Chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells in a three-dimensional environment

Salonius, Eve

2020-04

Salonius , E , Kontturi , L , Laitinen , A , Haaparanta , A-M , Korhonen , M , Nystedt , J , Kiviranta , I & Muhonen , V 2020 , ' Chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells in a three-dimensional environment ' , Journal of Cellular Physiology , vol. 235 , no. 4 , pp. 3497-3507 . https://doi.org/10.1002/jcp.29238

http://hdl.handle.net/10138/324359
https://doi.org/10.1002/jcp.29238

acceptedVersion

Chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells in a three-dimensional environment

Running title: Chondrogenic differentiation MSCs in 3D

Eve Salonius[1*], Leena Kontturi[2], Anita Laitinen[3], Anne-Marie Haaparanta[4], Matti Korhonen[3], Johanna Nystedt[3], Ilkka Kiviranta[1,5], Virpi Muhonen[1]

1 Department of Orthopaedics and Traumatology, Clinicum, University of Helsinki, Helsinki, Finland

2 Drug Research Program, Division of Pharmaceutical Biosciences, University of Helsinki, Helsinki, Finland

3 Finnish Red Cross Blood Service, Advanced Cell Therapy Centre, Helsinki, Finland

4 Department of Electronics and Communications Engineering, Tampere University of Technology and BioMediTech, Tampere, Finland

5 Department of Orthopaedics and Traumatology, Helsinki University Hospital, Helsinki, Finland

*Address correspondence to: Eve Salonius, University of Helsinki, Orthopaedics and Traumatology, Biomedicum Helsinki, Haartmaninkatu 8, 00290, Helsinki, Finland. Tel. +358-9-471-71919, eve.salonius@helsinki.fi

Abstract

Cell therapy combined with biomaterial scaffolds is used to treat cartilage defects. We hypothesized that chondrogenic differentiation bone marrow-derived mesenchymal stem cells (BM-MSCs) in three-dimensional biomaterial scaffolds would initiate cartilaginous matrix deposition and prepare the construct for cartilage regeneration *in situ*. The chondrogenic capability of human BM-MSCs was first verified in a pellet culture. The BM-MSCs were then either seeded onto a composite scaffold rhCo-PLA combining polylactide and collagen type II (C2) or type III (C3), or commercial collagen type I/III membrane (CG). The BM-MSCs were either cultured in a proliferation medium or chondrogenic culture medium. Adult human chondrocytes (ACs) served as controls. After 3, 14, and 28 days, the constructs were analyzed with qPCR and confocal microscopy and sulfated glycosaminoglycans (GAGs) were measured. The differentiated BM-MSCs entered a hypertrophic state by day 14 of culture. The ACs showed dedifferentiation with no expression of chondrogenic genes and low amount of GAG. The CG membrane induced the highest expression levels of hypertrophic genes. The two different collagen types in composite scaffolds yielded similar results. Regardless of the biomaterial scaffold, culturing BM-MSCs in chondrogenic differentiation medium resulted in chondrocyte hypertrophy. Thus, caution for cell fate is required when designing cell-biomaterial constructs for cartilage regeneration.

Key Words: biomaterial, cartilage, chondrogenesis, MSC, scaffold

1 Introduction

Articular cartilage is a highly specialized connective tissue covering joint surfaces. It provides a nearly frictionless gliding surface and load distribution for pain-free movement. Articular cartilage is well known for its poor repair capacity. More than half of cartilage defects are attributed to sports injuries (Falah, et al. 2010; Aroen et al. 2004) and they are prone to propagate into early-onset osteoarthritis (Carbone & Rodeo 2017). Cartilage repair techniques, such as autologous chondrocyte implantation, in which the patient's own cartilage cells are seeded under a periosteal patch or on a biomaterial membrane, are used in an attempt to relieve symptoms and potentially delay the onset of osteoarthritis (Brittberg et al. 1994; Bhosale 2008).

Cell-augmented cartilage repair techniques traditionally utilize the patient's own chondrocytes (Brittberg et al. 1994). An important limitation of these cartilage repair procedures is that the required cartilage biopsy causes additional morbidity, and the yield of chondrocytes is limited. Mesenchymal stromal cells (MSCs) are known to possess chondrogenic differentiation capacity (Dominici et al. 2006; Johnstone et al. 1998), they can be expanded through multiple passaging without losing their phenotype, and the clinical safety profile of these cells has been satisfactory (Lee et al. 2014; Lalu et al. 2012). MSCs have been successfully used as an alternative cell source in cartilage repair surgery (Wakitani et al. 2011). MSCs can be obtained from various adult tissue sources, but bone marrow has been the most frequently used source (Somoza et al. 2014). MSC collection

64 is less traumatic than chondrocyte harvest, larger cell quantities can be obtained, and the
65 cells can also be greatly expanded *in vitro* (Somoza et al. 2014; Yoo et al. 1998).

66 Cell therapies can benefit from cell delivery devices, such as biomaterial scaffolds that
67 provide the cells and the forming tissue with attachment surface and proper mechanical
68 support (Murphy & Barry 2015). Culturing regenerative cells on biomaterial scaffolds
69 prior to implanting the construct into the cartilage defect may improve the stability of the
70 construct as well as cell viability. However, the optimal conditions for differentiating
71 chondrogenic bone marrow-derived mesenchymal stromal cells (BM-MSCs) in a three-
72 dimensional environment are still poorly understood. Collagen is frequently used in
73 biomaterial scaffolds. As it is one of the main constituents of cartilage extracellular matrix,
74 it provides cells with a natural environment in biomaterial scaffolds. Type II collagen is
75 the most abundant collagen type in articular cartilage and recombinant human type II
76 collagen has been shown to improve cartilage regeneration (Pulkkinen et al. 2013). Type
77 III collagen yields good results when combined with type I collagen (Ebert et al. 2017;
78 Russlies et al. 2002) and it may have a role in cartilage healing (Wu et al. 2010).

79 Due to the mechanically demanding function and highly specialized tissue structure of
80 articular cartilage, biomechanical issues need to be taken into consideration when
81 designing scaffolds for cartilage tissue engineering (Pattappa et al. 2019). Additionally,
82 more understanding on the interactions of chondrogenic cells and biomaterials is needed
83 to enable the design of improved scaffolds. To contribute to this understanding, this study
84 was designed to explore the behavior of BM-MSCs in composite scaffolds, in which
85 poly(L/D)lactide fibers are carded and needle-punched into mesh and combined with
86 recombinant human collagen. This novel scaffold structure forms a biomechanically
87 improved scaffold structure (Gasik et al. 2018).

The purpose of this study was to explore chondrogenic differentiation of human BM-MSCs in three-dimensional biomaterial scaffolds intended for cartilage repair. In addition, we wanted to investigate whether the type of collagen used in the scaffold affects the chondrogenic potential of these cells. We hypothesized that chondrogenic differentiation of BM-MSCs in this biomaterial scaffold would enhance cartilaginous matrix deposition by the cells and thus prepare the cell─scaffold construct for cartilage regeneration *in situ*.

2 Materials and methods

2.1 Ethical considerations

The study protocol was approved by the Ethics Committee of the University of Helsinki (7/13/03/02/2014), and the permission to the study was given by the Department of Orthopaedics and Traumatology of Helsinki University Hospital. A voluntary and informed consent was obtained in written form from all bone marrow donors.

2.2 BM-MSC culture and proliferation

Bone marrow was harvested from three healthy voluntary donors, aged between 20 and 30 years, and the BM-MSCs were expanded as previously described using platelet lysate to replace fetal calf serum (Salmenniemi et al 2017). BM-MSCs were displayed a typical MSC phenotype and osteogenic and adipogenic differentiation capacity (methods, as described in (Laitinen et al. 2016)). For this study, BM-MSCs from passage 1 (p1) were frozen in human serum albumin (HSA, Albunorm 200g/L, Octapharma, Lachen, Switzerland) and 10% DMSO CryoSure, WAK-Chemie Medical GmbH, Germany) and stored in liquid nitrogen in aliquots.

For chondrogenic pellet testing, BM-MSCs were thawed and seeded at 1 000 cells/cm^2 for passage 2 (p2) in a MSC proliferation medium consisting of DMEM-GlutaMAX™-I (Life Technologies, Paisley, Scotland, UK), 10% pooled platelet lysate (Finnish Red Cross Blood Service), 1% PenStrep (Life Technologies), and 40 IU/ml heparin (LEO Pharma, Ballerup, Denmark). After 8 days, BM-MSCs were detached with trypsin (TrypLE Select CTS, Gibco, Invitrogen) and counted in Bürker's chamber after staining with Trypan Blue (Invitrogen).

2.3 Chondrogenic pellet test for BM-MSCs

Chondrogenic potential of BM-MSCs varies between individuals (Skog et al. 2015). Therefore, we tested the chondrogenic differentiation potential of the BM-MSCs from three individual donors in a pellet culture to determine the best BM-MSC batch for this study. The chondrogenic pellet differentiation test was performed by centrifuging 300 000 cells (in p2) at 200g for 5 minutes, as described by Skog and colleagues (Skog et al. 2015). Briefly, the cell pellets were cultured for 14 days in conical polypropylene tubes containing 400 µl of xeno-free chondrogenic differentiation medium comprised of Dulbecco's Modified Eagle Medium supplemented with 1% PenStrep (Life Technologies), 1.5 mg/ml human serum albumin (Sigma Aldrich), 40 µg/ml L-proline (Sigma Aldrich), 25 µg/ml ascorbic acid (Sigma Aldrich), 10 µg/ml insulin (Insuman Rapid, Sanofi Aventis), 8 µg/ml human transferrin (Sigma Aldrich), 5.5 µg/ml linoleic acid (Sigma Aldrich), 40 ng/ml dexamethasone (Oradexon, Shering-Plough), 10 ng/ml sodium selenite (Sigma Aldrich), and 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN, USA). The medium was changed twice a week.

After 14 days, the pellets were fixed with 10% buffered formalin, dehydrated in ascending alcohol series and embedded in paraffin. The blocks were cut into 5 µm thick sections and stained with Safranin-O for visualization of the proteoglycan content, and immunohistochemically for type II collagen, using previously described protocols (Muhonen et al. 2016).

2.4 Chondrocyte harvest and culture

Human articular chondrocytes (AC) were harvested from a male cadaver in collaboration with Department of Forensic Medicine at University of Helsinki. The cartilage harvest was performed within 34 hours after the death of the donor. The knees were opened through a medial parapatellar approach, the patella was laterally dislocated, and cartilage biopsies were harvested from the entire weight-bearing area of the femoral trochlea. A cylindrical cartilage sample with a diameter of 6 mm was taken from the trochlear cartilage in order to assess the histological appearance of the cartilage.

The cartilage biopsies were transported to laboratory in sterile phosphate buffered saline (PBS). The cartilage was minced and digested for 48 hours in chondrocyte proliferation medium consisting of DMEM-F12 supplemented with GlutaMax (Gibco, Invitrogen), 10% fetal bovine serum (HyClone, ThermoScientific), 1% PenStrep (Life Technologies), 1% Fungizone (Invitrogen), 0.05% sodium-L-ascorbate (Sigma Aldrich). In addition, 80 mg/ml of type II collagenase (Worthington) was added for the digestion. The cells were filtered through a 70 µm nylon mesh (Prinsal, Tuusula, Finland), collected by centrifugation and washed with sterile PBS.

2.5 Scaffolds

153 In this study, we used a recombinant human collagen–polylactide (rhCo-PLA) composite
154 scaffold developed free of animal-derived materials by our team (Muhonen et al. 2016).
155 Briefly, the medical grade polylactide (poly(L/D)lactide 96/4; Corbion Purac, Gorinchem,
156 The Netherlands) was used to manufacture the carded needle punched PLA felt. The PLA
157 felt was gamma irradiated (25 kGy) and cut into 8 mm diameter cylindrical discs. Two
158 types of recombinant human collagen were used in the rhCo-PLAs to produce two
159 different rhCo-PLA scaffolds: type II collagen (abbreviated C2; Fibrogen Europe Ltd.,
160 Helsinki, Finland) or type III collagen (abbreviated C3; Fibrogen Europe Ltd.). The PLA
161 felt was immersed into recombinant human collagen (type II or type III) solution and
162 freeze-dried into collagen–PLA composites. The scaffolds were cross-linked with 95%
163 ethanol solution with 14 mM EDC (N-[3-dimethylaminopropyl]-N´-ethylcarbodiimide
164 hydrochloride, Sigma-Aldrich, Helsinki, Finland) and 6 mM NHS (N-
165 Hydroxysuccinimide, Sigma-Aldrich, Helsinki, Finland), washed and freeze-dried again.
166 The scaffolds were manufactured and packed in a laminar flow chamber to prevent any
167 contamination.

168 Commercial Chondro-Gide® membrane (Geistlich Pharma AG, Wolhusen, Switzerland)
169 produced from porcine-derived type I/III collagen served as a control (abbreviated CG).
170 The membrane was cut into discs with a diameter of 8 mm.

171 To confirm sterility, all scaffolds were treated twice with 70% alcohol for 15 minutes and
172 subsequently washed three times in sterile PBS prior to use.

173 *2.6 Cell cultures in scaffolds*

174 The pellets in the pilot test stained similarly for Safranin-O and type II collagen. Based
175 on the size of the pellet, donor 1 was chosen for scaffold cultures. Passage 1 BM-MSCs

were then thawed and cultured as passage 2 cells in MSC proliferation medium. The control samples (day 0) were collected at this point.

Chondrocytes harvested from a cadaver donor were expanded in the above described chondrocyte proliferation medium in monolayer culture. The medium was changed twice a week. The chondrocytes were cultured until passage 3, and control samples (day 0) were collected.

All three types of scaffolds were placed on a 24-well plate and a suspension of 500 000 cells in 40 µl of medium was pipetted onto each scaffold. The cells were allowed to attach to the scaffolds for 1-2 minutes, after which 0.4 ml of medium was pipetted onto each well. BM-MSCs were either cultured in MSC proliferation medium (MSC-P) or in chondrogenic differentiation medium (MSC-C). The human adult chondrocytes (AC) were cultured in their proliferation medium. The study design is outlined in Figure 1.

2.7 Glycosaminoglycans

The amount of sulfated glycosaminoglycans (sGAGs) in each cell–scaffold-construct was quantified using Blyscan assay (Biocolor, UK) and the DNA amounts with Picogreen assay (Molecular Probes, Invitrogen) for normalization of sGAG amounts. The scaffolds containing the cells (MSC-P, MSC-C and AC) were washed twice with PBS, placed dry in Eppendorf tubes and stored immediately at –80°C. The cell-scaffold samples were cut into approximately 4 mm^2 pieces with a scalpel and digested with 1 mg/ml Proteinase K (Sigma-Aldrich) (1 ml of solution for each cell–scaffold sample) overnight at 60°C. After digestion, the samples were centrifuged for 10 min at 10 000g and the supernatants were used for the measurements.

198 *Blyscan assay*. The measurement was performed applying the manufacturer's protocol
199 using bovine tracheal chondroitin 4-sulfate to create the standard curve. Briefly, 100 µl
200 of samples were mixed with 1 ml of Blyscan reagent and incubated with gentle shaking
201 for 30 min. After incubation, samples were centrifuged for 10 min at 8 000 g and
202 supernatants were discarded. Precipitated sGAG pellets were dissolved by the addition of
203 0.5 ml of dissociation reagent and vortexing. 200 µl of samples was transferred to a 96-
204 well plate in duplicates, and the absorbance was measured with a microplate reader at 656
205 nm.

206 *Picogreen assay*. The measurement was performed following the manufacturer's protocol
207 using Lambda DNA as a standard. The samples were diluted 1:10 and 50 µl of the
208 dilutions were transferred to a 96-well plate in duplicates. 50 µl of Picogreen working
209 solution was added to each sample and incubated for 2–5 min at room temperature. The
210 fluorescence was measured with a microplate reader at 480/520 nm.

211 *2.8 RNA extraction and cDNA synthesis*

212 Eight biological parallel samples per study group were analyzed. In order to perform the
213 RNA extraction, the scaffolds were disrupted using Tissue Lyser II (Qiagen, Hilden,
214 Germany) with carbide beads. The samples were homogenized with QIAzol® Lysis
215 Reagent (Qiagen Sciences, Maryland, USA, cat. no. 79306) and the RNA was separated
216 from the organic phase with chloroform (Sigma-Aldrich). Further purification was
217 performed using RNeasy Mini Kit (Qiagen, 74104) following manufacturer's instructions.
218 Total RNA yield and RNA purity were measured with NanoDrop 1000
219 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the samples were

220 stored in –80°C until further processing. RNA samples with 260/280 values <1.6 and
221 260/230 values <0.1 were omitted from the final analyses.

222 Reverse transcription of RNA to single-stranded cDNA was carried out in PCR tubes
223 (Nippon Genetics Europe GmbH, Dueren, Germany) using a commercial kit (High
224 Capacity cDNA Reverse Transcription Kit with RNase Inhibitor; Applied Biosystems,
225 Foster City, CA, USA) according to the manufacturer's instructions. The total RNA used
226 in the reactions was equal in each sample. The reverse transcription program was as
227 follows: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min. The produced cDNA was
228 stored at –20°C.

229 *2.9 Real-time qPCR*

230 The real-time qPCR analysis was performed with iQ^{TM}5 Multicolor Real-Time PCR
231 Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For the reaction, a
232 commercial gene expression assay (TaqMan® Gene Expression Assay, Applied
233 Biosystems) was used according to the manufacturer's protocol. The assay codes are
234 presented in Table 1. The amplification reaction conditions were as follows: hold in 50°C
235 for 2 min, hold in 95°C for 10 min, cycle 95°C for 15 sec and 60°C for 1 min repeated 40
236 times. *GAPDH* was used as the housekeeping gene based on our previous stability tests
237 with mesenchymal stem cells. The gene expression levels were normalized with *GAPDH*
238 and day 0 samples were used as calibrators in the $\Delta\Delta C_T$ method (Livak & Schmittgen
239 2001).

240 *2.10 Confocal microscopy*

241 The cells in the scaffolds were fixed with 10% buffered formalin and stained with Hoechst
242 stain (Invitrogen). The cell nuclei were then imaged with a fluorescent confocal

243 microscope Leica TC SP8 CARS (Leica Microsystems) with 25x HCX IR APO L water
244 objective and Leica Application Suite Advanced Fluorescence software (version
245 3.3.0.10134). For each sample, three fields of view from the representative areas were
246 imaged and a maximum projection image was created with a mean stack size of 320 µm.

247 *2.11 Statistical analyses*

248 To avoid multiple testing, statistical analyses were performed to answer the original
249 research question on how the scaffolds affect cell behavior and different scaffolds were
250 compared to each other within the cell types. Differences between scaffolds were
251 determined with permutation type analysis of variance followed by pairwise multiple
252 comparisons with the Bonferroni procedure. *P* values ≤ 0.05 were considered statistically
253 significant. All the data are presented as mean ± standard error (SE).

254 **3 Results**

255 *3.1 Cell yields and cell types after chondrogenic differentiation*

256 The MSC counts obtained from eight day culture in p2 were 5.8×10^6 for donor 1, $3.0 \times$
257 10^6 for donor 2, and 0.8×10^6 for donor 3. Pellets of the cells of donor 1 showed good
258 Safranin-O uptake and positive immunohistological staining with type II collagen (Figure
259 2). Based on these results, donor 1 MSCs were chosen for the study. The chondrocyte
260 yield from the cadaver trochlear cartilage was 14.4×10^6. The histology of the cadaver
261 cartilage was healthy mature hyaline cartilage (Supplementary Figure S1).

262 *3.2 RNA and DNA contents*

The amounts of RNA and DNA were the highest in the MSC-C samples and lowest in the chondrocyte samples ($p<0.0001$) (Figure 3). On days 3 and 28, the RNA amounts did not differ between the scaffolds in any cell group. By contrast, on day 14, the MSC-Ps cultured in the C2 scaffold showed a higher RNA amount than those cultured in the C3 scaffold ($p=0.028$) and ACs on C2 showed higher RNA amount than ACs on CG ($p=0.007$). The DNA amounts in MSC-Cs and MSC-Ps did not change significantly between days 14 and 28 ($p=0.59$ for MSC-Cs and $p=0.07$ for MSC-Ps, respectively).

3.3 Amount of sulfated glycosaminoglycans

The amount of sGAG was analyzed and the differences between the scaffolds within each cell type were evaluated. MSC-Ps showed higher sGAG amount than MSC-Cs and ACs (Figure 4). The MSC-Ps cultured on CG showed higher sGAG than cells cultured on C2 or C3 ($p<0.0001$ for day 14 and $p<0.0001$ for day 28). By contrast, MSC-Cs on CG showed lower sGAG than C2 and C3 ($p=0.005$ for day 14 and $p<0.0001$ for day 28). Otherwise, no differences between the scaffolds were detected. The type of recombinant human collagen used in the rhCo-PLA scaffolds did not affect the results as there were no differences in sGAG content between the two rhCo-PLA scaffolds with any of the studied cell types.

3.4 No upregulation of chondrogenic genes during 3D chondrogenic differentiation

The mean relative gene expression levels of the measurements are shown in Figure 5. The statistical significances of gene expression levels between scaffolds were evaluated within the cell types. None of the studied genes were upregulated in the ACs or MSC-Ps at any time point.

ACAN was upregulated only in MSC-Cs grown on CG membrane on day 28 (3.88±0.8 fold) and the difference to the two rhCo-PLA scaffolds was statistically significant (p<0.0001) (Figure 5a). The indicator gene of chondrogenesis *SOX9* was not upregulated in any of the studied specimens. The chondrogenic gene *COL2A1* was upregulated only in a few MSC-C specimens on day 14 but variation between individual specimens was large (Figure 5b). Otherwise, no expression of *COL2A1* was detected.

The indicators of chondrocyte hypertrophy and osteogenic commitment, *RUNX2* and *COL10A1*, were only upregulated in the MSC-C samples. *RUNX2* was upregulated in the MSC-Cs grown on C3 and CG on day 14 (3.49±0.52 fold and 3.53±0.29 fold, respectively) (Figure 5c). The expression remained high on day 28 and the cells grown on CG showed the highest expression levels (6.55±0.84 fold; p<0.0001). *COL10A1* was greatly upregulated in MSC-Cs seeded on the CG membrane on day 14 (29.64±4.81 fold) and it remained high on day 28 (20.32±5.15 fold) (Figure 5d). A statistically significant difference for MSC-Cs was found between scaffolds C3 and CG on day 14 (p=0.0042).

The fibroblast/synovial marker *MFAP5* was greatly upregulated in the MSC-C samples on day 14 but not at the later time point or in any other samples (Figure 5e). For this gene, there was a statistically significant difference between C2 and CG scaffolds (p=0.011).

3.5 Confocal microscopy showed differences in cell distribution on the scaffolds

All of the CG membranes showed polarity in the cell distribution. One side of the membrane was nearly empty whereas the other side showed an even distribution of cells in the day 3 and day 14 samples. The day 28 samples showed very few cells on both sides of the membrane (Figure 6 a,b).

Both C2 and C3 scaffolds showed a substantial cell amount in all groups at all time points (Figure 6 a,b,c). The cells were relatively evenly distributed throughout the scaffolds with some larger cell clusters in all groups.

4 Discussion

In this study, we investigated the effect of three different biomaterial scaffolds intended for cartilage repair on the chondrogenic differentiation capacity of human BM-MSCs.

Composite scaffold rhCo-PLA has been proven to enhance cartilage repair *in vivo* (Muhoen et al. 2016). In this study, we investigated whether the collagen type used in the rhCo-PLA scaffold has an effect on the chondrogenic differentiation of BM-MSCs. The commercial collagen membrane Chondro-Gide® was chosen as the control material, as it is well established in human articular cartilage repair (Kon et al. 2011).

The results of the DNA and RNA measurements showed that BM-MSCs cultured with chondrogenic medium (MSC-C) exhibited the highest cell count after 14 days in biomaterial scaffolds. The scaffold type did not have an effect on the cell amount in MSC-C. However, the chondrogenic medium maintained cell viability when the cells were seeded on biomaterial scaffold compared to the undifferentiating culture conditions (MSC-P). The fact that the total DNA amount remained the same between 14 to 28 days in culture in both chondrogenic and proliferation media indicates either a steady turnover in cell population or general survival of seeded cells between the time periods. Either way, this indicates that the MSCs or ACs seeded on the investigated biomaterial scaffolds do not proliferate and produce ECM sufficiently to completely fill the scaffold spaces.

328 Surfaces coated with bovine type I collagen have been shown to promote human BM-MSC
329 proliferation (Somaiah et al. 2015). In a study by Amalki and Agrawal (2016) an increased matrix
330 metalloproteinase-1 (MMP-1) level was shown to promote MSC proliferation. Type III collagen
331 is mainly cleaved by MMP-1, and type II collagen by MMP-13. Recombinant human type II
332 collagen hydrogel has shown to transiently increase the expression of MMP-13, MMP-14 and
333 MMP-8 in BM-MSC cultures (Muhonen et al. 2017). To our knowledge, the effect of type II
334 collagen on MSC proliferation has not been determined, but it is possible that the cleavage of type
335 II collagen in the scaffolds is conveyed by MMP-13, and that its presence promotes BM-MSC
336 proliferation more than the type III collagen cleaving MMP-1. Further studies are required to
337 confirm this hypothesis.

338 Cell distribution, proliferation and tissue-specific ECM production is clinically
339 significant for cartilage restoration. Even though the biomaterial scaffold type did not
340 have an effect on cell number, it did have a clear effect on cell distribution within the
341 scaffold. As seen in confocal microscopy, the cells residing on the CG scaffold showed a
342 more polarized distribution, in which the cells were located on one side of the membrane,
343 than cells seeded onto the rhCo-PLA scaffolds. The cells residing in the rhCo-PLA
344 scaffolds were more evenly distributed throughout the scaffold.

345 Primary chondrocytes seeded on the biomaterial scaffolds showed neither expression of
346 chondrocyte specific genes *SOX9* or *COL2A1*, nor the synovial/fibroblast marker *MFAP5*
347 on 3 or 14 days in culture. This is a clear indication of dedifferentiation of these cells. It
348 is likely that the off-routing from chondrocyte phenotype had taken place during the
349 monolayer culture, before cell seeding into the scaffolds (Benya et al. 1978). Based on
350 our results, we can conclude that seeding monolayer expanded passage 3 primary

chondrocytes on a biomaterial scaffold and culturing these cell–scaffold constructs *in vitro*, is not sufficient for re-routing these cells to the chondrocyte phenotype.

Monolayer expanded passage 3 primary chondrocytes are used in a clinical setting in matrix-induced autologous chondrocyte implantation (MACI®) cartilage repair therapy that has been reported with good outcomes and is approved by the US Food and Drug Administration (FDA) (US Food & Drug Administration 2016; Corbett et al. 2017). However, the fate of the scaffold-laden cells and their role in cartilage regeneration remains to be elucidated. Interestingly, our results demonstrate that the biomaterial scaffold, *i.e.*, the cell attachment surface, does not have a significant effect on overall chondrocyte survival or gene expression of the studied marker genes.

The chondrogenic pellet test verified that the cells used in the studies had the capacity to differentiate into chondrocytes. In previous studies, undifferentiated MSCs in hydrogel constructs have resulted in higher glycosaminoglycan content than chondrogenically predifferentiated MSCs both *in vitro* (Grayson et al. 2010) and *in vivo* (Dashtdar et al. 2011) although opposite *in vitro* results have also been presented (Tay et al. 2013; Li et al. 2014). Contrary to our expectations, undifferentiated MSCs (MSC-Ps) exhibited the highest amount of sulfated GAGs. This finding might be explained by the substantial expression of glycosylated adhesion proteins that is typical for MSCs (Nystedt et al. 2010; Silva et al. 2003), providing a possible explanation to the high sGAG amount in the MSC-Ps. On the other hand, the low amount of sGAG in the MSC-Cs might be due to the prolonged *in vitro* differentiation leading to diminished chondrogenic potential of the MSC-Cs (Li et al. 2014; Huang et al. 2004), or the delayed GAG deposition of MSC-Cs caused by the scaffold collagen (Muhonen et al. 2017).

Based on our qPCR results, the chondrogenically differentiated MSCs (MSC-Cs) seemed to enter endochondral ossification regardless of the scaffold type. Chondrogenic predifferentiation of MSCs in poly(ε-caprolactone) scaffolds prior to subcutaneous implantation in nude mice was recently shown to diminish GAG content and increase mineralization of the constructs (Larson 2019). In our study, the osteoblast differentiation markers *RUNX2* and *COL10A1* were upregulated on day 14, indicating that the hypertrophic pathway was determined at an early phase of the culture.

In a study on human BM-MSCs by Herlofsen and colleagues, *RUNX2* remained stable on days 0, 7, 14 and 21, whereas *type X collagen* increased on day 7 of *in vitro* culture and remained high (Herlofsen et al. 2011). Moreover, our results indicate that the collagen type in the scaffold does not play a significant role in the process, since there were no statistically significant differences in the expression of the markers of terminal differentiation between the cells seeded on different rhCo-PLA scaffolds. This supports the hypothesis that BM-MSCs might have a predefined fate of endochondral ossification (Somoza et al. 2014; Vinardell et al. 2012; Xu et al 2017). In addition, even though TGF-β is one of the most important factors in chondrogenesis, it later inhibits type II collagen and promotes osteogenic differentiation in chondrogenic MSCs (Nazirkar et al. 2014). Therefore, the bone marrow origin of these cells, together with the 28-day exposure to TGF-β, might be the reason for the tendency of these cells to become hypertrophic.

Dashtdar and colleagues discussed that culturing rabbit MSCs in alginate gel might have induced chondrogenic differentiation in the undifferentiated stem cells, even without chondrogenic culture medium (Dashtdar et al. 2011). In gel environment, the cells are surrounded by matrix and therefore microscopically in 3D environment. By contrast, in the scaffolds investigated in this study, the cells attach to the fiber structures and hence,

may experience a 2D environment. Thus, the lack of cues from surrounding cells and ECM components might have inhibited chondrogenesis of MSCs when seeded on the various biomaterial scaffolds (Chen et al. 2014). Both the rhCo-PLA and CG scaffolds have been shown to promote chondral repair *in vivo* (Brittberg et al. 1994; Muhonen et al. 2016), but this capability does not extend to static *in vitro* conditions.We have seen with scanning electron microscopy that chondrocytes seeded onto the rhCo-PLA scaffolds attach to the collagen network in between the PLA fibers and that this attachment takes place within minutes (unpublished results). The rhCo-PLA scaffold with the cells residing in their individual collagen pouches might hinder cell–cell contacts and cell condensation, which is a crucial part of chondrogenic differentiation *in vitro* (Lee et al. 2014; Yoo et al. 1998; Kim et al. 2016; Hsu & Huang. 2013). This might explain the differences in proteoglycan content of pelleted and scaffold-seeded cells, and between the CG membrane and rhCo-PLA scaffolds. The membranous structure of the CG allows for cell–cell contact and thus, promotes chondrogenesis and osteogenesis (Zhu et al. 2016). Thus, the relative cell number in the C2 and C3 scaffolds was smaller than in the CG scaffold although the initial cell numbers were equal in each scaffold.

CG scaffolds are manufactured with collagen types I and III. Type I collagen is the most abundant collagen type in bone and frequently used in scaffolds developed for bone regeneration. Although we believe that the differences between the rhCo-PLA scaffolds and the CG membrane are mainly due to scaffold architecture, the collagen type might have some effect as well. Although a previous study showed that when meniscal chondrocytes were cultured in type I or II collagen scaffolds, the type of collagen did not affect the results (Tebb et al. 2006), a study investigating BM-MSCs on scaffolds manufactured with type I or type II collagen (Tamaddon et al. 2017) proved the opposite:

Type II collagen promoted Safranin-O staining and type II collagen staining in immunohistology, whereas very little Safranin-O uptake was detected with type I collagen scaffolds. However, both type I and type II collagen scaffolds lead to calcium deposition, possibly indicating chondrocyte hypertrophy. However, the expression of *SOX9* and *RUNX2* was unaffected by the collagen type in the study by Tamaddon and colleagues.

Combining the mechanical structure of PLA and the cell-friendly environment of rhCo, the rhCo-PLA scaffold has shown promising results in articular cartilage repair *in vivo* (Muhonen et al. 2016). Although it has been thought that type II collagen is superior over other collagen types in cartilage repair solutions, this study suggests that the structural architecture of the scaffold is more important than the type of collagen used in the manufacturing of the scaffold.

As the expression levels of the chondrogenic genes were very low in all of the studied groups and the scaffolds were mechanically disrupted, the scaffolds might have interfered with the qPCR results, leading to high variation between individual specimens in the gene expression. Additionally, the limitations of a static cell culture have possibly affected these results. Hypertrophy is a common phenomenon in *in vitro* chondrogenesis of MSCs (Chen et al. 2015), whereas MSCs in articular cartilage defects *in vivo* have not shown upregulation of hypertrophic markers (Frisbie et al. 1999; Giovannini et al. 2010). In joints, the cells are under cyclic mechanical loading that provides the joint with a flux of nutrients and differentiation cues. The rhCo-PLA scaffold has been shown to promote cartilage repair in a porcine model (Muhonen et al. 2016) but static predifferentiation of BM-MSCs in rhCo-PLA or CG *in vitro* does not seem to be beneficial. Further research is needed to evaluate the effect of BM-MSC predifferentiation in these scaffolds *in vivo*.

In conclusion, these studies show that chondrogenic differentiation of human BM-MSCs leads to cell hypertrophy in the rhCo-PLA scaffolds and on the collagen membrane. The type II collagen promotes BM-MSC proliferation but the collagen type used in the composite scaffold rhCo-PLA does not affect MSC differentiation during *in vitro* culture.

Acknowledgements

The authors wish to thank Adjunct professor Philippe Lunetta (Department of Forensic Medicine, University of Helsinki) and Professor Minna Kellomäki (BioMediTech, Tampere University of Technology) for their valuable help in providing materials for the study. Biomedicum Imaging Unit (University of Helsinki) is acknowledged for providing imaging equipment. The authors wish to thank the technical personnel at the FRCBS Advanced Cell Therapy Centre. The authors wish to thank Helsinki University Hospital (HUS) for funding the work [grant TYH2013317].

Availability of data and materials

The datasets used or analyzed (or both) during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ES designed and performed the experiments, analyzed the data and wrote the manuscript. LK performed part of the experiment and wrote the manuscript. AL provided the MSCs, wrote and corrected the manuscript. AMH provided the scaffolds and corrected the manuscript. MK, JN, IK and VM designed the study and participated

in revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval

The study protocol was approved by the Ethics Committee of the University of Helsinki (7/13/03/02/2014), and the permission to the study was given by the Department of Orthopaedics and Traumatology of Helsinki University Hospital. A voluntary and informed consent was obtained in written form from all bone marrow donors.

Competing interests

V. Muhonen and A.-M. Haaparanta are the majority owners of a company (Askel Healthcare Ltd) commercializing a product based on the rhCo-PLA scaffold described herein for veterinary medicine. Other authors have no financial or personal disclosures that would pose potential conflicts of interests.

References

Amalki SG, Agrawal DK. (2016). Effects of matrix metalloproteinases on the fate of mesenchymal stem cells. Stem Cell Res Ther. 7, 129. https://doi.org/10.1186/s13287-016-0393 -1 [doi]

Aroen A, Loken S, Heir S, Alvik E, Ekeland A, Granlund OG et al. (2004) Articular cartilage lesions in 993 consecutive knee arthroscopies. Am J Sports Med 32,211-5. https://doi.org/10.1177/0363546503259345

Benya PD, Padilla SR, Nimni ME. (1978) Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 15,1313-21. 0092-8674(78)90056-9

Bhosale AM. (2008). Articular cartilage: structure, injuries and review of management. Br Med Bull 87,77-95.

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med, 331, 889-95. https://doi.org/10.1056/NEJM199410063311401.

Carbone A, Rodeo S. (2017). Review of current understanding of post-traumatic osteoarthritis resulting from sports injuries. J Orthop Res, 35:397-405. https://doi.org/10.1002/jor.23341

Chen S, Fu P, Cong R, Wu H, Pei M. (2015). Strategies to minimize hypertrophy in cartilage engineering and regeneration. Genes Dis, 2, 76-95. https://doi.org/10.1016/j.gendis.2014.12.003

Corbett MS, Webster A, Hawkins R, Woolacott N. (2017). Innovative regenerative medicines in the EU: a better future in evidence? BMC Med, 15, 49,017-0818-4. https://doi.org/10.1186/s12916-017-0818-4

Dashtdar H, Rothan HA, Tay T, Ahmad RE, Ali R, Tay LX et al. (2011). A preliminary study comparing the use of allogenic chondrogenic pre-differentiated and undifferentiated mesenchymal stem cells for the repair of full thickness articular cartilage defects in rabbits. J Orthop Res ,29, 1336-42. https://doi.org/10.1002/jor.21413

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy ,8 ,315-7. Q2183N8UT042W62H [pii]

Ebert JR, Fallon M, Wood DJ, Janes GC. (2017). A Prospective Clinical and Radiological Evaluation at 5 Years After Arthroscopic Matrix-Induced Autologous Chondrocyte Implantation. Am J Sports Med , 45, 59-69. https://doi.org/10.1177/0363546516663493

Falah M, Nierenberg G, Soudry M, Hayden M, Volpin G. (2010). Treatment of articular cartilage lesions of the knee. Int Orthop, 34,621-30. https://doi.org/10.1007/s00264-010-0959-y

Frisbie DD, Trotter GW, Powers BE, Rodkey WG, Steadman JR, Howard RD et al. (1999). Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. Vet Surg, 28, 242-55. S0161349999000290 [pii]

Gasik M, Zühlke A, Haaparanta AM, Muhonen V, Laine K, Bilotsky Y et al. (2018). The Importance of Controlled Mismatch of Biomechanical Compliances of Implantable Scaffolds and Native Tissue for Articular Cartilage Regeneration. Frontiers in bioengineering and biotechnology, 6, 187. doi:10.3389/fbioe.2018.00187

Giovannini S, Diaz-Romero J, Aigner T, Heini P, Mainil-Varlet P, Nesic D. (2010). Micromass co-culture of human articular chondrocytes and human bone marrow

mesenchymal stem cells to investigate stable neocartilage tissue formation in vitro. Eur Cell Mater, 20, 245-59. vol020a20 [pii]

Grayson WL, Bhumiratana S, Grace Chao PH, Hung CT, Vunjak-Novakovic G. (2010). Spatial regulation of human mesenchymal stem cell differentiation in engineered osteochondral constructs: effects of pre-differentiation, soluble factors and medium perfusion. Osteoarthritis Cartilage, 18,714-23. https://doi.org/10.1016/j.joca.2010.01.008

Herlofsen SR, Kuchler AM, Melvik JE, Brinchmann JE. (2011). Chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in self-gelling alginate discs reveals novel chondrogenic signature gene clusters. Tissue Eng Part A 17,1003-13. https://doi.org/10.1089/ten.TEA.2010.0499

Hsu SH, Huang GS. (2013). Substrate-dependent Wnt signaling in MSC differentiation within biomaterial-derived 3D spheroids. Biomaterials, 34, 4725-38. https://doi.org/10.1016/j.biomaterials.2013.03.031

Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS. (2004). Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. Anat Rec A Discov Mol Cell Evol Biol ,278, 428-36. https://doi.org/10.1002/ar.a.20010

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238,265-72. S0014-4827(97)93858-1 [pii]

Kim IG, Ko J, Lee HR, Do SH, Park K. (2016). Mesenchymal cells condensation-inducible mesh scaffolds for cartilage tissue engineering. Biomaterials, 85l, 18-29. https://doi.org/10.1016/j.biomaterials.2016.01.048 [doi]

Kon E, Filardo G, Condello V, Collarile M, Di Martino A, Zorzi C et al. (2011). Second-generation autologous chondrocyte implantation: results in patients older than 40 years. Am J Sports Med, 39, 1668-75. https://doi.org/10.1177/0363546511404675 [doi]

Laitinen A, Oja S, Kilpinen L, Kaartinen T, Moller J, Laitinen S et al. (2016) A robust and reproducible animal serum-free culture method for clinical-grade bone marrow-derived mesenchymal stromal cells. Cytotechnology, 68, 891-906. https://doi.org/10.1007/s10616-014-9841-x [doi]

Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC et al. (2012). Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS One, 7:e47559. https://doi.org/10.1371/journal.pone.0047559 [doi]

Larson BL, Yu SN, Park H, Estes BT, Moutos FT, Bloomquist CJ, et al. (2019). Chondrogenic, hypertrophic, and osteochondral differentiation of human mesenchymal

stem cells on three-dimensionally woven scaffolds. J Tissue Eng Regen Med https://doi.org/10.1002/term.2899.

Lee JK, Responte DJ, Cissell DD, Hu JC, Nolta JA, Athanasiou KA. (2014). Clinical translation of stem cells: insight for cartilage therapies. Crit Rev Biotechnol, 34, 89-100. https://doi.org/10.3109/07388551.2013.823596 [doi]

Li YY, Cheng HW, Cheung KM, Chan D, Chan BP. (2014). Mesenchymal stem cell-collagen microspheres for articular cartilage repair: cell density and differentiation status. Acta Biomater, 10,1919-29. https://doi.org/10.1016/j.actbio.2014.01.002 [doi]

Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-8. https://doi.org/10.1006/meth.2001.1262 [doi]

Muhonen V, Narcisi R, Nystedt J, Korhonen M, van Osch GJ, Kiviranta I. (2017). Recombinant human type II collagen hydrogel provides a xeno-free 3D micro-environment for chondrogenesis of human bone marrow-derived mesenchymal stromal cells. J Tissue Eng Regen Med, 11, 843-54. https://doi.org/10.1002/term.1983 [doi]

Muhonen V, Salonius E, Haaparanta AM, Jarvinen E, Paatela T, Meller A et al. (2016). Articular cartilage repair with recombinant human type II collagen/polylactide scaffold in a preliminary porcine study. J Orthop Res 34,745-53. https://doi.org/10.1002/jor.23099 [doi]

Murphy M, Barry F. (2015). Cellular chondroplasty: a new technology for joint regeneration. J Knee Surg, 28,45-50. https://doi.org/10.1055/s-0034-1390329 [doi]

Nazirkar G, Singh S, Dole V, Nikam A. (2014). Effortless effort in bone regeneration: a review. J Int Oral Health 6,120-4.

Nystedt J, Anderson H, Hirvonen T, Impola U, Jaatinen T, Heiskanen A et al. (2010). Human CMP-N-acetylneuraminic acid hydroxylase is a novel stem cell marker linked to stem cell-specific mechanisms. Stem Cells 28,258-67. https://doi.org/10.1002/stem.250 [doi]

Pattappa G, Zellner J, Johnstone B, Docheva D, Angele P. (2019) Cells under pressure - the relationship between hydrostatic pressure and mesenchymal stem cell chondrogenesis. Eur Cell Mater, 37, 360-381. https://doi.org10.22203/eCM.v037a22. [doi]

Pulkkinen HJ, Tiitu V, Valonen P, Jurvelin JS, Rieppo L, Toyras J et al. (2013). Repair of osteochondral defects with recombinant human type II collagen gel and autologous chondrocytes in rabbit. Osteoarthritis Cartilage, 21, 481-90. https://doi.org/10.1016/j.joca.2012.12.004 [doi]

Russlies M, Behrens P, Wunsch L, Gille J, Ehlers EM. (2002) A cell-seeded biocomposite for cartilage repair. Ann Anat, 184, 317-23.

Salmenniemi U, Itala-Remes M, Nystedt J, Putkonen M, Niittyvuopio R, Vettenranta K et al. (2017). Good responses but high TRM in adult patients after MSC therapy for GvHD. Bone Marrow Transplant, 52,606-8. https://doi.org/10.1038/bmt.2016.317 [doi]

Silva WA,Jr, Covas DT, Panepucci RA, Proto-Siqueira R, Siufi JL, Zanette DL et al. (2003). The profile of gene expression of human marrow mesenchymal stem cells. Stem Cells 21,661-9. https://doi.org/10.1634/stemcells.21-6-661 [doi]

Skog M, Muhonen V, Nystedt J, Narcisi R, Kontturi LS, Urtti A et al. (2015). Xeno-free chondrogenesis of bone marrow mesenchymal stromal cells: towards clinical-grade chondrocyte production. Cytotechnology, 67,905-19. https://doi.org/10.1007/s10616-014-9721-4 [doi]

Somaiah C, Kumar A, Mawrie D, Sharma A, Patil SD, Bhattacharyya J, et al. (2015). Collagen Promotes Higher Adhesion, Survival and Proliferation of Mesenchymal Stem Cells. PLoS One, 12:e e0145068. https://doi.org/10.1371/journal.pone.0145068 [doi]

Somoza RA, Welter JF, Correa D, Caplan AI. (2014). Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. Tissue Eng Part B Rev 20,596-608. https://doi.org/10.1089/ten.TEB.2013.0771 [doi]

Tamaddon M, Burrows M, Ferreira SA, Dazzi F, Apperley JF, Bradshaw A, et al. (2017). Monomeric, porous type II collagen scaffolds promote chondrogenic differentiation of human bone marrow mesenchymal stem cells in vitro. Sci Rep, 3, 7, 43519. https://doi.org/10.1038/srep43519 [doi]

Tay LX, Lim CK, Mansor A, Kamarul T. (2013). Differential protein expression between chondrogenic differentiated MSCs, undifferentiated MSCs and adult chondrocytes derived from Oryctolagus cuniculus in vitro. Int J Med Sci, 11,24-33. https://doi.org/10.7150/ijms.7244 [doi]

Tebb TA, Tsai SW, Glattauer V, White JF, Ramshaw JA, Werkmeister JA. (2006). Development of porous collagen beads for chondrocyte culture. Cytotechnology 52, 99–106. https://doi.org/10.1007/s10616-006-9034-3 [doi]

US Food & Drug Adminstration. FDA approves first autologous cellularized scaffold for the repair of cartilage defects of the knee [FDA News Release]. Retreived from https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm533153.htm; 2016, December 13 [accessed July 23. 2018]

Wakitani S, Okabe T, Horibe S, Mitsuoka T, Saito M, Koyama T et al. (2011). Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. J Tissue Eng Regen Med, 5, 146-50. https://doi.org/10.1002/term.299 [doi]

Vinardell T, Sheehy EJ, Buckley CT, Kelly DJ. (2012). A comparison of the functionality and in vivo phenotypic stability of cartilaginous tissues engineered from

different stem cell sources. Tissue Eng Part A, 18, 1161-70. https://doi.org/10.1089/ten.TEA.2011.0544 [doi]

Wu JJ, Weis MA, Kim LS, Eyre DR. (2010). Type III collagen, a fibril network modifier in articular cartilage. J Biol Chem, 285, 18537-44. https://doi.org/10.1074/jbc.M110.112904 [doi]

Xu L, Liu Y, Sun Y, Wang B, Xiong Y, Lin W et al. (2017). Tissue source determines the differentiation potentials of mesenchymal stem cells: a comparative study of human mesenchymal stem cells from bone marrow and adipose tissue. Stem Cell Res Ther 8:,75,017-0716-x. https://doi.org/10.1186/s13287-017-0716-x [doi]

Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM et al. (1998). The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am, 80, 1745-57.

Zhu M, Lin S, Sun Y, Feng Q, Li G, Bian L. (2016). Hydrogels functionalized with N-cadherin mimetic peptide enhance osteogenesis of hMSCs by emulating the osteogenic niche. Biomaterials,77, 44-52. https://doi.org/10.1016/j.biomaterials.2015.10.072 [doi]

Figure legends

Figure 1. Study design showing the cell types and scaffolds with white background and different time points with gray background. For the study, human bone marrow-derived stem cells were used in passage 2 and cultured in chondrogenic differentiation medium (MSC-C) or proliferation medium (MSC-P), and human cadaveric articular chondrocytes (AC) were used in passage 3. Each cell type was seeded onto three different biomaterials and cultured for 3 and 14 days (AC), or 14 and 28 days (MSC-C and MSC-P).

Figure 2. Safranin-O and type II collagen stained histological sections of bone marrow-derived mesenchymal stem cell pellets cultured in chondrogenic differentiation medium for 14 days. Scale bar: 200 µm.

Figure 3. The results of the RNA (a) and DNA (b) measurements in each group. The error bars represent standard error. *Bonferroni procedure was used to correct significance levels for post hoc testing ($p<0.05$). a) A statistically significant difference was found between scaffolds C2 and C3 in proliferated MSCs (MSC-P) on day 14 ($p=0.028$), and between scaffolds C2 and CG in chondrocytes (AC) on day 14 ($p=0.007$). b) The DNA amounts in each cell group differed from one another ($p<0.0001$). No statistically significant differences in DNA amounts were found between days 14 and 28.

Figure 4. The results of the sGAG/DNA measurements in each group. The error bars represent standard error. Statistically significant differences were found on day 14 and day 28 in proliferated and chondrogenically differentiated MSCs. All the statistically significant differences were located between the CG scaffold and the two rhCO-PLA

scaffolds (C2 and C3). *Bonferroni procedure was used to correct significance levels for post hoc testing (p<0.05).

Figure 5. Relative gene expression levels for the studied genes in proliferated (MSC-P) and chondrogenically differentiated (MSC-C) mesenchymal stromal cells and the control cells. Statistical significances between the scaffolds within each cell type are marked above the cell group. The bars represent average relative gene expression in each group and the whiskers represent standard error. *Bonferroni procedure was used to correct significance levels for post hoc testing (p<0.05). a) In MSC-Cs on day 14, *ACAN* was higher in CG than in C3 scaffold (p=0.001), and on day 28 all scaffolds differed from one another (p<0.0001). b) *COL2A1* was only upregulated in MSC-Cs but variation between individual samples was large. c) *RUNX2* was upregulated in MSC-Cs, where C2 showed lower expression values than the two other scaffolds on day 14 (p=0.001) and CG showed higher expression levels than the two other scaffolds on day 28 (p<0.0001). d) *COL10A1* was upregulated in MSC-Cs, where the only statistically significant difference between the scaffolds was found between C3 sand CG on day 28 (p=0.004). e) *MFAP5* showed highest expression levels in MSC-Cs on scaffold C2, which differed from CG (p=0.011).

Figure 6. Maximum projection of confocal microscopy images of the Hoechst stained nuclei in proliferated MSCs (a), differentiated MSCs (b) and adult chondrocytes (c). Each image is from a representative site of the scaffold. Each row represents a time point and each column represents a scaffold type. Scale bars: 50 µm.

Figure S1. Safranin-O stained histological section of the cadaver donor cartilage from which the chondrocytes were obtained. Scale bar: 200 µm.