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

Running title: Chondrogenic differentiation MSCs in 3D

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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

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

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

Due to the mechanically demanding function and highly specialized tissue structure of articular cartilage, biomechanical issues need to be taken into consideration when designing scaffolds for cartilage tissue engineering (Pattappa et al. 2019). Additionally, more understanding on the interactions of chondrogenic cells and biomaterials is needed to enable the design of improved scaffolds. To contribute to this understanding, this study was designed to explore the behavior of BM-MSCs in composite scaffolds, in which poly(L/D)lactide fibers are carded and needle-punched into mesh and combined with recombinant human collagen. This novel scaffold structure forms a biomechanically 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, Alunorm 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/cm² 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 μ m 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 μ m nylon mesh (Prinsal, Tuusula, Finland), collected by centrifugation and washed with sterile PBS.

2.5 Scaffolds

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

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

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

2.6 Cell cultures in scaffolds

The pellets in the pilot test stained similarly for Safranin-O and type II collagen. Based 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 μ l 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 mm² 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.

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

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

2.8 RNA extraction and cDNA synthesis

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

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

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

2.9 Real-time qPCR

The real-time qPCR analysis was performed with iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For the reaction, a commercial gene expression assay (TaqMan® Gene Expression Assay, Applied Biosystems) was used according to the manufacturer's protocol. The assay codes are presented in Table 1. The amplification reaction conditions were as follows: hold in 50°C for 2 min, hold in 95°C for 10 min, cycle 95°C for 15 sec and 60°C for 1 min repeated 40 times. *GAPDH* was used as the housekeeping gene based on our previous stability tests with mesenchymal stem cells. The gene expression levels were normalized with *GAPDH* and day 0 samples were used as calibrators in the $\Delta\Delta\text{C}_\text{T}$ method (Livak & Schmittgen 2001).

2.10 Confocal microscopy

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

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

2.11 Statistical analyses

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

3 Results

3.1 Cell yields and cell types after chondrogenic differentiation

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

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.

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

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

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

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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.

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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 μ m.

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 and 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 .