Primary chondrocytes enhance cartilage tissue formation upon co-culture with a range of cell types†

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Co-culture models have been increasingly used in tissue engineering applications to understand cell–cell interactions and consequently improve regenerative medicine strategies. Aiming at further elucidating cartilage tissue formation, we co-cultured bovine primary chondrocytes (BPCs) with human expanded chondrocytes (HECs), human dermal fibroblasts (HDFs), mouse embryonic stem cells (MESCs), or mouse-3T3 feeder cells (M3T3s) in micromasses. BPCs were either co-cultured (1 : 5 ratio) with all cell types allowing direct cell–cell contacts or as separate micromasses in the same well with HECs. In co-culture groups with direct cell–cell contacts cartilaginous tissue was formed in all experimental groups. *In situ* hybridization showed that only 16–27% of the cells expressed type II collagen mRNA. Corresponding with the fact that micromasses consisted for approximately 20% only of BPCs, the amount of GAG was similar between 100% BPC micromass and the co-culture groups with HECs and HDFs. Therefore, co-culture micromasses support cartilage tissue formation predominantly originating from primary chondrocytes in direct contact with a variety of cell types. These findings potentially could be applied to optimize cell-therapy treatments for cartilage regeneration.

1. Introduction

Over the past decades, co-culture has been extensively used in biological research to study cell–cell interactions.[1–3] Cell–cell interactions play a major role in tissue and organ engineering as shown by co-culture experiments.[4,5] Interestingly, in some studies co-cultured cells influenced each other's behaviour without losing their own phenotypic characteristics.[6] In contrast, other co-culture experiments showed that one cell population can adapt to the phenotype of the other cell population.[7,8] These observations highlight the importance of further understanding the role of cell–cell communication parallel to cell–material interactions, if regenerative medicine treatments are desired to reach steadily the clinics.

In the last two decades co-culture was introduced in cartilage research mainly to study cellular interaction of chondrocytes with synovial cells lining the joint cavity and with osteoblasts residing in the subchondral bone.[9] Cartilage is a unique tissue in that it consists of only one cell type, chondrocytes. It holds no vasculature or nerve system and therefore has limited capacity for self-repair. Cell–cell interactions between chondrocytes and other cell populations mainly take place at the interface of cartilage. Yet, these cells can behave differently depending on the cartilaginous region where they are located.[10] Accordingly, co-culture has been used to study interactions between articular chondrocytes and synovial cells in relation to the development of osteoarthritis.[9,11,12] In addition, co-culture has been used to study interactions between articular chondrocytes and osteogenic cells at the bone–cartilage interface.[13,14]

Recently, we have shown that co-culture or co-implantation of primary chondrocytes with expanded chondrocytes or mesenchymal stem cells (MSCs) enhanced cartilage tissue formation.[15] It is unclear whether this effect is restricted to co-cultures of chondrocytes with MSCs or also chondrocytes in combination with other cell types increase cartilage tissue formation. Furthermore, it is unclear which interactions underlie the increased cartilage tissue formation in co-cultures of primary chondrocytes and MSCs. This may involve direct cell–cell contacts between chondrocytes and MSCs. Alternatively, cell communication between MSCs and primary chondrocytes may occur *via* release and binding of growth factors or other cues that enhance cartilage tissue formation. In this study, we addressed whether the increased cartilage tissue formation in co-culture systems required direct cell–cell contacts or is caused by cell–cell communication *via* factor(s) that are secreted by either one of the cell types when in the co-culture. Secondly, we tested whether other cell types than MSCs can also increase cartilage tissue formation in micromass co-cultures. Finally, we evaluated which cell type in the micromasses contributed to cartilage tissue formation using collagen 2 *in situ* hybridization (ISH) and species-specific antibody staining. In a previous review it was assessed that co-culture of cell types originating from different species interact similarly than cell types from the same species. In

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order to distinguish one cell type from the other during the course of time in co-culture we have combined cell types from different specie origin. The specificity of the *in situ* hybridization probes allowed us to apply cells originating from bovine, human, and mouse origin. Next to primary chondrocytes from bovine origin, our aim was to include cell types with an anticipated difference in multipotency and/or ability to provide trophic factors to the system. For these studies, we have used human expanded chondrocytes (HECs), human dermal fibroblasts (HDFs), mouse 3T3 feeder cells (M3T3s) and mouse embryonic stem cells (MESCs). The ability to re-differentiate into the chondrogenic lineage of expanded human chondrocytes has been shown in abundance and in addition it was observed these cells secrete a variety of cytokines and growth factors.[16–18] Futhermore, chondrogenic differentiation capacity of dermal fibroblast has been demonstrated.[19–21] In contrast, 3T3s are commonly used as a feeder cell line[22,23] being a strong potential source of trophic mediators, supporting proliferation and maintenance of other cell types[24,25] with limited or no capacity to differentiate. Finally on the other side of the potency spectrum, embryonic stem cells still have the potency to form any tissue in the body.[26,27]

2. Experimental

2.1 Cell isolation and culture

The same batch of foetal bovine serum (FBS) was used for the culture of all cells and during micromass culture.

2.1.1 Chondrocytes. In co-culture experiments BPCs were used immediately after isolation. For isolation of these cells, full thickness articular cartilage was dissected from the patellofemoral groove of adult bovine. HECs were obtained from full thickness cartilage dissected from knee biopsies of patient undergoing total knee replacement (# CARTD-K011) after obtaining consent from the local medical ethical committee. Dissected cartilage was incubated for 20–22 h in collagenase type II solution containing 0.15% collagenase (Worthington, UK), Dulbecco's modified Eagle's medium (DMEM, Gibco, The Netherlands) supplemented with penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). The suspension was filtered through a 100 μm mesh nylon filter (Nucleon) and cells were washed 2 times with phosphate buffered saline (PBS) supplemented with penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}).

For expansion, HECs were plated at a density of 3.5×10^4 cells per cm^2 and cultured in medium 1 (CM1) consisting of DMEM medium supplemented with 10% FBS, 1× non-essential amino acids (NEAA) (Sigma-Aldrich, The Netherlands), 10 mM HEPES buffer (Biowhittaker, USA), 0.2 mM ascorbic acid 2-phosphate (Sigma-Aldrich), 0.4 mM proline (Sigma-Aldrich), 100 U ml^{-1} penicillin (Invitrogen) and 100 μg ml^{-1} streptomycin (Invitrogen). Cells were released with trypsin–EDTA and viable cells were counted with trypan blue staining and a Burker-Turk counting chamber. Cells were replated at a density of 3.5×10^4 cells per cm^2. After 2–3 passages, expanded cells were mixed with primary chondrocytes for micromass culture.

2.1.2 Mouse 3T3 feeder cell line (M3T3s). After thawing, ATCC-3T3 feeder cells were plated at a density of 5×10^3 cells per cm^2 and cultured in medium 2 (CM2) consisting of α MEM (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich) and 10% FBS. Medium was refreshed every 2–3 days and cells were sub-cultured as described in the "Chondrocytes" section above. After a total of 3–4 passages, 3T3 feeder cells were mixed with primary chondrocytes for micromass culture.

2.1.3 Human dermal fibroblasts (HDFs). Human dermal fibroblasts were isolated from dermis of adult human breast tissue by enzymatic digestion. Isolation and culture protocols were adapted from Wang *et al.*[28] Briefly, dermis was minced into small pieces and digested with 0.25% (w/v) collagenase type II (Worthington) and 0.25% (w/v) dispase (Worthington) at 37 °C for 2.5 h. After isolation cells were cultured in medium three (CM3) consisting of DMEM, 10% FBS (Sigma-Aldrich), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). Cultures were refreshed every 2–3 days and after 1 passage suspended in freezing medium containing 10% DMSO and 20% FBS and frozen in liquid nitrogen. After thawing, cells were seeded at a density of 5×10^3 cells per cm^2 and cultured as described above. After 3–4 passages, HDFs were mixed with primary chondrocytes for micromass culture.

2.1.4 Mouse ES cell culture. Murine ES cell line E14, subclone IB10 was cultured as described previously.[34] In brief, cells were plated at a density of 5000–10 000 cells per cm^2 on gelatin-coated tissue culture flasks. MESCs were cultured in 50% MES proliferation medium consisting of DMEM (BioWhittaker) containing 4.5 mg ml^{-1} D-glucose, 10% FBS (selected batch for MES cell culture, Greiner), 0.1 mM NEAA (Sigma-Aldrich), 4 mM L-glutamine (Invitrogen), 100 U ml^{-1} penicillin (Invitrogen), 100 μg ml^{-1} streptomycin (Invitrogen) and 50% of buffalo rat liver cell-conditioned MES proliferation medium. Prior to use 1000 U ml^{-1} Leukaemia Inhibitory Factor (Esgro, Chemicon International) and 50 μM 2-mercapto-ethanol (Gibco) were added to the medium. Cells were grown at 37 °C in a humidified 5% CO_2 incubator and passaged with 0.05% trypsin/EDTA before reaching confluence.

2.1.5 Micromass culture. 100 000 BPCs were mixed with 400 000 HECs, HDFs, MESCs or M3T3s. The cells were centrifuged at 500*g* for 2 min in 3 ml of CM1 in a polypropylene Falcon centrifuge tubes to form a micromass. The micromass were cultured in CM1 for 3 weeks and medium was refreshed every 3–5 days. Each experimental group ($n = 9$) was processed for histology ($n = 3$), *in situ* hybridization ($n = 3$), immunohistochemistry or quantitative biochemical analysis ($n = 3$).

2.2 Histology

Micromass cultures were fixed with 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH = 7.2–7.4). Samples were washed in PBS, dehydrated and embedded in paraffin. Sections (5 μm) were cut with a microtome, stained for sulfated glycosaminoglycans (GAGs) with safranin O, and counterstained with haematoxylin (Gill #3) and fast green to visualize nuclei and cytoplasm respectively.

2.3 *In situ* hybridization (ISH)

ISH was performed essentially as described previously.[29] In short, micromass cultures were fixed with 4% paraformaldehyde for 10 minutes washed with PBS and subsequently embedded in paraffin. Sections (6 μm) were cut with a microtome, deparaffinised, and rehydrated. The sections were treated with 5 μg ml^{-1} proteinase K (Sigma-Aldrich) at 37 °C in Tris–HCl (pH 8.0) containing 50 mM EDTA and post-fixed at room temperature (RT) with 4% paraformaldehyde for 5 minutes. After washing with PBS, endogenous alkaline phosphatase was removed applying 0.2 M HCl for 10 minutes at RT. The slides were acetylated with acetic anhydride in 0.1 M triethanolamine (TEA) at pH 8.0 and washed in 2× sodium chloride/sodium citrate buffer (SSC). Sections were hybridized overnight at 60 °C with 1 μg μl^{-1} probe solution consisting of 1 μg μl^{-1} yeast tRNA, 50% formamide, 2× SSC, 1× Denhardt's solution, and 10% dextrane sulfate. The next day, slides were washed twice in 2× SSC, 50% formamide at 60 °C, and again in 2× SSC. Sections were treated with RNase A solution (20 μg ml^{-1}) containing 10 mM Tris (pH 8.0), 0.5 mM NaCl and 1 mM EDTA for 30 minutes at 37 °C to digest unbound probe. Slides were washed twice in 2× SSC. To block a specific antibody binding, sections were incubated for 30 minutes in blocking buffer-containing 10× Tris Buffered Saline (TBS), 10% sheep serum and 0.3% Triton. Anti-DIG antibody (1 : 1250) was incubated overnight at 4 °C in blocking buffer. The next day slides were washed 3 times in TBS-I and once in TBS-II (0.1 M Tris–HCl pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$). Slides were stained for 5½ hours with nitroblue tetrasodium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) solution (0.375 mg ml^{-1}/0.188 mg ml^{-1}) containing 59 mg ml^{-1} polyvinyl alcohol (PVA), 75 mM NaCl, 75 mM Tris–HCl pH 0.5, 0.19 M $MgCl_2$ and 0.2× TBS-II. Subsequently, slides were washed in Tris/EDTA buffer (pH 8.0), TBS-I, counter stained with methyl green (2%) for 25 seconds and embedded in Euparal (Bio Quip Products, Rancho Dominguez, CA, USA). Tris, SSC and TBS buffers are made according to protocols described.[30] Sequence of type I collagen and II probes was previously described by van der Eerden *et al.*[29] In previous experiments, it was shown that the type II collagen mRNA probe cross-reacted with human, bovine and mouse chondrocytes (data not shown). The percentage of type II collagen expressing cells was determined by the number of labelled cells divided by the total number of cells counted in five ad random selected fields in each of the ISH sections and multiplied by 100%.

2.4 Immunohistochemistry

Micromasses were embedded in OCT™ compound (Tissue-Tek) and immediately frozen at −80 °C for immunostaining. Sections (5 μm) were cut with a cryotome and fixed with acetone for 10 min. Cryo-sections were stained overnight at 4 °C for type II collagen (1 : 100, DSHB # II-II6B3, USA). Blocking was done with 10% human serum. Goat anti-mouse (1 : 100, DAKO) was used as a secondary antibody. Staining was visualized with 3-diaminobenzidine (DAB)-solution (DAKO) for 10–20 minutes.

Specificity of human specific MHC Class I antibody (1 : 100) was verified with human chondrocytes. This antibody did not cross-react with bovine chondrocytes. The antibody was diluted in washing buffer (PBS containing 10% blocking buffer DAKO Cytomation X0909). Slides were pre-blocked in 100% blocking buffer for 1 hour and incubated with the first antibody overnight. The next day, slides were washed 3 times in washing buffer and incubated with the second antibody goat anti-mouse (1 : 100, DAKO) for 1 hour. Slides were washed 3 times with PBS and staining was visualized with fluorescent microscope.

2.5 Quantitive GAG and DNA assay

Micromasses used for quantitative analysis of GAGs and cell number were washed with PBS and frozen at −80 °C. Subsequently, they were digested with 1 mg ml^{-1} proteinase K (Sigma-Aldrich) in Tris/EDTA buffer (pH 7.6) containing 18.5 μg ml^{-1} iodoacetamide and 1 μg ml^{-1} pepstatin A (Sigma-Aldrich) for >16 h at 56 °C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (DMMB, Sigma-Aldrich) staining in PBE buffer (14.2 g l^{-1} Na_2HPO_4 and 3.72 g l^{-1} Na_2EDTA at pH 6.5) with a microplate reader (Bio-TEK instruments) at an absorbance of 520 nm. The standard curve for the GAG analysis was generated using chondroitin sulfate A. Cell number was determined *via* quantification of total DNA with CyQuant DNA kit according to the manufacturer description (Molecular probes) in a fluorescent plate reader (Perkin-Elmer). The standard curve for DNA analysis was generated with λ DNA provided with the CyQuant DNA kit.

2.6 Statistical analyses

Normality of the data was analyzed by determining skewness, which was between −3 and 3 and showed a normal distribution in all data groups. Data were analyzed for differences of the means with ANOVA and $p < 0.05$ was considered statistically significant. Statistical significance of $p \leq 0.01$ was indicated in the figure legends. The lack of significant differences compared to the 100% primary chondrocyte group was indicated in the legend of Fig. 1. Data are presented as the mean with standard deviation.

3. Results

3.1 Direct cell–cell contacts are required for increased cartilage tissue formation in micromass co-cultures

In this experiment, it was determined whether mixing of primary and expanded chondrocytes or the secretion of growth factor(s) by either cell type enhanced cartilage tissue formation. Results showed that expanded chondrocytes (p3) did not produce GAG or type II collagen in micromass culture, but did produce type I collagen (Fig. 1A). In contrast, BPCs produced abundant GAGs and type II collagen and expressed type I collagen only in the outer rim of the micromass (Fig. 1B). A co-culture experiment was performed in which primary or expanded chondrocytes micromass were cultured in the same well without allowing direct cell contact between these 2 cell types (Fig. 1C) or as a single micromass consisting of both cell populations in a 1 : 1 ratio (Fig. 1D). When primary and expanded chondrocytes were cultured as individual micromasses in the same well, no GAG or type II collagen was found in the micromass consisting of 100% expanded chondrocytes. Only type I collagen was detected

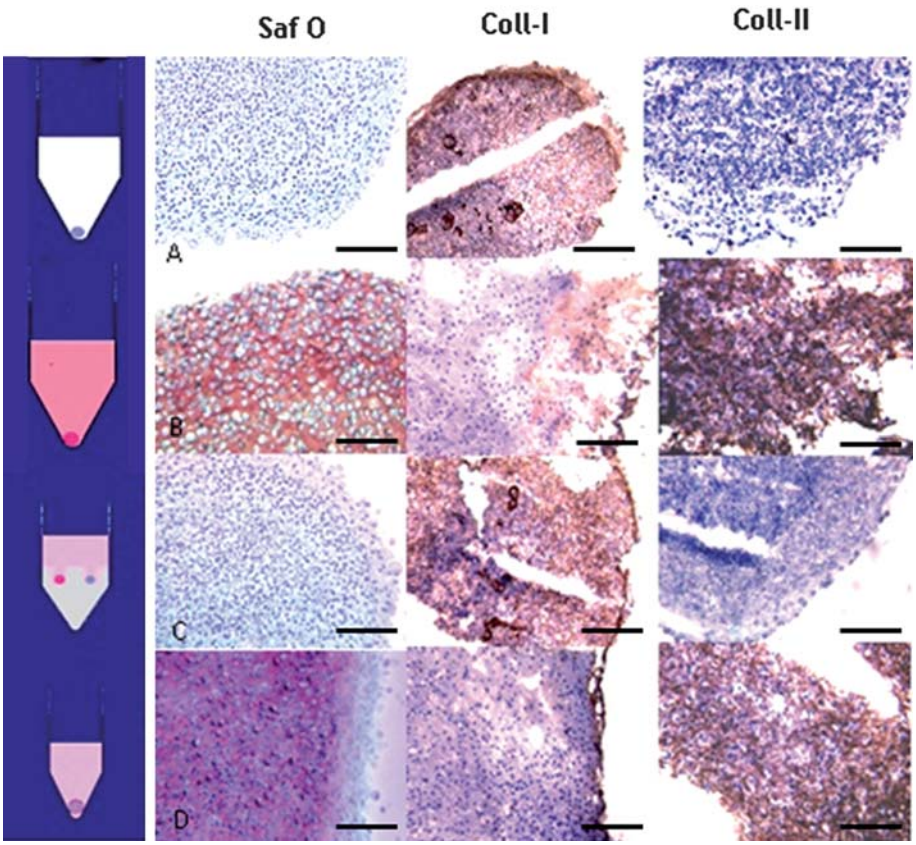

Fig. 1 Primary and expanded chondrocytes were co-cultured where either cell types were combined in a single micromass or (D) either cell type was co-cultured in 2 distinct micromass in the same medium not allowing direct contact between micromass (C). Controls are micromass cultures of expanded (A) or primary chondrocytes (B) (n= 3). All micromass cultures were analyzed for sulfated proteoglycans with safranin O (Saf O) and type I (Coll-I) and II collagen(Coll-II) with immunostaining. Scale bar reflects 100 μm.

(Fig. 1C). Whereas the primary chondrocytes micromass in this co-culture showed abundant GAG and collagen type II staining but no collagen type I similarly as in Fig. 1B (data not shown). In remarkable contrast, when primary chondrocytes were mixed with expanded chondrocytes in the same micromass, intense GAG as well as type II collagen staining was detected. This staining was homogenously distributed over the cell micromass (Fig. 1D). In these micromass, type I collagen was only detected in the outer rim of the micromass, where cells displayed a fibroblastic morphology (Fig. 1D).

3.2 Cartilaginous tissue formation in co-cultures of BPCs with HDFs, HECs, M3T3s, and MESCs

Micromass co-cultures of primary chondrocytes with different cell types were analyzed for cartilagenous tissue formation. Abundant safranin O staining was shown in all co-culture groups (1 : 5) (Fig. 2A and C, 3A and C and 4A). When primary chondrocytes were not included in the culture systems (0 : 5) GAGs were absent (Fig. 2B and D, 3B and 4B), with the exception of MESCs cultures, as shown by safranin O staining (Fig. 3C). The micromass' of all co-culture groups contained type 2 collagen. There was, however, a considerable difference in the distribution of type II collagen between the various experimental groups. In the co-culture group of BPCs with HDFs, type II collagen was found throughout the micromass except for the outer cell layers (Fig. 2E). In the co-culture group of BPCs with MESCs, type II collagen was preferentially found in the extensions at the periphery of the micromass (Fig. 3E). In this group, histological analysis clearly indicated the formation of other tissue types than cartilage. The structures morphologically resembled vessels and columnar epithelia and other tissues (Fig. 3C). Surprisingly, these structures were not found in the control group consisting solely of MESCs (Fig. 3B and D). In the co-culture group of BPCs with M3T3s, type II collagen was detected in intensely stained patches throughout the micromass and was again not present in the outer rim (Fig. 4C). No type II collagen was detected in the control groups with only HDF's (Fig. 2E), MESCs (Fig. 3F) or M3T3s (Fig. 4C). Previous data showed that co-culture of primary with expanded chondrocytes support cartilage tissue formation and the formed tissue expressed hyaline cartilage specific markers.[15]

3.3 Origin of cartilaginous extracellular matrix when BPCs are co-cultured with HDFs, HEC, M3T3s and MESCs

In none of the control group micromass cells expressing type II collagen mRNA were detected (data not shown). After 3 weeks of culture, 27.3 ± 11.1% of the cells in the BPCs/HECs group were found to express type II collagen mRNA (Fig. 5A). Interestingly, human specific antibody staining showed that expanded chondrocytes could be found throughout the micromasses also in

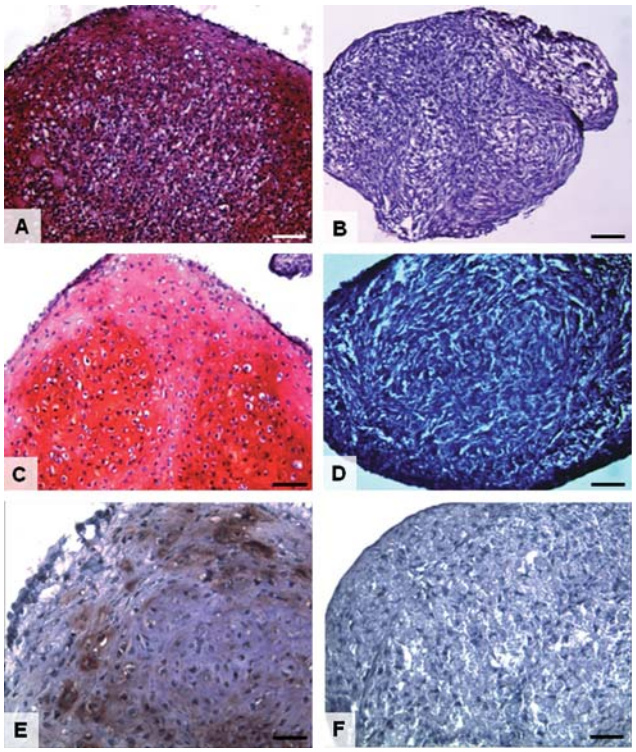

Fig. 2 Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with human expanded chondrocytes (HECs) and stained for sulfated proteoglycans (A), or human dermal fibroblasts (HDFs) and stained for proteoglycans with safranin O staining (C) or collagen type II with immunohistochemistry (E). HECs only cultured in micromass for 4 weeks serve as a negative control for safranin O staining (B). HDFs only cultured in micromass for 4 weeks serve as negative control for safranin O staining (D) or for collagen type II immunohistochemistry (F). Scale bar reflects 100 μm.

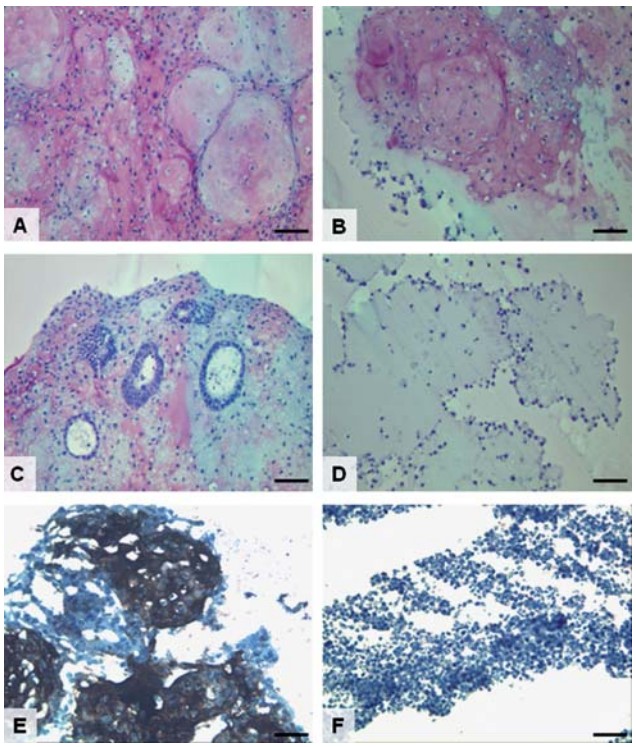

Fig. 3 Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with mouse embryonic stem cells (MESCs) and stained for sulfated proteoglycans (A and C) or for collagen type II with immunohistochemistry (E), MESCs only cultured in micromass for 4 weeks serve as negative control for safranin O staining (D) or collagen type II immunohistochemistry (F). Scale bar reflects 100 μm.

areas containing no type II collagen mRNA positive cells (Fig. 5C). Fig. 5D shows the accompanying light microscopic picture of fluorescent Fig. 5C. Cells expressing type I collagen mRNA were not detected (Fig. 5B). In the BPCs/HDFs co-culture group, 24 ± 3% of the cells expressed type II collagen (Fig. 6A). Type I collagen mRNA was not detected (Fig. 6B). Interestingly, antibody-staining specific for human cells showed co-localization of areas lacking dermal fibroblasts with intense safranin O stained areas (Fig. 6D, inlet). Thus, areas intensely stained for proteoglycans with safranin O were rich in BPCs (Fig. 6C and D, inlet). Fig. 6D shows accompanying light microscopic of Fig. 6C. In the BPCs/MESCs co-culture group *in situ* hybridization demonstrated distinct areas in the micromass that expressed type II collagen mRNA (Fig. 7A), whereas in the complementary regions no collagen II mRNA was detected. In this group, 30.8% of the total amount of cells contributed to type II collagen mRNA expression. Type I collagen specific mRNA was detected in specific areas (Fig. 7B). Analysis of subsequent sections suggested that the presence of collagen I or II mRNA was mutually exclusive. When BPCs were co-cultured with M3T3s, 16.3 ± 11.5% of the cells actively contributed to type II collagen mRNA expression (Fig. 7C). Large standard deviation reflected that some areas contained a high number of type II collagen mRNA expressing cells while in other areas none of the

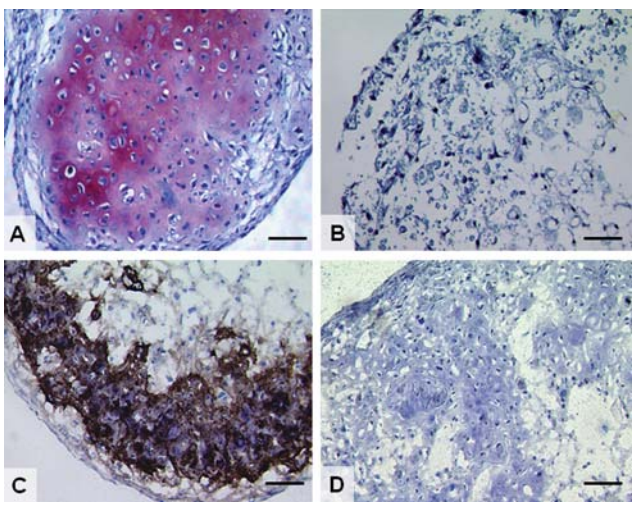

Fig. 4 Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with Mouse 3T3 cells (M3T3s) and stained for sulfated proteoglycans (A) or collagen type II with immunohistochemistry (C). M3T3s only cultured in micromass for 4 weeks serve as a negative control for safranin O staining (B) or for collagen type II immunohistochemistry (D). Scale bar reflects 100 μm.

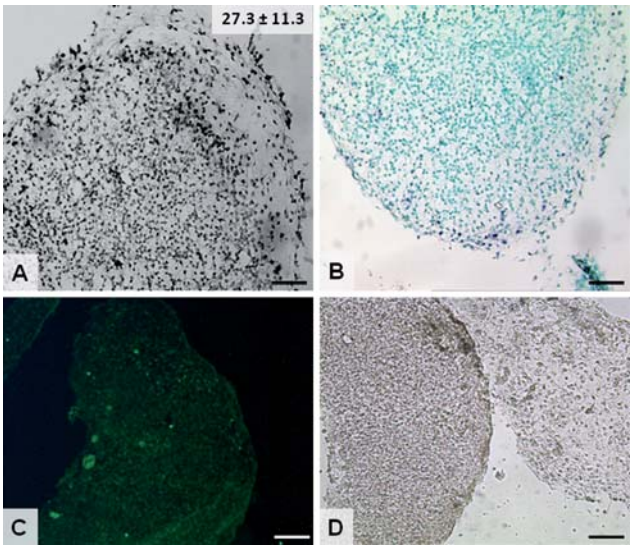

Fig. 5 Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with human expanded chondrocytes (HECs) and analysed for either collagen type II (A) or I (B) mRNA expressing cells with *in situ* hybridization. In the inlet the average percentage of collagen type II expressing cells is indicated. Cells expressing collagen type II or I mRNA are coloured black. Co-culture micromasses of BPCs with HECs, in addition, were stained for human cell specific antigens with fluorescent immunohistochemistry (C). (D) is the light microscopic equivalence of the fluorescent image depicted in (C). Scale bar reflects 100 μm.

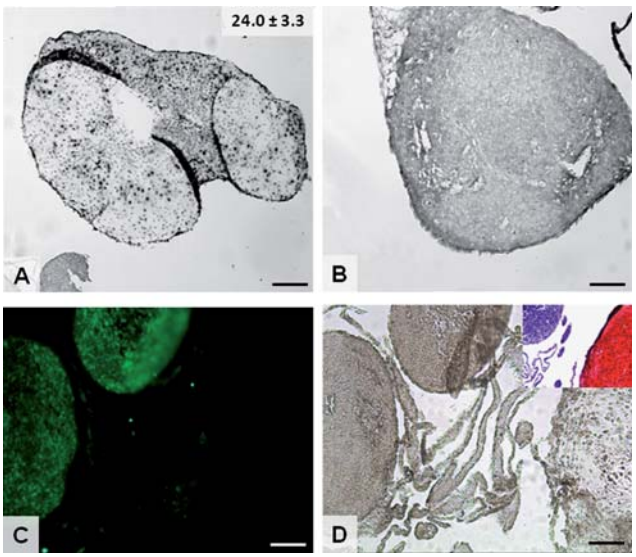

Fig. 6 Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with human dermal fibroblasts (HDFs) and analysed for either collagen type II mRNA expressing cells. In the inlet the average percentage of collagen type II expressing cells is indicated (A) or collagen type I expressing cells (B) with *in situ* hybridization. Cells expressing collagen type II mRNA are coloured black. Co-culture micromass of BPCs with HDFs, in addition were stained for human cell specific antigens with fluorescent immunohistochemistry (C). (D) is the light microscopic equivalence of the fluorescent image depicted in (C). In the inset of image (D) the safranin O stained equivalence of the human specific antigen sample (C and D) is depicted. Scale bar reflects 100 μm.

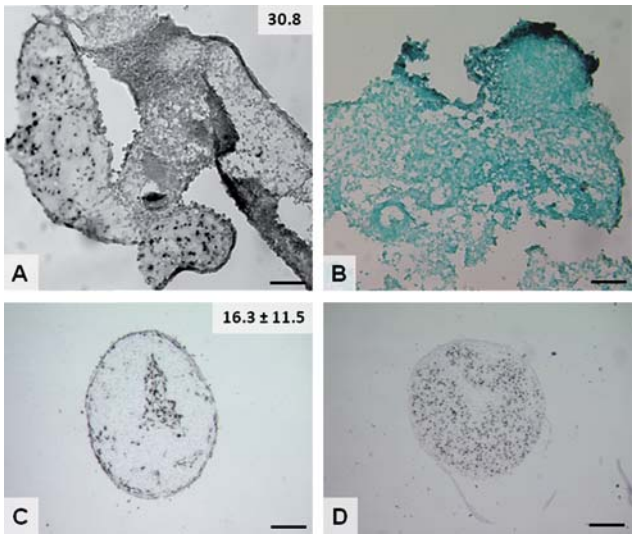

Fig. 7 Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with Mouse embryonic cells (MESCs) and analysed for either collagen type II mRNA expressing cells (A) or collagen type I expressing cells (B) with *in situ* hybridization. Bovine primary chondrocytes (BPCs) were co-cultured for 4 weeks in a single micromass at 1 : 5 ratio with Mouse 3T3s (M3T3s) and analysed for either collagen type II mRNA expressing cells (C) or collagen type I expressing cells (D) with *in situ* hybridization. Scale bar reflects 100 μm.

cells expressed type II collagen mRNA. Furthermore, ISH showed that type I collagen mRNA expressing cells were present throughout the micromass except in the outer rim and the middle of the micromass. Again, collagen I and II mRNA expression were mutually exclusive with cells expressing either one or neither of the respective mRNAs (Fig. 7D).

3.4 Enhanced cartilage tissue formation by BPCs after co-culture with HDFs, M3T3s, MESCs and HECs

After 4 weeks of culture, we quantitatively determined GAG production and DNA content in each micromass (Fig. 8 and

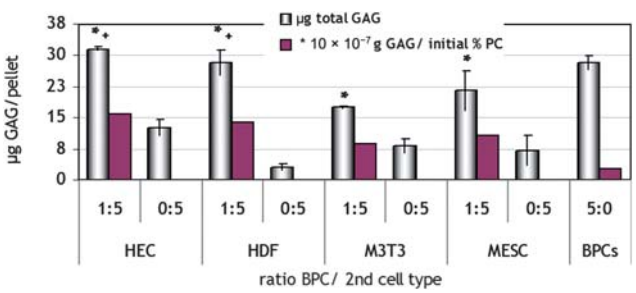

Fig. 8 BPCs were co-cultured at a 1 : 5 ratio with HECs, HDFs, MESCs and M3T3s in micromass culture for 4 weeks. Micromass cultures were analyzed for total glycosaminoglycans (GAGs) and total DNA. Graph depicts total GAG (μg) per micromass culture for all groups and GAG (10^{-7} g) per initial percentage of primary chondrocyte for co-cultured groups. Table shows total GAG (μg), total DNA (μg) for all groups and GAG per primary chondrocytes (10^{-7} g GAG/initial % PC) for co-cultured groups and BPC control group (5 : 0). * = significantly different from 0 : 5 control, + = not significantly different from 5 : 0 control (100% primary chondrocytes) ($p < 0.05$).

Table 1 Total glycosaminoglycan (GAG) and DNA analysis of micromass co-cultures of bovine primary chondrocytes (BPCs) with either human expanded chondrocytes (HECs) or human dermal fibroblasts (HDFs) or mouse 3T3 cells (M3T3s) or mouse embryonic stem cells (MESCs)

Cells co-cultured	Ratio cells (day 0)	Total GAG/μg	Total DNA/μg	μg GAG/initial % BPC
BPC/HEC	1 : 5	31.5 ± 0.4	20.32 ± 4.06	1.58
	0 : 5	12.5 ± 1.9	22.32 ± 4.36	
BPC/HDF	1 : 5	28.2 ± 3.1	29.40 ± 3.95	1.07
	0 : 5	3,1 ± 0.7	3.46 ± 0.58	
BPC/M3T3	1 : 5	17.5 ± 0.4	19.58 ± 2.20	0.88
	0 : 5	8.1 ± 1.6	13.71 ± 2.54	
BPC/MESC	1 : 5	21.5 ± 4.8	41.57 ± 3.74	1.41
	0 : 5	7.2 ± 3.5	7.45 ± 0.75	
BPC	5 : 0	28.1 ± 1.7	34.16 ± 6.88	0.28

Table 1). Control micromass consisting of 100% HDF, M3T3, and MESCs contained significantly less DNA than the BPCs micromass. 100% HECs followed a similar trend. Furthermore, they contained significantly less GAG ($p < 0.05$). Co-culture of BPCs with M3T3s or HECs slightly reduced GAG content in the micromass compared to 100% BPCs ($p < 0.05$). In contrast, co-culture with HDFs or MESCs had no significant effect on GAG content. Remarkably, the total amount of GAG produced in the co-culture micromass of human cells with BPCs did not differ from the production in the micromass consisting of 100% BPCs. Compared to the 100% BPC micromass, total GAG production in the co-culture groups with mouse cells was 30 to 40% reduced. The ISH results suggested that only a minority of the cells contributed to collagen II production in the co-culture groups. The number of type II collagen mRNA positive cells varied between 16 and 30%. These numbers roughly corresponded to the initial seeding density of BPCs in the co-culture micromass. This suggested that the BPCs only contributed to the formation of a cartilaginous matrix, while the co-cultured human or mouse cells contributed only marginally to this process. When the total GAG production in the co-culture micromass was corrected for the percentage of BPCs initially seeded in the cell micromass, the normalized GAG production increased between 3.1 (MESCs) and 5.6 (HDFs) fold compared to the 100% BPCs.

4. Discussion

Different studies showed enhanced cartilage tissue formation in co-cultures of primary and expanded chondrocytes or MSCs.[15,31] The mechanisms underlying this phenomenon are unknown. In this study, we have investigated if this phenomenon is limited to certain cell types and which cell types when in co-culture contribute to cartilage tissue formation. The most important findings of our experiments are: (i) in co-culture experiments, mixing of primary with expanded chondrocytes in the same micromass is required to support increased cartilage tissue formation; (ii) tissue formation is not restricted to co-cultures with MSCs or expanded chondrocytes but is found in co-cultures of primary chondrocytes with a variety of cell types; (iii) cartilage tissue formation is predominantly due to increased activity of the primary chondrocytes rather than (trans) differentiation of the co-cultured cells towards the chondrogenic lineage.

Analysis of primary and expanded chondrocytes co-cultured in two distinct micromass in the same medium showed no hyaline cartilage formation in expanded chondrocyte micromass whereas cartilage tissue formation micromass was observed with primary chondrocytes only. When the two cell types were combined in a single micromass thus allowing cell–cell contact, abundant cartilage tissue formation was observed throughout the micromass. This experiment rendered it unlikely that secreted factors were involved in the increased formation of a cartilaginous matrix when primary and expanded chondrocytes are co-cultured. Instead, mixing of both cell types in the same micromass appeared to be a prerequisite to enhance cartilage tissue formation. Results suggest that direct cell signal exchange *e.g. via* gap-junctions is necessary to elicit the observed enhanced tissue formation. The functional units forming gap junctions are known as connexons. It has been shown that chondrocytes in culture express connexon type 43.[32,33] However, the involvement of secreted factors cannot be completely ruled out. It seems plausible that medium conditioning is insufficient due to limited secretion of factors. This can be explained by the notion that various chondrogenic growth factors tend to stick to the extracellular matrix instead of being secreted into the surrounding culture medium. Thus, only when cells are in close vicinity, the concentration of these factors may become sufficiently high to observe the stimulatory effect on cartilage tissue formation. To distinguish between these two mechanisms, more experiments are needed in which for example specific blockers of either pathway are included. Experiments providing answers to cell signalling mechanisms underlying the observed phenomenon here could include the application of antibodies against connexon type 43. However, in these experiments the possibility this signalling pathway is made redundant through activation of alternative intercellular communication pathway involving other connexons or other intercellular communication pathways needs to be carefully considered. In addition unravelling of which cell signalling pathways are involved in enhancing cartilage formation when different cell types are co-cultured with primary chondrocytes, would greatly contribute to the understanding of the underlying mechanisms.

The chondrogenic potential of expanded chondrocytes, embryonic stem cells as well as dermal fibroblasts has been extensively shown.[19,34,35] Most of these experiment comprised 3D culture and some chondrogenic stimulation in the form of growth factors and/or extracellular matrix proteins. When we individually cultured these cell types in a micromass, we did not obtain evidence for cartilage tissue formation. Only in micromass consisting of 100% MESCs, some safranin O staining was present. This did not coincide with type II collagen mRNA and protein expression. It remained, therefore, questionable whether the safranin O staining in these micromass was indicative for cartilage specific tissue formation. In contrast, when we mixed the cells with primary bovine chondrocytes in a 4 to 1 ratio abundant cartilage tissue formation was observed based on strong safranin O staining, type II collagen mRNA expression, and quantitative GAG measurements. In the co-cultures with mouse cells, the amount of GAG formation was somewhat lower compared to the co-cultures with human dermal fibroblasts. The amount of GAGs produced in the micromass consisting of 80% dermal fibroblasts was comparable to the amount produced in

the micromass consisting of 80% expanded chondrocytes and to the micromass consisting solely of bovine primary chondrocytes.

Histological and quantitative analysis of the cell micromass provided evidence for communication between the co-cultured cells and the primary chondrocytes and *vice versa*. When cultured as individual cell micromass, HDFs, MESCs, and to a lesser extent M3T3s hardly grew in size. This is indicative for either impaired growth or increased cell death when these cells were cultured in micromasses. Since we only analysed end stages, we were not able to distinguish between these two possibilities. Remarkably, mixing the cells with 20% of BPCs resulted in cell micromass and DNA contents that were indistinguishable from the micromass consisting of 100% BPCs. Thus, the presence of as less as 20% of BPCs created already a suitable environment, which sustained growth of HDFs and MESCs in a micromass. In addition, histological analysis of the micromasses consisting of BPCs and embryonic stem cells revealed that also other tissues were formed besides cartilage. Unexpectedly, this diversity in tissue formation was less pronounced in the control group consisting of embryonic stem cells only. This suggested that co-culture with BPCs favours the differentiation of embryonic stem cells not only into chondrocytes but also into other tissue types. In summary, BPCs provided signals that supported cell growth and/or survival of co-cultured cells. Furthermore, they might induce differentiation of pluripotent MESCs into a variety of cell lineages.

On the other hand, the co-cultured cells also provided support to the BPCs by producing factors that stimulated the chondrogenic phenotype of these cells resulting in increased production of GAGs. Various lines of evidence support this conclusion. In the cell micromass in which HDFs were mixed with BPCs, sequential sections of safranin O staining and human specific antibody staining suggested that a cartilaginous matrix was predominantly present in regions that hardly contained human cells. Likewise, type II collagen mRNA as detected by ISH was almost exclusively present in these regions. Similar findings were made in the micromasses consisting of BPCs co-cultured with mouse cells. Cell counts demonstrated that in the mixed cell micromass approximately 20% of the cells in the micromass expressed type II collagen mRNA. Combination of these data suggests that only a minority of the cells in the mixed micromass contributed to cartilage tissue formation. Since the number of type II collagen mRNA positive cells was approximately 20% and this number corresponded to the initial seeding density of the BPCs in the micromass, it appears highly likely that the BPCs in the cell micromass are those cells that contributed to cartilage tissue formation. This is in line with findings from Tsuchiya *et al.*,[8] who showed that in micromass co-cultures of chondrocytes with bone marrow mesenchymal stem cells the proportion of the two different cell types did not change during the culture period. We cannot, however, completely exclude (trans) differentiation of the co-cultured cells into chondrocytes. Yet, this will be a rare event—if it occurred at all—based on cell counts in our experiments. This observation contrasts earlier findings in co-cultures of bone marrow mesenchymal stem cells (MSCs) and embryonic stem cells with primary cell types from different tissues. In these experiments it was shown that MSCs acquired the phenotype of the cell type included in the co-culture.[36–40] Other studies have shown that in co-cultures of MSCs with primary cells from adult tissues, MSCs only supported the tissue specific physiology of the co-cultured cells, possibly by release of trophoblastic factors, but did not showed evidence for differentiation.[18,36,41] Similar results have been found in co-cultures in which M3T3s were included. In these experiments, the M3T3s only supported the physiology of the cell type they were co-cultured with.[42–44] In skin tissue engineering the co-culture of dermal fibroblasts with keratinocytes was found beneficial for both cell types.[24,45,46] Combined with our results, it appears that the effect of co-culture on the behaviour of a particular cell type is dependent on the cell types included in the experiment. Furthermore, (trans) differentiation in co-culture experiments may be restricted to pluripotent or multipotent cell types that have the intrinsic capacity of changing cell fate.

Finally, quantitive analysis of GAG production showed that the total amount of GAG was the same (in co-cultures with human cells) or slightly reduced (in co-cultures with mouse cells) than the total amount of GAG produced by the control group consisting of 100% primary chondrocytes. Since our evidence suggests that only the BPCs contributed to cartilage tissue formation in the mixed cell micromass, this analysis indicated that the production of GAGs per BPC must be increased between 5-fold (in co-cultures with human cells) and 3- to 4-fold (in co-cultures with mouse cells). Thus HECs, HDFs, M3T3s, and MESCs each stimulate the production of a chondrogenic matrix by BPCs. This effect requires mixing of both cell types in the same micromass, suggesting that a common mechanism may underlie this phenomenon for each of these cell types. The underlying mechanisms are subject of further study.

In summary, our results showed that primary chondrocytes enhanced cartilage tissue formation when co-cultured with a variety of human and mouse cell types. In a review by Caplan and Dennis,[18] the role of bone marrow MSCs was discussed as supportive to the proliferation and differentiation of other cell types by the release of trophoblastic factors. Our results suggest that this effect is not limited to MSCs, but can be observed with other cell types as well. These findings might provide the basis for investigating the possible application of these cell types in cartilage tissue engineering.

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