* 698: 3-8.

14. Chen FH, Rousche KT, Tuan RS (2006) Technology insight: Adult stem cells in cartilage regeneration and tissue engineering. *Nat Clin Pract Rheumatol* 2: 373-382.
15. Danišović L, Varga I, Polák S (2012) Growth factors and chondrogenic differentiation of mesenchymal stem cells. *Tissue cell* 44: 69-73.
16. Madry H, Kaul G, Zurakowski D, Vunjak-Novakovic G, Cucchiari M (2013) Cartilage constructs engineered from chondrocytes overexpressing IGF-I improve the repair of osteochondral defects in a rabbit model. *Eur Cell Mater* 25: 229-247.
17. Vinatier C, Bouffi C, Merceron C, Gordeladze J, Brondello JM, et al. (2009) Cartilage tissue engineering: towards a biomaterial-assisted mesenchymal stem cell therapy. *Curr Stem Cell Res Ther* 4: 318-329.
18. Grigore ME (2017) Biomaterials for cartilage tissue engineering. *J Tissue Sci Eng* 8: 1-5.
19. Zhang Z, McCaffery JM, Spencer RGS, Francomano C, et al. (2007) Hyaline cartilage engineered by chondrocytes in pellet culture: Histological, immunohistochemical and ultrastructural analysis in comparison with cartilage explants. *J Anat* 205: 229-237.
20. Kim J, Lin B, Kim S, Choi B, Evseenko D, et al. (2015) TGF- β 1 conjugated chitosan collagen hydrogels induce chondrogenic differentiation of human synovium-derived stem cells. *J Biol Eng* 9: 1.
21. Pasold J, Zander K, Heskamp B, Grüttner C, Lüthen F, et al. (2015) Positive impact of IGF-1-coupled nanoparticles on the differentiation potential of human chondrocytes cultured on collagen scaffolds. *10*: 113-1143.
22. Jonitz A, Lochner K, Peters K, Salamon A, Pasold J, et al. (2011) Differentiation capacity of human chondrocytes embedded in alginate matrix. *Connect Tissue Res* 52: 503-511.
23. Mulisch M, Welsch U (2010) *Romeis - mikroskopische Technik*. Spektrum Akademischer Verlag, Heidelberg.
24. Heinrich G (1957) *Fibel der histologischen Technik*, (2nd edn), Gustav Fischer Verlag, Jena.
25. Ahamed M (2013) Silica nanoparticles-induced cytotoxicity, oxidative stress and apoptosis in cultured A431 and A549 cells. *Hum Exp Toxicol* 32: 186-195.
26. Freshney RI (2005) Culture of animal cells. *Differentiation*.
27. Haleem AM, Chu CR (2010) Advances in tissue engineering techniques for articular cartilage repair. *Operative Techniques in Orthopaedics* 20: 76-89.
28. Zhang L, Su P, Xu C, Yang J, Yu W, et al. (2010) Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Biotechnol Lett* 32: 1339-1346.
29. Knutsen G, Engebretsen L, Ludvigsen TC, Drogset JO, Grøntvedt T, et al. (2004) Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. *J Bone Joint Surg Am* 86-86A: 455-464.
30. Bai XH, Wang DW, Luan Y, Yu XP, Liu CJ, et al. (2009) Regulation of chondrocyte differentiation by ADAMTS-12 metalloproteinase depends on its enzymatic activity. *Cell Mol Life Sci* 66: 667-680.
31. Li S, Sengers BG, Oreffo ROC, Tare RS (2015) Chondrogenic potential of human articular chondrocytes and skeletal stem cells: A comparative study. *J Biomater Appl* 29: 824-836.
32. Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noël D (2009) Cartilage engineering: A crucial combination of cells, biomaterials and biofactors. *Trends Biotechnol* 27: 307-314.
33. Collins MN, Birkinshaw C (2013) Hyaluronic acid based scaffolds for tissue engineering—a review. *Carbohydr Polym* 92: 1262-1279.
34. Matsiko A, Levingstone TJ, O'Brien FJ, Gleeson JP (2012) Addition of hyaluronic acid improves cellular infiltration and promotes early-stage chondrogenesis in a collagen-based scaffold for cartilage tissue engineering. *J Mech Behav Biomed Mater* 11: 41-52.
35. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ (2005) Comparison of effect of BMP-2, -4 and -6 on *in vitro* cartilage formation of human adult stem cells from bone marrow stroma. *Cell Tissue Res* 320: 269-276.
36. Matsuda C, Takagi M, Hattori T, Wakitani S, Yoshida T (2005) Differentiation of human bone marrow mesenchymal stem cells to chondrocytes for construction of three-dimensional cartilage tissue. *Cytotechnology* 47: 11-17.