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Trophic Effects of Mesenchymal Stem Cells Increase Chondrocyte Proliferation and Matrix Formation

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Previous studies showed that coculture of primary chondrocytes (PCs) with various sources of multipotent cells results in a higher relative amount of cartilage matrix formation than cultures containing only chondrocytes. The aim of this study was to investigate the mechanism underlying this observation. We used coculture pellet models of human mesenchymal stem cells (hMSCs) and human PCs or bovine PCs (bPCs) and studied the fate and the contribution to cartilage formation of the individual cell populations during coculture. Enhanced cartilage matrix deposition was confirmed by histology and quantification of total glycosaminoglycan deposition. Species-specific quantitative polymerase chain reaction demonstrated that cartilage matrix gene expression was mainly from bovine origin when bPCs were used. Short tandem repeat analysis and species-specific quantitative polymerase chain reaction analysis of genomic DNA demonstrated the near-complete loss of MSCs in coculture pellets after 4 weeks of culture. In coculture pellets of immortalized MSCs and bPCs, chondrocyte proliferation was increased, which was partly mimicked using conditioned medium, and simultaneously preferential apoptosis of immortalized MSCs was induced. Taken together, our data clearly demonstrate that in pellet cocultures of MSCs and PCs, the former cells disappear over time. Increased cartilage formation in these cocultures is mainly due to a trophic role of the MSCs in stimulating chondrocyte proliferation and matrix deposition by chondrocytes rather than MSCs actively undergoing chondrogenic differentiation.

Introduction

Articular cartilage repair is a challenge due to the inability of cartilage to repair itself after damage. Autologous chondrocyte implantation (ACI) has become the golden-standard treatment for large-size cartilage defects.[1,2] However, ACI creates donor-site injury and is dependent on two-dimensional expansion of isolated chondrocytes resulting in chondrocyte dedifferentiation.[3]

To reduce the number of chondrocytes needed in ACI, a partial substitution of chondrocytes with pluripotent stem cells is a promising strategy. It has been reported that coculture of bone marrow mesenchymal stem cells (MSCs) and articular chondrocytes enhanced matrix deposition[4–6] even in the absence of the chondrogenic factors transforming growth factor-β and dexamethasone.[7] Increased cartilage matrix formation was also found in coculture of chondrocytes with other cell types, such as adipose-tissue derived stem cells, human embryonic stem cells, and meniscus cells.[8–11]

MSCs are promising for tissue repair because of their multilineage differentiation capacity.[12] Because of their importance in the development of articular cartilage, MSCs are a potential source for coculture with chondrocytes. It is hypothesized that MSCs repair damaged tissue by differentiating into tissue-specific cells replacing lost cells.[13] However, evidence suggests that differentiation into tissue-specific cells cannot fully explain the benefits of transplanted MSCs in remodeling and recovery of damaged or lost tissue.[14–16] These studies point to a central role of MSCs in tissue repair as trophic mediators, secreting factors promoting tissue-specific cells to restore the damaged or lost tissue.[17,18]

Two explanations have been proposed to explain increased cartilage formation in cocultures of MSCs and articular chondrocytes. First, it has been suggested that increased cartilage formation in cocultures is due to chondrogenic differentiation of MSCs stimulated by factors secreted by chondrocytes. Indeed, chondrocyte-conditioned medium can induce chondrogenic differentiation of MSCs directly and in transwell cultures.[19,20] However, it is unclear whether such an effect also occurs in cocultures in which the cells are in direct cell–cell contact. Second, studies have hypothesized that the increased cartilage matrix formation is due to stimulation of the chondrocytes by MSCs.[6] Scientific evidence for this hypothesis is rather limited due to the inability to distinguish between the contributions of the individual cell populations to cartilage formation.

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In this study we have addressed these issues by setting up pellet coculture models of human MSCs (hMSCs) and either human primary chondrocytes (hPCs) or bovine PCs (bPCs). Using a xenogenic system allowed us to determine the contribution of each cell population to the increased cartilage formation by using species-specific gene expression analysis, whereas xenogenic-specific effects were excluded in the human coculture system. We examined chondrogenic gene expression, cell apoptosis, and cell proliferation in human and bovine cell populations. Our data clearly demonstrate that the increased cartilage deposition in cocultures is mainly due to a trophic role of the MSCs in stimulating chondrocyte proliferation and matrix deposition rather than MSCs actively undergoing chondrogenic differentiation.

Materials and Methods

Cell culture and expansion

bPCs were isolated from full-thickness cartilage knee biopsies of female calves of ~6 months old. Cartilage was separated and digested as previously described.[21] hPCs were obtained from full-thickness cartilage dissected from knee biopsies of a patient undergoing total knee replacement as published previously.[11] MSCs were isolated from aspirates as described previously.[22] The use of bone marrow aspirates and human knee biopsies was approved by a local Medical Ethics Committee. Donor information of human primary cells is listed in Supplementary Table T1 (Supplementary Data are available online at www.liebertonline.com/tea). We define the "primary" cells (bPCs, hPCs, and hMSCs) in this article as cells with a low passage number without immortalization. Immortalized MSCs (iMSCs) were kindly provided by Dr. O. Myklebost (Oslo University Hospital, Norway). Culture condition and characteristics of iMSCs are described in Supplementary Figure S1.

To form high-density micromass cell pellets, 200,000 cells per well were seeded in a round-bottom 96-wells plate in the chondrocyte proliferation medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1× nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate, 0.4 mM proline, 100 U penicillin/mL, and 100 μg/mL streptomycin) or chondrogenic differentiation medium (see Supplementary Fig. S1) and centrifuged for 3 min at 2000 rpm. The medium was refreshed twice a week. For cocultures, iMSCs or hMSCs and bPCs or hPCs were mixed at ratios of 80%/20% and 50%/50%.

All reagents used for were purchased from Invitrogen unless otherwise stated. Common chemicals were purchased from Sigma-Aldrich.

Histology

Cell pellets were fixed with 10% formalin for 15 min and embedded in paraffin using routine procedures. Sections of 5 μm were cut and stained for sulfated glycosaminoglycans (GAGs) with alcian blue combined with counterstaining of nuclear fast red to observe nuclei, or stained with toluidine blue alone.

Quantitative GAG and DNA assay

Cell pellets ($n=6$) were washed with phosphate-buffered saline and frozen overnight at −80°C. Subsequently, they were digested and measured for GAG quantification as previously reported.[11] Relative cell number was determined by quantification of total DNA using a CyQuant DNA Kit, according to the manufacturer's instructions.

DNA isolation, RNA isolation, and quantitative polymerase chain reaction

Total DNA was isolated from pellet cultures with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA was isolated from pellet culture with the RNeasy Mini Kit (Qiagen). One microgram of total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad). The primers for quantitative polymerase chain reaction (qPCR) are listed in Supplementary Tables ST2 and ST3. Detailed description for qPCR can be found in the Supplementary Methods.

Cell tracking with organic fluorescent dyes

The organic fluorescent dyes CM-DiI and CFSE were used for cell tracking in cocultures. Cells were labeled according to the manufacturer's protocols.

5-ethynyl-2′-deoxyuridine and TUNEL staining

For labeling of newly synthesized DNA, 5-ethynyl-2′-deoxyuridine (EdU) was added to the culture media at a concentration of 10 μM, 24 h before harvesting the samples. Cell pellets were then washed with phosphate-buffered saline and fixed with 10% formalin for 15 min. Samples were embedded in cryomatrix, and cut into 10 μM sections with a cryotome (Shandon). Sections were permeabilized and stained for EdU with Click-iT® EdU Imaging Kit. Cryosections were also stained for DNA fragments with DeadEnd Fluorometric TUNEL System (Promega). Nuclei were counterstained with Hoechst 33342.

Image acquisition and analysis

All fluorescent images were taken with a BD pathway 435 confocal microscope (BD Biosciences), unless otherwise stated. Using montage capture, images of high resolutions were obtained covering the entire section of a pellet. Separate images were captured using BP536/40 (Alexa 488), BP593/40 (DiI), and LP435 (Hoechst 33342) and pseudocolored green, red, and blue, respectively. ImageJ software[23] was used for cell counting. Briefly, we manually set a threshold to avoid artifacts. The number of green cells, red cells, green + red cells, and total cells were counted by running plug-ins written in macro language of ImageJ (available on request). Values represent the mean ± standard error of at least three biological replicates.

Preparation of conditioned medium

For the conditioned medium, Dulbecco's modified Eagle's medium was incubated with iMSCs of 90% confluence for 48 h, passed through a 0.22 mm filter, and stored at −20°C. Upon use, the conditioned medium was thawed, transferred to Amicon Ultra-15 Centrifugal Filter Unites (Millipore) with a cut off of 3000D Nominal Molecular Weight Limit, and centrifuged at 4000 *g* for 40 min. The concentrated solute (still named conditioned medium) was supplemented with

all chemicals needed for chondrocyte proliferation medium (see Cell culture and expansion).

Short tandem repeats analysis

Genomic DNA samples were extracted from pellets with the QIAamp DNA Mini Kit (Qiagen). The sixteen loci of the kit PowerPlex 16 System (Promega) were amplified, typed, sequenced, and analyzed by ServiceXS B.V. Specific alleles for the donor of hMSCs and the donor of hPCs were found in six loci (D7S820, D5S818, D13S317, D16S539, CSF1PO, and Penta_D). These alleles were used to define the origin of cells in allogeneic coculture of hMSCs and hPCs. The amount of DNA present for each donor was calculated from the areas of the electropherogram for each locus of hMSCs' or hPCs' specific alleles and the ratio of hMSCs and hPCs was determined.

Statistical analysis

For the experiments using primary human cells (hMSCs and hPCs), three donors were tested, which showed similar results. Each experiment was performed at least in triplicate. So, only data from one representative donor are shown. Experiments using iMSCs and bPCs were performed at least in triplicate with similar data. A representative experiment is shown. Differences between different ratios of cocultures of MSCs and PCs were examined for statistical significance with one-way analysis of variance followed by Tukey HSD Tests. Comparisons between iMSCs and bPCs in the same conditions were made using the Student's t test. p-values of <0.05 were considered significant.

Results

Coculturing hMSCs with hPCs enhanced cartilage matrix formation

To study the contribution of MSC and chondrocytes on cartilage matrix formation, we cocultured hMSCs with hPCs from different donors. After 4 weeks coculture in the chondrogenic differentiation medium, histology (Fig. 1A) and GAG assay (Fig. 1B) indicated that coculture of hMSCs and hPCs increased cartilage formation. To determine the ratio of MSC and PC after prolonged coculture, we isolated genomic DNA, and short tandem repeat (STR) loci with different repeat sizes in the different donors were analyzed. The results of locus D7S820 (Fig. 1C) as well as analysis of other five STR loci (Supplementary Table S4) indicated that the proportion of hMSCs decreased significantly.

To elucidate the mechanisms behind the apparent loss of MSC in our coculture system, we used xenogenic cocultures of hMSCs and bPCs to enable identification of the role of each of the cell types in coculture in pellet cultures. An advantage of these xenogenic cocultures is that this system is more stable than coculture systems that depend on donor hPCs isolated after total knee replacement surgery.

Xenogenic coculture of hMSC and bPCs show enhanced chondroinduction

To allow long-term cell tracking in cocultures, we set up a pellet coculture model of hMSCs and bPCs. Cells were mixed in different ratios and pellet culture was performed in the chondrocyte proliferation medium lacking transforming growth factor-β and dexamethason. After 4 weeks, histology and GAG quantification were performed to evaluate cartilage formation. Alcian blue staining indicated the presence of GAG in all experimental groups except in the 100% hMSCs pellets (Fig. 2A, left panel) in concordance with the absence of chondrogenic factors in the medium. In the positively stained areas at higher magnification (Fig. 2A, right panel), cells showed a typical chondrocyte morphology and embedding in lacunae. Similar data were obtained by toluidine blue staining (Supplementary Fig. S2). GAG quantification showed a trend of decreased total GAG with increasing seeding percentage of hMSCs. However, when total GAG content was normalized to DNA or to the initial seeding percentage of bPCs, coculture pellets showed significantly higher GAG content. Similar data were obtained when different MSC donors were used (data not shown).

To avoid the effects of donor variation of primary cultured MSCs,[24] we replaced hMSCs with a telomerase immortalized hMSC cell line (iMSCs). This cell line resembled primary cultured MSCs in their ability to differentiate into the adipogenic, osteogenic, and chondrogenic lineages (Supplementary Fig. S1), but had relatively low capacity of chondrogenic differentiation. As shown in Figure 2C and D, coculture of bPCs with iMSCs for 4 weeks increased cartilage formation after correction for DNA content or initial seeding percentage of bPCs as compared with hMSCs. Despite the relatively low chondrogenic potential of iMSCs, increased cartilage matrix formation was observed in coculture of bPCs and iMSCs. This demonstrated that iMSCs show comparable behavior to hMSCs in cocultures with regard to enhanced cartilage formation, indicating that it is not the chondrogenic capacity of the MSCs that is responsible for enhanced chondroinduction.

Chondrocytes are located at the periphery of the cell pellet

We used organic fluorescent dyes to label individual cell populations in pellet cocultures for short-term cell tracking. Pellets were formed after 1 day of culture (Fig. 3A). Rather than forming a homogenous pellet, both cell populations tended to segregate. This process continued in the following days and the dynamic cell pellets became more and more stable. After 4 days of coculture, pellets were organized in a layer-like structure in which iMSCs resided predominantly in the core of the pellet and the bPCs, mixed with a subfraction of iMSCs, were predominantly found at the periphery. These observations are in agreement with the differential adhesion hypothesis, which stipulates that mixed heterotypic cells rearrange to adopt a combination-specific anatomy.[25] From day 5 onward, fluorescent dye transfer between labeled and nonlabeled cells in the pellets became apparent as reported previously.[26] This made it impossible to perform long-term cell tracking in coculture pellets using CM-DiI and/or CFSE labeling of cell populations.

Enhanced cartilage matrix formation originates from bPCs

After 1 day and 4 weeks of culture we isolated genomic DNA from the cell pellets and performed species-specific qPCR for genomic GAPDH. As shown in Figure 3B, after 1 day the ratio of genomic human and bovine DNA was in line with the seeding percentage of both cell populations. The percentage of human DNA was slightly higher, which is most likely explained by faster aggregation of the iMSCs in

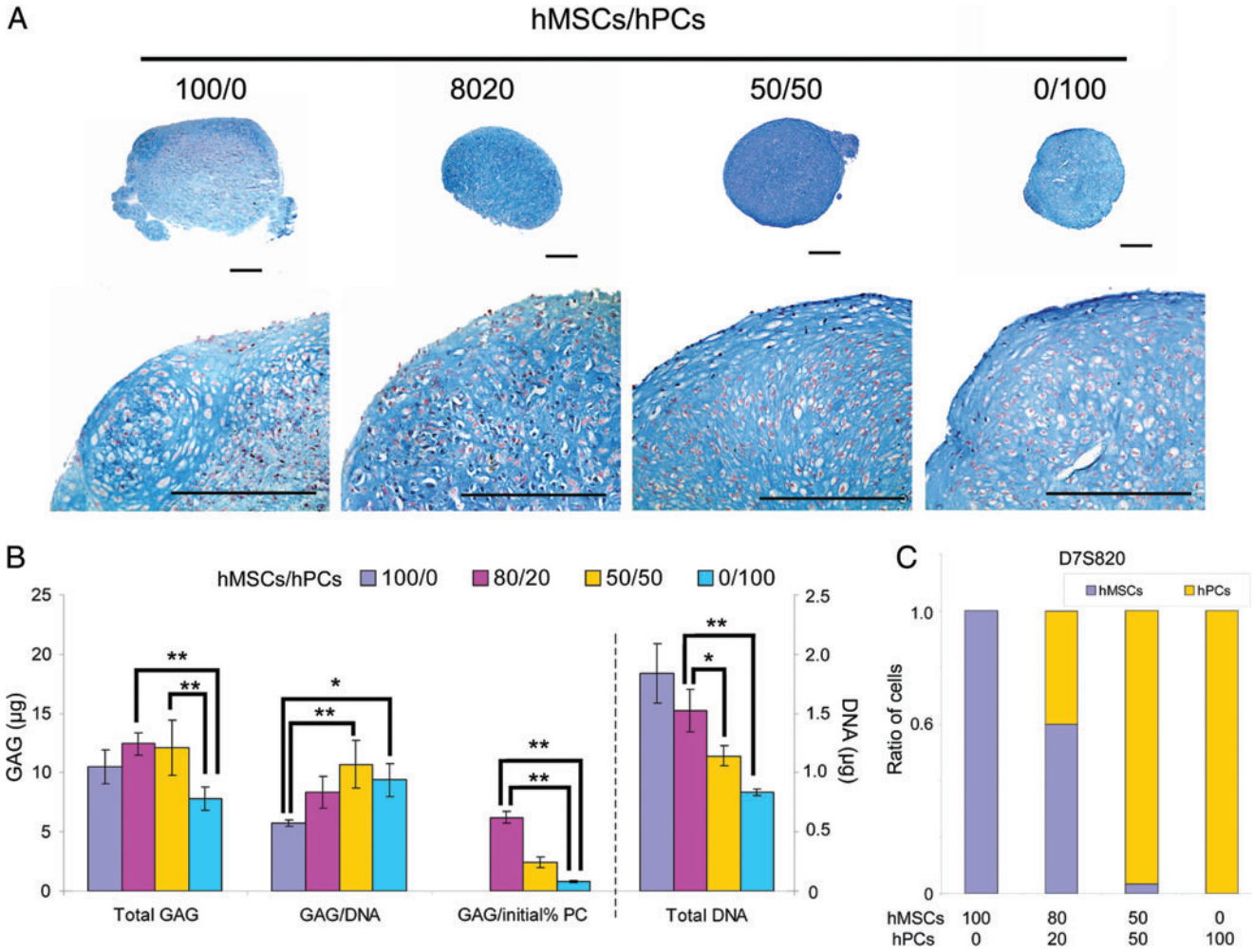

FIG. 1. Human mesenchymal stem cells (hMSCs)/human primary chondrocytes (hPCs) cocultures enhance cartilage matrix formation and show decrease of MSCs after 4 weeks of culture. **(A)** Alcian blue staining indicates the presence of glycosaminoglycan (GAG). Pellets were cultured in the chondrogenic differentiation medium (as described in Supplementary Data) for 4 weeks before examination. Scale bar = 200 μm. **(B)** Biochemical assay shows an increase in GAG in coculture pellets. Amount of GAG and DNA of pellets ($n = 6$) were measured 4 weeks after culture in the chondrogenic differentiation medium. Ratios of hMSCs and bovine PCs (bPCs) are indicated by different colors of bars. Scale on the left is for Total GAG, GAG/DNA, and GAG/initial% PC, whereas scale on the right is for Total DNA. Asterisk represents $p < 0.05$. Double asterisk represents $p < 0.01$. Error bar reflects standard deviation (SD). **(C)** Analysis of short tandem repeat locus D7S820 reflects ratios of hMSCs and hPCs after 4 weeks of coculture. Initial ratio of hMSCs and hPCs are indicated at the bottom of the bar chart. Color images available online at www.liebertonline.com/tea

pellets. Remarkably, after 4 weeks of culture, the coculture pellets contained predominantly DNA of bovine origin indicative for an overgrowth of bovine cells or a loss of human cells during the 4-week cell culture period. DNA analysis of coculture pellets at 1, 2, and 3 weeks of culture demonstrated a steep drop in human DNA between 1 and 2 weeks with a further gradual decline at weeks 3 and 4 (data not shown).

An even more striking difference was found in mRNA isolated at 4 weeks. GAPDH mRNA in the coculture pellets was from bovine origin (Fig. 3C). Even in cell pellets with an initial seeding of 80% iMSCs, hardly any human mRNA was detected. Similar data were found in coculture pellets of hMSCs and bovine chondrocytes after 4 weeks of culture also demonstrating the near absence of human DNA in the cell pellets, which is fully in line with the data obtained in coculture pellets of iMSCs and bPCs (Fig. 3D) and hMSC and hPC (Fig. 1C).

We next performed species-specific qPCR to study the origin of the mRNA expression of chondrogenic genes in coculture pellets (Fig. 3F, G). At week 4, only expression of chondrogenic genes from bovine origin were detected in coculture pellets. These data suggested that the cartilaginous matrix in coculture pellets is from bovine origin. This observation, combined with the observation that in allogeneic cocultures the percentage of MSCs decreased during prolonged culturing, suggests that the enhanced contribution of chondrocytes in the matrix formation may be due to PCs proliferation or MSC cell death.

iMSCs cocultured with bPCs die via apoptosis

To determine whether MSC undergo apoptosis during prolonged cell culture, we performed a fluorescent TUNEL

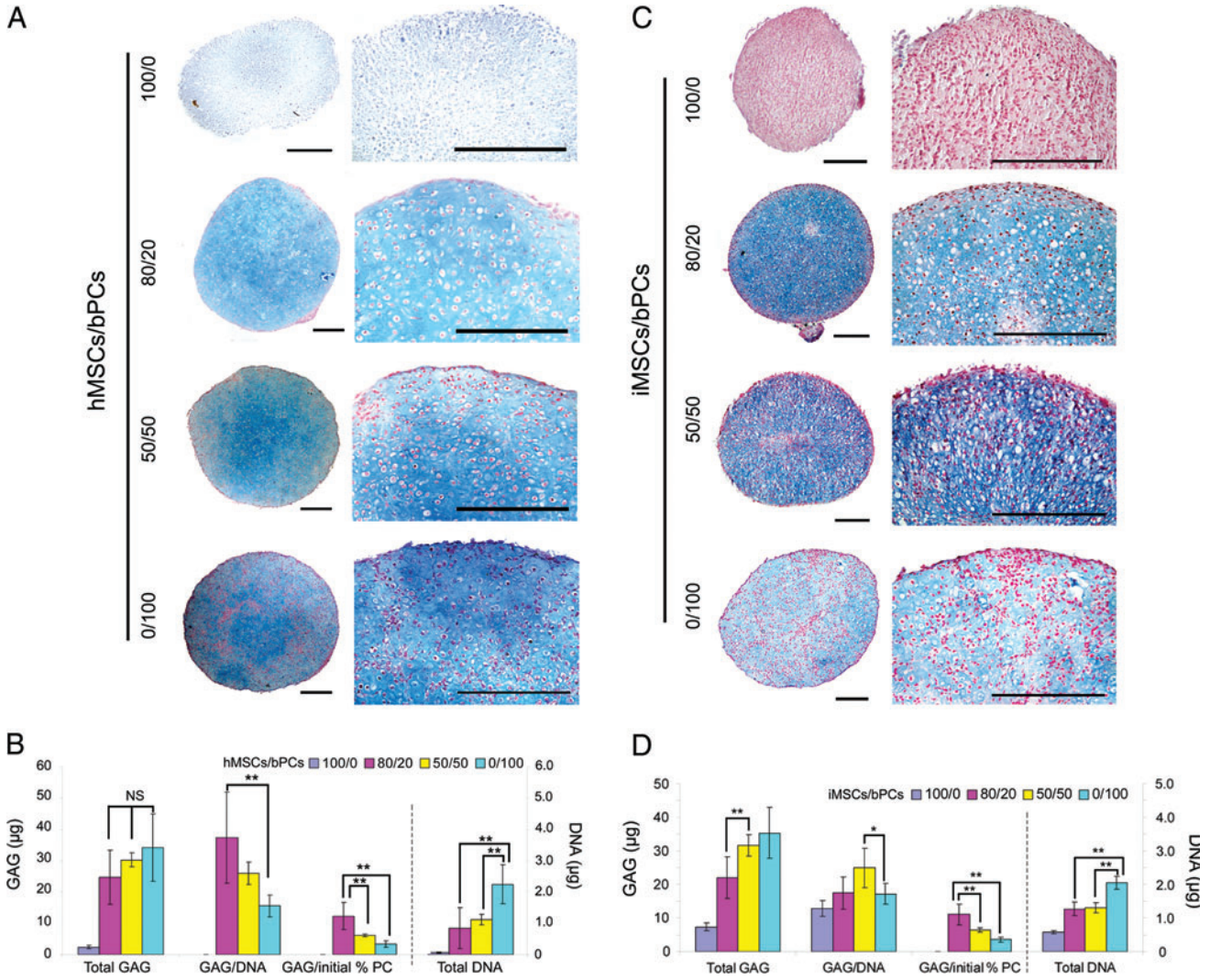

FIG. 2. Coculture of MSCs and chondrocytes increases cartilage matrix formation. **(A)** Alcian blue staining shows the presence of GAG in pellets cultured in the chondrocytes proliferation medium. Ratios of hMSCs and bPCs are indicated on the left of the images. The left panel shows overviews of pellets, whereas the right panel shows magnified pictures. Scale bar = 200 μm. **(B)** Biochemical assay shows an increase in GAG in coculture pellets. Amount of GAG and DNA of pellets ($n = 6$) was measured 4 weeks after culture in the chondrocyte proliferation medium. Ratios of hMSCs and bPCs are indicated by different colors of bars. Scale on the left is for Total GAG, GAG/DNA, and GAG/initial 10%PC, whereas scale on the right is for Total DNA. Double asterisk represents $p < 0.01$. NS, not significant. Error bar reflects SD. **(C)** Alcian blue staining of pellets cultured in the chondrocyte proliferation medium. Ratio of immortalized MSCs (iMSCs) and bPCs is indicated on the left of the images. The left panel shows overviews of pellets, whereas the right panel shows magnified pictures. Scale bar = 200 μm. **(D)** Biochemical assay of pellets ($n = 6$) at 4 weeks after culture in the chondrocyte proliferation medium. Ratios of iMSCs and bPCs are indicated by different colors of bars. Scale on the left is for Total GAG, GAG/DNA, and GAG/initial PC, whereas scale on the right is for Total DNA. Asterisk represents $p < 0.05$. Double asterisk represents $p < 0.01$. Error bar reflects SD. Color images available online at www.liebertonline.com/tea

assay. At weeks 1 and 2, high numbers of TUNEL-positive cells were found in all cell pellets containing iMSCs, but not in pure bPCs cell pellets (Fig. 4). TUNEL-positive cells were predominantly present in the periphery of the pellets, which is mostly composed of bPCs mixed with iMSCs (Fig. 3A). Fewer TUNEL-positive cells were found in the core of the pellet. Cell death in iMSC-containing pellets started to increase significantly from day 5 onward. From this time point cell tracking results by fluorescent labeling of cell populations became unreliable due to nonspecific dye transfer. Since the TUNEL-positive cells are predominantly found in iMSCs containing cell pellets and human DNA over time disappears from the cell pellets, we concluded that cell death by apoptosis at least partially explains the disappearance of human DNA from coculture cell pellets.

iMSCs stimulate chondrocyte proliferation in pellet cocultures

We then examined cell proliferation in coculture pellets using EdU incorporation. We focused on time points up to

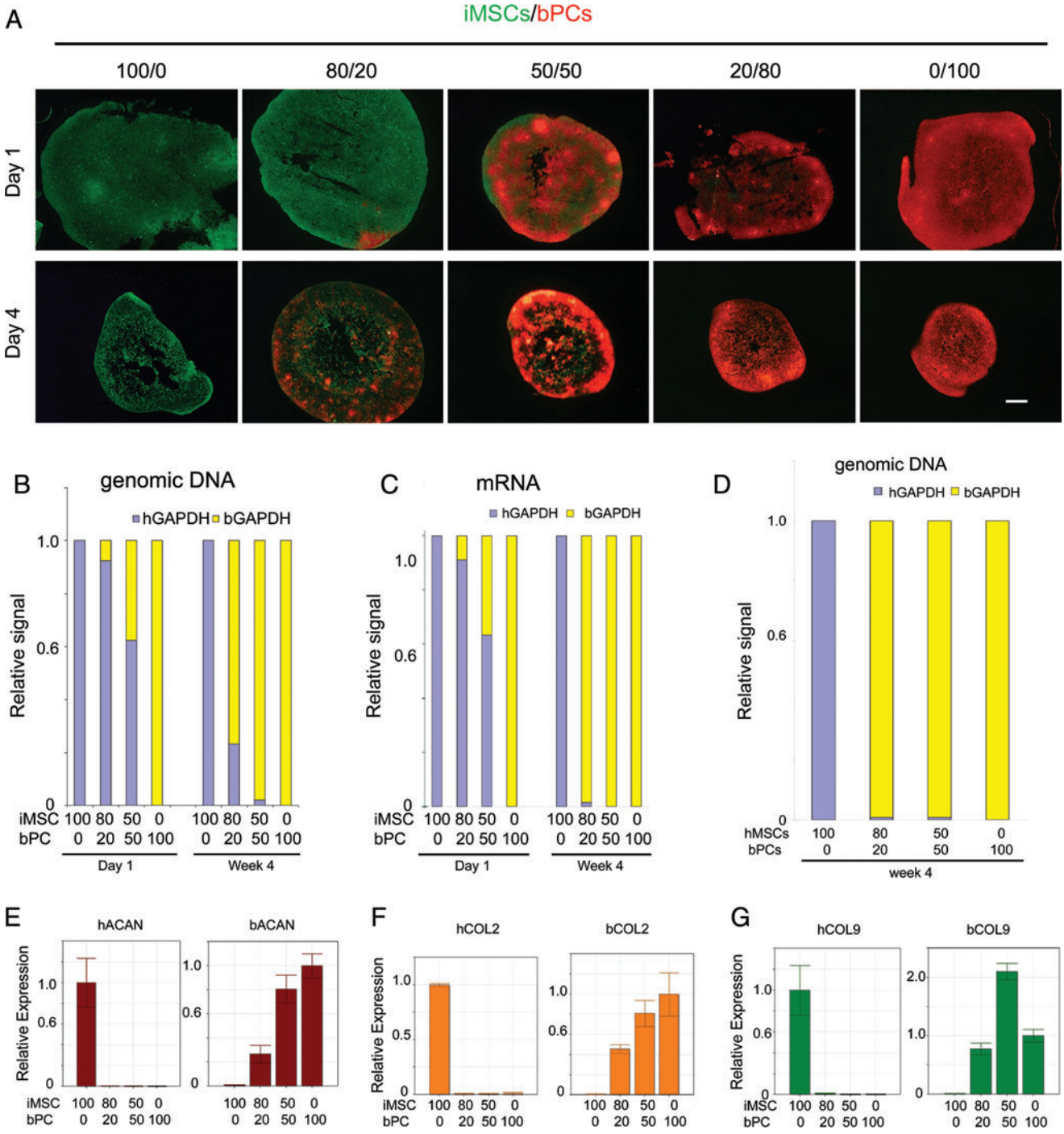

FIG. 3. Cartilage matrix is from bovine origin. **(A)** Cell assembly of iMSCs and bPCs in coculture pellets. iMSCs and bPCs were labeled with CFSE O (green) and CM-DiI (red), respectively, mixed at different ratios and then cultured in the chondrocyte proliferation medium. At days 1 and 4, pellets were harvested for cryosection. Images were made directly on the sections without any treatment, using a Nikon E300 fluorescent microscope. Scale bar = 200 μm. **(B)** Species-specific quantitative polymerase chain reaction (qPCR) of GAPDH in cocultures of iMSCs and bPCs at genomic DNA level. Genomic DNA was extracted from pellets ($n = 3$) at day 1 and week 4. **(C)** Species-specific qPCR of GAPDH in cocultures of iMSCs and bPCs at mRNA level. RNA was extracted from pellets at day 1 and week 4. **(D)** Species-specific qPCR of GAPDH in cocultures of hMSCs and bPCs at genomic DNA level. Genomic DNA was extracted from pellets ($n = 3$) at week 4. **(E–G)** Expression levels of ACAN **(E)**, COL2 **(F)**, and COL9 **(G)** were examined by species-specific qPCR. RNA samples were extracted from pellets ($n = 3$) cultured in the chondrocyte proliferation medium for 4 weeks. Relative expression levels were obtained by normalization of human- or bovine-specific signals to cross species-specific GAPDH and β-actin signals. For human-specific genes, values are relative amounts to 100/0 iMSC/bPC group. For bovine-specific genes, values are relative amounts to 0/100 iMSC/bPC group. Error bar reflects SD. Color images available online at www.liebertonline.com/tea

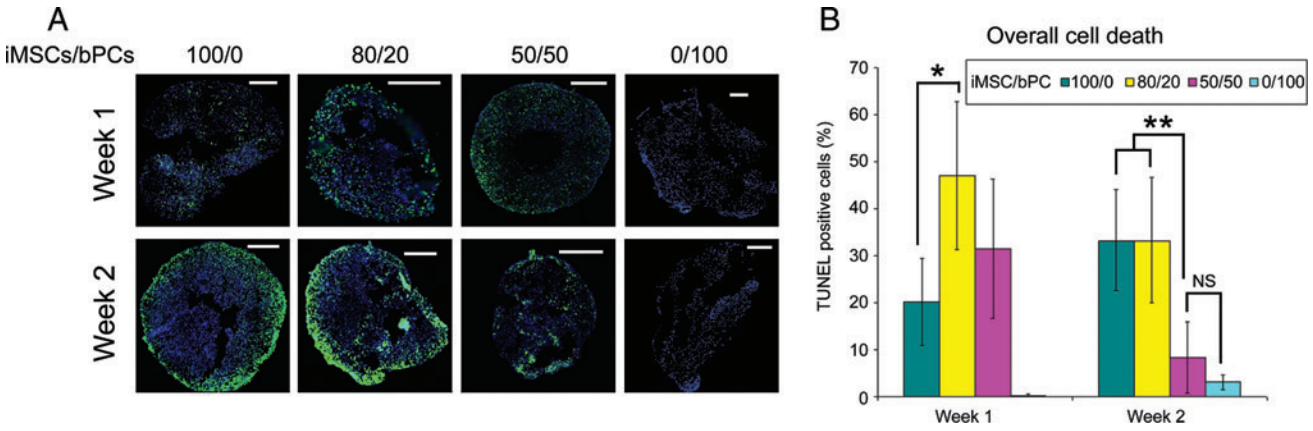

FIG. 4. Preferential cell deaths of MSCs by apoptosis. **(A)** TUNEL staining of pellets. Cell pellets were cultured in the chondrocyte proliferation medium for 1 or 2 weeks before harvesting for cryosection. TUNEL-positive cells were observed with fluorescent labeling (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 200 μm. **(B)** Quantification of TUNEL-positive cells. Ratios of iMSCs and bPCs are indicated by bar colors. Data from 3 pellets were analyzed for statistic significance. Asterisk represents $p < 0.05$. Double asterisk represents $p < 0.01$. NS, not significant. Error bar reflects SD. Color images available online at www.liebertonline.com/tea

3 days, in which organic fluorescent dyes are highly reliable for cell tracking.[27] bPCs were labeled with CM-DiI (red) to distinguish them from iMSCs. At day 1, EdU-positive cells were evenly distributed over the pellet. At days 2 and 3, EdU-positive cells were predominantly found at the periphery of the pellets where red-labeled bPCs resided (Fig. 5A). We determined the percentage of EdU-positive iMSCs or bPCs in cocultures. Generally, coculture increased the proliferation of both iMSCs and bPCs (Fig. 5B, C). Interestingly, the percentage of EdU-positive bPCs was higher than that of iMSCs in cocultures of 80% iMSCs and 20% bPCs starting from day 2 onward (Fig. 5B).

Similar data were obtained in dye swap experiments in which iMSCs instead of bPCs were labeled with CM-DiI, demonstrating that enhanced proliferation of bPCs in coculture pellets was not an artifact of cell labeling (Supplementary Fig. S3).

These data show that the change in ratio between MSC and PC during prolonged coculturing is in addition to apoptosis also due to increased proliferation of chondrocytes in pellet cultures.

iMSC-conditioned medium increases bPCs proliferation and matrix formation

To examine the effects of secreted factors, we compared proliferation and matrix formation of bPCs when cultured in the proliferation medium or in the 50–100-times concentrated iMSC conditioned medium. The concentrate was dissolved in the chondrocyte proliferation medium. Pellets of bPCs cultured for 1 week in the iMSC conditioned proliferation medium showed higher EdU incorporation than cells cultured in the nonconditioned proliferation medium (Fig. 6A, B). Like in coculture pellets EdU-positive cells were predominantly found in the periphery of the pellet. Higher EdU incorporation was associated with increased DNA content. Additionally, total GAG content showed an increase, but GAG corrected for DNA was not significantly different between the two conditions (Fig. 6C).

Discussion

It has been shown that the conditioned medium of chondrocytes induced osteo-chondrogenic differentiation of MSCs[19] and coculture of MSCs and chondrocytes in three-dimensional environments induced chondrogenic gene expression in MSCs.[28] Based on these studies, it was suggested that the beneficial effects of coculturing chondrocytes and MSCs in cartilage matrix formation are largely due to the differentiation of MSCs into chondrocytes. In this study, we show that pellet cocultures of chondrocytes and MSCs in chondrocyte proliferation medium benefit cartilage formation. Further, we observe a significant decrease in MSCs caused by a preferential cell death of MSC. After 4 weeks of culture, this results in an almost homogeneous cartilage construct, in which mainly chondrocyte-derived cells reside. The beneficial effects of the pellet coculture are largely due to stimulation of proliferation and matrix formation of chondrocytes induced by a trophic effect of the MSCs. Our investigation distinguishes itself from comparable studies, the design of which did not allow discrimination between the contributions of individual cell populations to cartilage matrix formation.[5,6] Although we cannot completely rule out the possibility that a subset of MSCs differentiated into chondrocytes and directly participated in cartilage formation, our data suggest that this may apply to a minority of cells only.

In pellet cocultures of hMSCs and bovine chondrocytes, one may argue that our observations are due to a species difference, which may hamper the response of bovine chondrocytes to hMSCs and vice-versa. However, species specificity cannot explain our findings since similar observations were made in a fully human coculture model, indicating that in both models comparable mechanisms are likely operational. In addition, we show that the mechanisms underlying these observations are not donor specific, but are due to cell type-specific contribution of MSCs as well as the chondrocytes. As shown in this study, as well as in many other studies,[5,6,11] coculture of hMSCs or other cell types[29,30] with xenogenic chondrocytes appears a good model to study cell-specific contributions to tissue formation.

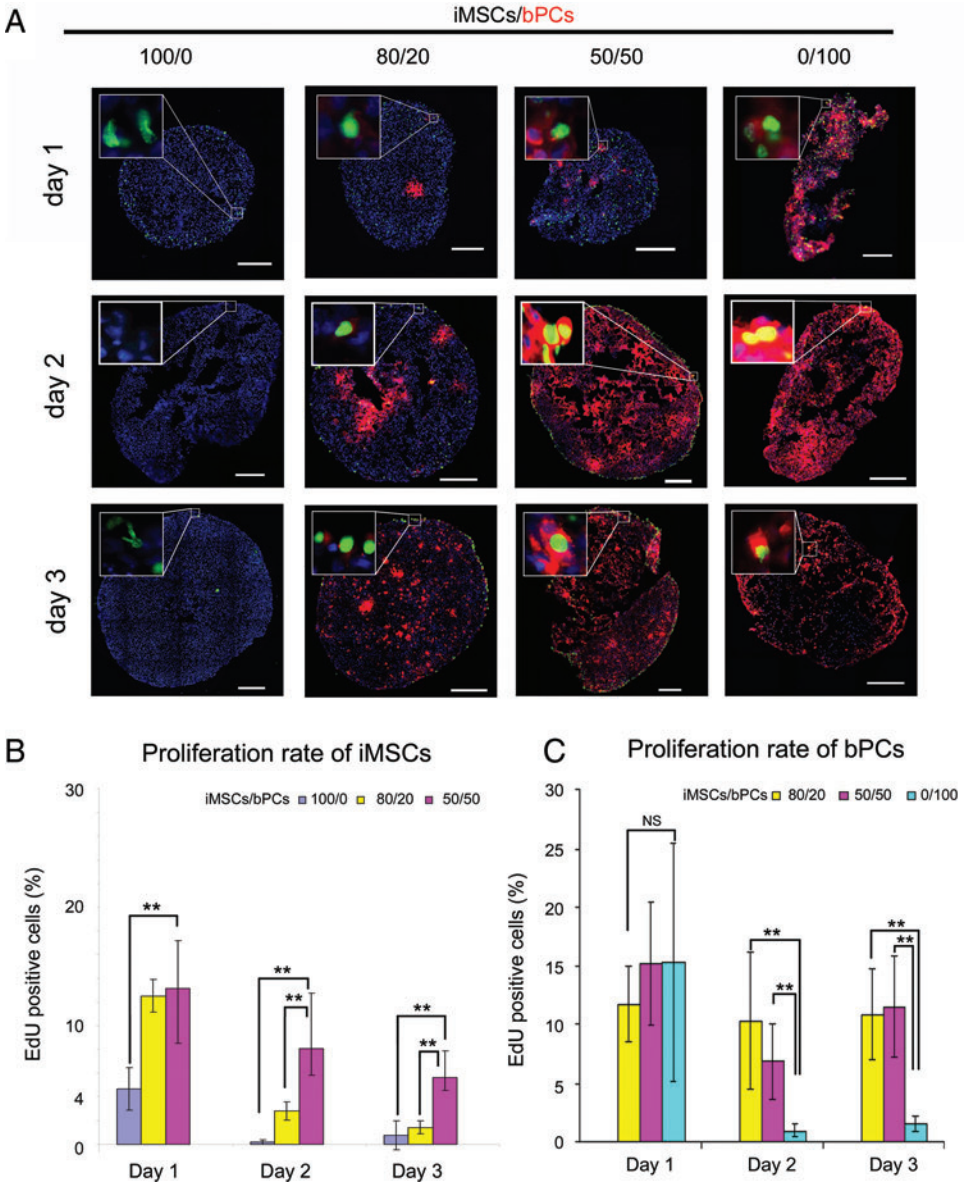

FIG. 5. MSCs stimulate chondrocyte proliferation in coculture pellets. **(A)** 5-Ethynyl-2′-deoxyuridine (EdU) staining of pellets at days 1, 2, and 3. bPCs were labeled with CM-DiI (red). EdU incorporation into newly synthesized DNA was observed by Alexa 488 (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 200 μm. Inserts show enlarged images. **(B)** Quantification of EdU-positive iMSCs in all conditions. The initial ratios of MSCs and bPCs are indicated by bar colors. Data from three pellets were analyzed for statistic significance. Double asterisk represents $p < 0.01$. Error bar reflects SD. **(C)** Quantification of EdU-positive bPCs in all conditions. The initial ratios of MSCs and bPCs are indicated by bar colors. Data from three pellets were analyzed for statistic significance. Double asterisk represents $p < 0.01$. NS, not significant. Error bar reflects SD. Color images available online at www.liebertonline.com/tea

In our studies, we have used human telomarase reverse transcriptase (hTERT) immortalized hMSCs[31] as well as primary hMSCs. The iMSCs used in this study had a reduced chondrogenic potential. This lack of chondrogenic capacity did not impair their ability to stimulate cartilage formation in pellet cocultures, further providing evidence that chondrogenic differentiation of MSC does not significantly contribute significantly to the enhanced cartilage formation. Further, similar results were obtained with primary hMSCs. Our data do indicate that chondrogenic capacity of cells used in cocultures is not essential for stimulation of cartilage formation by chondrocytes in line with previous observations.[11] In addition, our data suggest that the relatively old age (60+ years) of the MSC donors does not affect their ability to simulate cartilage formation in coculture.

Cell proliferation in pellet cocultures was studied using EdU incorporation in DNA of proliferating cells. Cell proliferation was significantly increased in coculture pellets compared with pellets of pure cell populations. By using cell-specific labeling techniques and dye swap experiments, it was shown that EdU was preferentially incorporated in chondrocytes, which reside predominantly in the periphery of the cell pellet. This suggests that the MSCs are potent stimulators of chondrocyte proliferation in pellet cocultures. Limited proliferation of cells was found in the core of the cell pellet in which predominantly MSCs resided. Since EdU is extremely small (252 Da), this molecule is likely to penetrate with high efficiency in the pellet,[32] suggesting that the preferential EdU labeling of cells in the periphery of the pellets is not an artifact caused by diffusion limitation. It is assumed that absence of proliferating MSCs in the center of the pellets is likely due to space limitation in the compacted core creating an environment, which is not permissive for cell division.[33,34]

In coculture pellets significant numbers of TUNEL-positive MSCs were observed after 1 and 2 weeks of culture, suggesting that MSCs most likely died via apoptosis. Also, in pellets composed of 100% MSCs but not 100% bPCs, significant TUNEL staining was observed. Cell labeling experiments

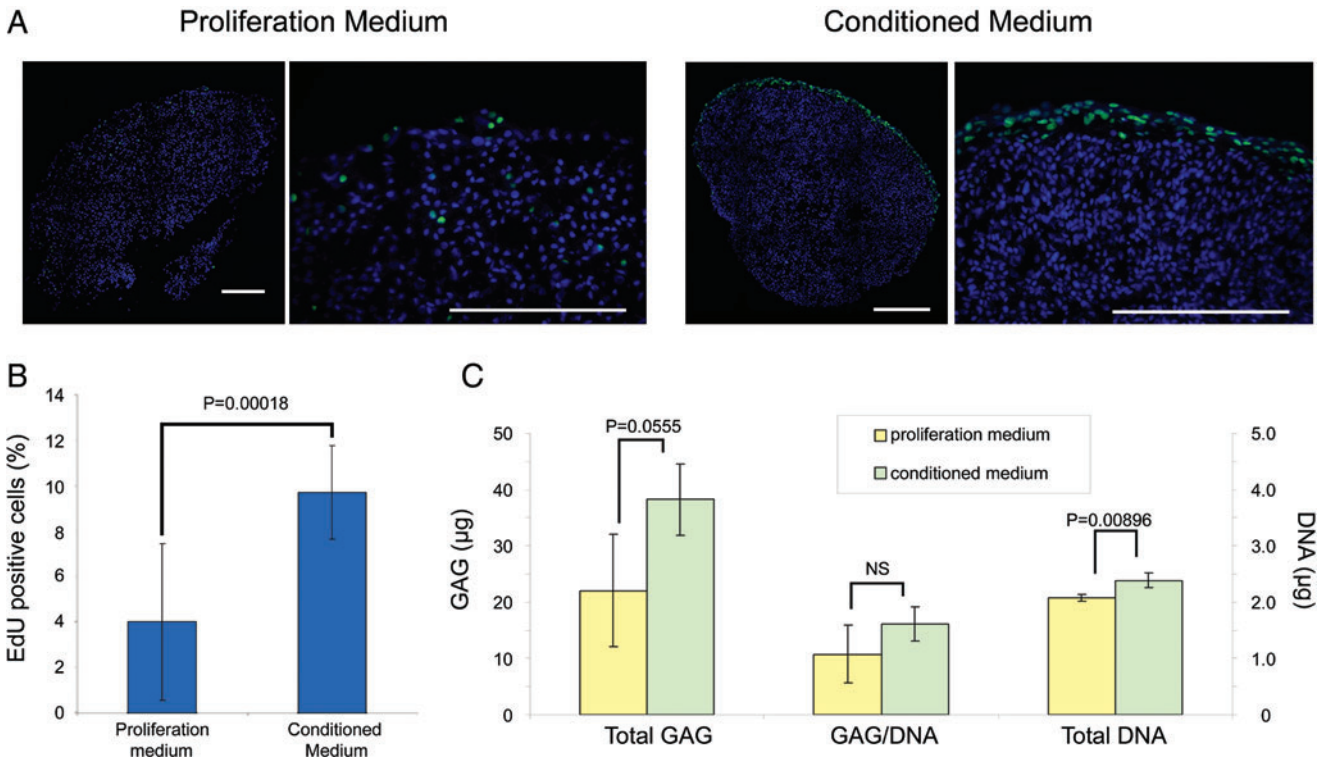

FIG. 6. The conditioned medium of iMSCs enhances proliferation of chondrocytes. **(A)** EdU staining of bPCs pellets at day 2 after culturing in the chondrocyte proliferation medium or conditioned medium of iMSCs. EdU incorporation into newly synthesized DNA was observed by Alexa 488 (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 200 μm. **(B)** Quantification of EdU-positive cells. Data from 3 pellets were analyzed for statistic significance. *p*-Value indicated in the bar chart is calculated by Student's *t*-test. **(C)** GAG and DNA assay were performed at week 1 after culturing in the chondrocyte proliferation medium or conditioned medium of iMSCs. The left scale is for Total GAG and GAG/DNA, whereas the right scale is for Total DNA. *p*-Values indicate on the graph were calculated with the Student's *t*-test. NS, not significant. Error bar reflects SD. Color images available online at www.liebertonline.com/tea

in pellet cocultures demonstrated that the majority of the TUNEL-positive cells were hMSCs. This is in line with the STR and genomic DNA analysis at the end of the culture period showing the disappearance of the MSCs from the cocultures over time. Our data suggest that the disappearance of MSCs in pellet cocultures is most likely caused by apoptosis. Interestingly, TUNEL-positive cells were predominantly found in the periphery of the pellet in which MSCs coresided with chondrocytes. TUNEL positivity was higher in coculture pellets than in pellets of pure cell populations. This suggested that in addition to suboptimal culture conditions of MSCs in pellets, the presence of chondrocytes may have contributed to the death of MSCs. This may be caused by secreting apoptosis-inducing cytokines.[35] Further, changes in extracellular matrix in pellet cultures as compared with natural extracellular matrix of MSCs may influence the fate of MSCs,[36–38] and this may have contributed to the increased cell death. Other explanations for death of MSCs in pellets could be cell compaction, and nutrition or space limitation in pellets.[39,40] However, the relatively low levels of TUNEL-positive cells in the core of the pellet compared with the periphery suggest that nutrient or oxygen limitation, which are likely most pronounced in the core of the pellet, are insufficient to induce cell death.

We provide evidence that the induction of chondrocyte proliferation by MSCs is most likely caused by secreted factors, since this effect was at least partly mimicked by using the MSC-conditioned medium. It has been reported that MSCs secrete a broad range of growth factors and cytokines, including interleukin-6, hepatocyte growth factor, and vascular endothelial growth factor, which enhance cell viability and proliferation *in vitro* and restore functions of damaged tissue *in vivo*.[41,42] Interleukin-6, for example, has been described to induce cartilage repair by increasing chondrocyte proliferation and stimulation of expression of cartilage matrix proteins and BMP-7.[43] On the other hand secreted factors by MSCs cannot fully explain increased cartilage formation in coculture pellets, since the relative deposition of GAGs per DNA was not significantly different between pellets cultured in the proliferation medium and that in the conditioned medium. This indicates that the conditioned medium only stimulated chondrocyte proliferation but not relative GAG amount per DNA, such as observed in coculture pellets. This is in line with other reports demonstrating a role of cell–cell contact in cartilage formation improvement in cocultures.[44–46] Therefore, it is likely that in addition to the trophic effects of MSCs mediated by secreted factors, enhanced cartilage formation in coculture with chondrocytes is due to additional stimuli such as direct cell–cell contact or other secreted factors.

Such a role of the MSCs as trophic mediators in cartilage formation in coculture pellets is in line with their proposed

role in tissue repair in other tissues, such as brain,[47,48] heart,[49–52] and kidney regeneration.[53,54] By providing nutrients and growth factors, MSCs increase proliferation and differentiation of host-derived cells to help them to repair damaged tissues.[55] The results of the present study are in line with and extent these observations to cartilage tissue formation. We are the first to show that MSCs have a prominent role as trophic mediators to stimulate cartilage matrix formation in pellet cocultures with chondrocytes.

Despite the success of ACI in treatment of large-size cartilage defects, the requirement of two operations separated by several weeks' expansion of chondrocytes *in vitro* is a major drawback of this procedure.[56] The results of this study imply that culture expansion of chondrocytes may benefit from coculturing with MSCs. The MSCs may not only stimulate proliferation, thereby shortening culture time, but simultaneously may help the chondrocytes to retain their phenotype by counteracting chondrocyte dedifferentiation.[41,42] They further imply that a substantial part of the chondrocytes needed for ACI may be substituted with MSCs without decrease in cartilage matrix formation. This may pave the road for a single step surgery to repair large-size cartilage defects, in which chondrocytes are isolated, mixed with bone marrow cells from the same patient, loaded on a scaffold, and directly re-implanted into the patient. Based on our *ex vivo* results, one may expect that in a few weeks the implant will consist mainly of chondrocytes and cartilage-specific matrix.

In conclusion, our data clearly demonstrate that in pellet cocultures of MSCs and PCs, MSCs disappear over time. Increased cartilage formation in these cocultures is mainly due to a trophic role of the MSCs in stimulating chondrocyte proliferation and matrix deposition by chondrocytes rather than MSCs actively undergoing chondrogenic differentiation.

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Disclosure Statement

No competing financial interests exist.

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