

ORIGINAL ARTICLE

Direct Cell–Cell Contact with Chondrocytes Is a Key Mechanism in Multipotent Mesenchymal Stromal Cell-Mediated Chondrogenesis

Tommy S. de Windt, MD, PhD,¹ Daniel B.F. Saris, MD, PhD,^{1,2} Ineke C.M. Slaper-Cortenbach, PhD,³ Mattie H.P. van Rijen, BSc,¹ Debby Gawlitta, PhD,¹ Laura B. Creemers, PhD,¹ Roel A. de Weger, PhD,⁴ Wouter J.A. Dhert, MD, PhD,⁵ and Lucienne A. Vonk, PhD¹

Using a combination of articular chondrocytes (ACs) and mesenchymal stromal cells (MSCs) has shown to be a viable option for a single-stage cell-based treatment of focal cartilage defects. However, there is still considerable debate whether MSCs differentiate or have a chondroinductive role through trophic factors. In addition, it remains unclear whether direct cell–cell contact is necessary for chondrogenesis. Therefore, the aim of this study was to investigate whether direct or indirect cell–cell contact between ACs and MSCs is essential for increased cartilage production in different cellular environments and elucidate the mechanisms behind these cellular interactions. Human ACs and MSCs were cultured in a 10:90 ratio in alginate beads, fibrin scaffolds, and pellets. Cells were mixed in direct cocultures, separated by a Transwell filter (indirect cocultures), or cultured with conditioned medium. Short tandem repeat analysis revealed that the percentages of ACs increased during culture, while those of MSCs decreased, with the biggest change in fibrin glue scaffolds. For alginate, where the lack of cell–cell contact could be confirmed by histological analysis, no difference was found in matrix production between direct and indirect cocultures. For fibrin scaffolds and pellet cultures, an increased glycosaminoglycan production and type II collagen deposition were found in direct cocultures compared with indirect cocultures and conditioned medium. Positive connexin 43 staining and transfer of cytosolic calcein indicated communication through gap junctions in direct cocultures. Taken together, these results suggest that MSCs stimulate cartilage formation when placed in close proximity to chondrocytes and that direct cell–cell contact and communication through gap junctions are essential in this chondroinductive interplay.

Introduction

TECHNIQUES FOR ARTICULAR cartilage repair have rapidly continued to develop. Where once debridement and microfracture were the only options to treat focal cartilage defects, several cell and cell-free options have been introduced.¹ The increasing patient population that has high demands in terms of treatment of symptoms and return to normal (sports) activity along with the rapid developments in regenerative medicine stimulates this evolution. Cartilage defects are recognized as injuries that can cause symptoms, such as pain, swelling, and locking, which have been documented to impair quality of life as much as osteoarthritis.²

Cell-based treatments, such as autologous chondrocyte implantation (ACI), typically used for larger defects (>2 cm²) have first been introduced in 1985.³ Since then, different generations of ACI have been developed and promising (long-term) results up to 20 years have supported the implementation of this advanced therapy medicinal product (ATMP) in specialized knee centers.^{4–6} However, the high costs and demands along with the burden of having to undergo two separate surgical procedures have incited the development of single-stage ATMPs. In recent years, different groups have focused on using a coculture approach to achieve this goal.⁷ The primary rationale behind this method is that the need for expansion of chondrocytes becomes

¹Department of Orthopaedics, University Medical Center Utrecht, Utrecht, The Netherlands.

²MIRA Institute for Biotechnology and Technical Medicine, University Twente, Enschede, The Netherlands.

³Cell Therapy Facility, Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht, The Netherlands.

⁴Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands.

⁵Faculty of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands.

redundant as these can be (partly) replaced by cells with multilineage potential such as mesenchymal stromal cells (MSCs). Indeed, when MSCs of different origins such as bone marrow, adipose tissue, or synovium are cocultured with chondrocytes, reproducible enhancement of chondrogenesis has been shown.⁸ However, although there is vast literature describing the use of cell combinations, the exact mechanisms that lay at the foundation of coculture-induced chondrogenesis remain unclear. Many authors have suggested that differentiation of MSCs lies at the basis of coculture-induced chondrogenesis, while there is little evidence to support this hypothesis.⁸ Meanwhile, recent studies have found MSCs to stimulate chondrocyte proliferation while slowly disappearing from the culture (chondroinduction).^{9,10} In addition, the necessity of cell-cell contact in cocultures remains unclear. In fact, although the majority of studies concluded that cell-cell contact is essential for chondrogenesis, two separate studies indicated that cocultures without direct cell-cell contact or conditioned medium can achieve similar chondrogenesis as direct cocultures.^{11,12} Still, a comprehensive study that takes different conditions into account such as cellular environments and scaffolds and that compares direct and indirect cell-cell contact using the same cell count in each condition is lacking. Therefore, questions remain as to what conditions are needed for optimal chondrogenesis and how this can be translated to new single-stage approaches. Therefore, the purpose of this study was to investigate whether direct or indirect cell-cell contact between articular chondrocytes (ACs) and MSCs is essential for chondrogenesis in different cellular environments and to elucidate the mechanisms behind these cellular interactions.

Material and Methods

Experimental design and study outline

To test the hypothesis that for coculture-induced chondrogenesis, a cellular environment is necessary that allows

close cell proximity, alginate beads (close, but no direct contact), fibrin glue scaffolds (cell-cell contact by allowing migration and aggregate formation), and cell pellets (direct cell-cell contact) were chosen. For each condition, both direct (two cell types mixed in the same culture system) and indirect (two cell types cultured in the same system, but separated by cell culture inserts) cocultures were used with 2.5×10^6 cells/mL at a 10% chondrocyte and 90% MSC ratio.¹³ To investigate the role of MSCs in cocultures, MSCs were replaced by dermal fibroblasts (DFs). Conditioned medium from both chondrocytes and MSCs was used to investigate the effect on chondrogenesis by either cell type. Figure 1 provides a schematic overview of the different study conditions. The difference in biochemically measured glycosaminoglycan (GAG) production, gene expression, and histology was compared between conditions. Using short tandem repeat (STR) analysis, the cell ratios were determined after the culture period. Besides, the hypothesis that ACs and MSCs communicate through gap junctions was assessed by staining gap junctions and a cytosolic dye transfer test.

Donors

Cartilage was obtained from redundant material from five patients who had undergone total knee arthroplasty (three males, two females, age 56–79 years, average 66 years). The anonymous collection of this material was performed according to the Medical Ethics regulations of the University Medical Center Utrecht and the guideline “good use of redundant tissue for research” of the Dutch Federation of Medical Research Societies.^{14,15}

Cell isolation and expansion

Cartilage samples were rinsed in phosphate-buffered saline (PBS), cut into small pieces, and digested in 0.15% collagenase (CLS-2; Worthington) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies) with

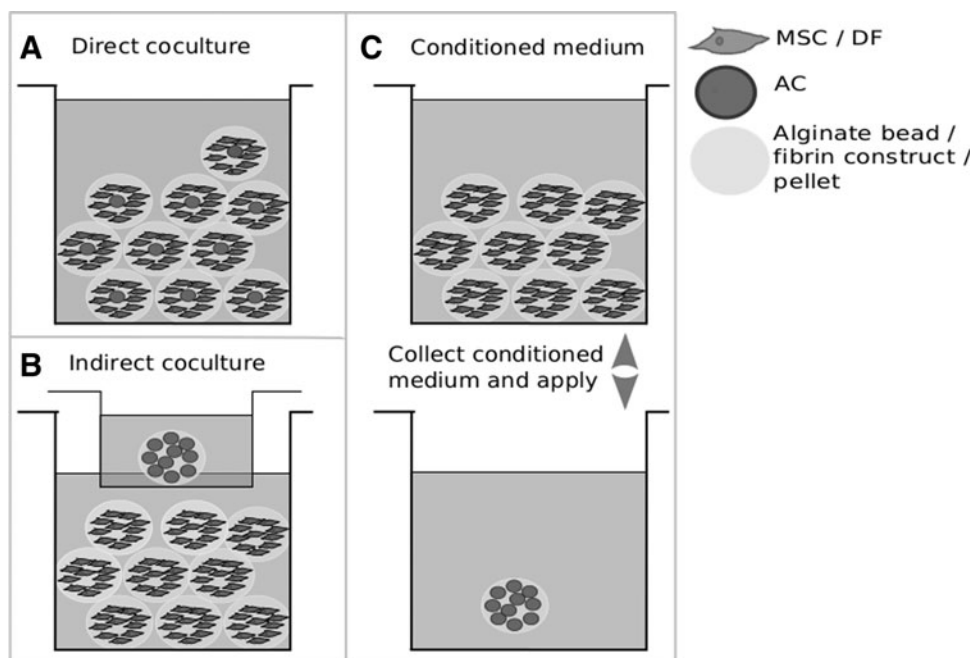


FIG. 1. Schematic overview of the different culture conditions. Articular chondrocytes (ACs) and mesenchymal stromal cells (MSCs) were cultured in alginate beads, fibrin glue, and cell pellets. For each culture condition, both direct (**A**, two cell types mixed in the same culture system) and indirect (**B**, two cell types cultured in the same system, but separated by cell culture inserts) cocultures were used with 2.0×10^6 cells/mL at a 10% chondrocyte and 90% MSC ratio. To investigate the role of MSCs in cocultures, MSCs were replaced by dermal fibroblasts (DFs). Conditioned medium (**C**) from both chondrocytes and MSCs was used to investigate the effect on chondrogenesis by either cell type.

penicillin (100 U/mL; Gibco) and streptomycin (100 µg/mL; Gibco) at 37°C overnight. Chondrocytes were expanded for two passages in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, and 100 µg/mL streptomycin. The MSCs used are classified as ATMPs and manufactured in the GMP-licensed Cell Therapy Facility of the UMC Utrecht. Briefly, bone marrow aspirates were obtained from third-party non-HLA-matched healthy donors as approved by the Dutch central Committee on Research Involving Human Subjects (CCMO, Biobanking bone marrow for MSC expansion, NL41015.041.12). The bone marrow donor or the parent or legal guardian of the donor signed the informed consent approved by the CCMO. Bone marrow was separated using a density gradient centrifugation (Lymphoprep, Axis Shield). MSCs were isolated by plastic adherence and expanded using the MC3 systems and α -minimal essential medium (α -MEM) with L-glutamine from Macopharma supplemented with 5% platelet lysate and 3.3 IU/mL heparin up to passage 3.¹⁶ Characterization of MSCs fits the internationally convened minimal criteria for these cells.¹⁷ The ATMP MSCs used in this study were obtained from surplus cells of one male donor used for the IMPACT trial (NCT02037204) and cultured for one additional passage (passage 4) in α -MEM supplemented with 10% FBS, 2% L-ascorbic acid-2-phosphate (ASAP; Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin to obtain sufficient cells. DFs isolated from foreskin were cultured to passage 4 in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin before use.

Cell culture

Cells were cultured in alginate beads, fibrin glue constructs, or pellets.

Per donor (or donor combination for cocultures) the following was performed:

For the alginate beads (0.25×10^6 cells/bead), cells were suspended in a 1.2% alginate solution (Keltone LVCR, Monsanto) and dripped into a 102 mM calcium chloride solution with a 23G needle and washed thrice with a standard saline solution (NaCl).

For the fibrin glue constructs, cells were resuspended in a 1:50 diluted fibrinogen component of Tissucol (Baxter) using PBS and 50 µL was injected in a 96-well plate and combined with a 1:15 diluted component of thrombin, resulting in 100 µL constructs containing 0.25×10^6 cells per construct.

For the pellet cultures, 100 µL of a cell suspension of 2.5×10^6 cells/mL was seeded into V-bottom 96-well plates (Nunc) and centrifuged for 5 min at 250 g to form pellets containing 0.25×10^6 cells/pellet.^{18,19}

For direct cocultures, 10 alginate beads, 10 fibrin constructs, or 10 pellets containing ACs and MSCs or ACs and DFs mixed in a 10:90 ratio were cultured in 1 well of a 24-well plate in 1 mL culture medium (DMEM supplemented with 2% insulin–transferrin–selenium (ITS)-X (Invitrogen), 2% ASAP, 2% human serum albumin (Sanquin Blood Supply Foundation), and 1% penicillin/streptomycin (100 U/mL, 100 µg/mL). For indirect cocultures, 1 AC-containing alginate bead, fibrin glue construct, or pellet, and 9 MSC-containing alginate beads, fibrin glue constructs, or pellets were cultured in one well, separated from each other by cell culture inserts (24-well plate format, 0.4 µm PFTE [Millipore]) in the same culture medium. For

conditioned medium, the conditioned medium was harvested from alginate bead, fibrin glue, or pellet cultures of MSCs or ACs, diluted with fresh culture medium (1:1), and added to ACs or MSCs, respectively, in the same culture system.

GAG analysis

After 4 weeks of culture, samples were digested overnight in a papain digestion buffer (250 µg/mL papain [Sigma-Aldrich], 0.2 M NaH₂PO₄, 0.1 M EDTA, 0.01 M cysteine) at 60°C before quantification of the GAG content with a dimethylmethylene blue (DMMB) assay. The absorption ratio was set at 540–595 nm using chondroitin-6-sulfate (Sigma-Aldrich) as a standard. DNA content in the papain digests was determined using a Picogreen DNA assay (Invitrogen) according to the manufacturer's instructions.

Gene expression

Total RNA was isolated from the cells using TRIzol (Invitrogen) as described by the manufacturer. Total RNA (750 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Biorad). Real-time polymerase chain reactions (PCRs) were performed using LightCycler Mastermix (LightCycler 480 SYBR Green I Master; Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics) according to the manufacturer's instructions. Primers (Invitrogen) used for real-time PCR are listed in Table 1.²⁰ The amplified PCR fragment extended over at least one exon border (except for 18S, which was used as the housekeeping gene).

Histological analysis

Samples were dehydrated using graded alcohol steps, immersed in xylene, embedded in paraffin, and cut in 5-µm sections. To evaluate the proteoglycan content, 0.125% safranin-O (Merck counterstained with Weigert's hematoxylin [Klinipath], 0.4% fast green [Merck]) and 0.4% toluidine blue (Merck, counterstained with 0.02% Fast Green [Merck]) stainings were used. Type II collagen deposition and connexin 43 expression were determined by immunohistochemistry. Antigen retrieval was performed by subjecting the sections to 1 mg/mL pronase (Sigma-Aldrich) for 30 min at 37°C, followed by 10 mg/mL hyaluronidase (Sigma-Aldrich) incubation for 30 min at 37°C. Subsequently, the sections were blocked using a 5% bovine serum albumin (BSA) in PBS solution for 1 h, followed by an overnight incubation at 4°C with a primary antibody against human type II collagen (mouse anti-human type II collagen, II-II6B3, 1/100 dilution in PBS-BSA-5%; Developmental Studies) or against connexin 43/GJA1 (rabbit anti-connexin 43, ab66151, 1/50 dilution in PBS-BSA 5%; Abcam). After washing, the slides were incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1/100 dilution in PBS-BSA-5%) for 60 min at ambient temperature. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). The sections were counterstained with Mayer's hematoxylin.

The histological slides were scored by two independent researchers according to the Bern score.^{21,22} The Bern score has been validated for the histological grading of pellet-cultured cartilage and was adapted to include the type II collagen immunostaining.^{21,22}

TABLE 1. OLIGONUCLEOTIDE SEQUENCES USED FOR REAL-TIME PCR

Target gene	Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)
<i>18S</i>	Fw: 5' GTAACCCGTTGAACCCCAT 3' Rev: 5' CCATCCAATCGGTAGTAGCG 3'	57	151
<i>ACAN</i>	Fw: 5' CAACTACCCGGCCATCC 3' Rev: 5' GATGGCTCTGTAATGGAACAC 3'	57	160
<i>COL1A1</i>	Fw: 5' TCCAACGAGATCGAGATCC 3' Rev: 5' AAGCCGAATTCCTGGTCT 3'	57	191
<i>COL2A1</i>	Fw: 5' AGGGCCAGGATGTCCGGCA 3' Rev: 5' GGGTCCCAGGTTCTCCATCT 3'	56	195
<i>COL10A1</i>	Fw: 5' CACTACCCAACACCAAGACA 3' Rev: 5' CTGGTTCCCTACAGCTGAT 3'	56	225
<i>SOX9</i>	Fw: 5' CCCAACGCCATCTTCAAGG 3' Rev: 5' CTGCTCAGCTCGCCGATGT 3'	60	242

Forward (Fw) and reverse (Rev) primers for *18S*.

ACAN, aggrecan; *COL1A1*, α 1(I)procollagen; *COL2A1*, α 1(II)procollagen; *COL10A1*, α 1(X)procollagen; *SOX9*, SRY (sex-determining region Y)-box 9.

STR analysis

Genomic DNA was isolated from the ACs, MSCs, and DFs before use in the present study and from the coculture samples after the 4-week culture. Ten loci were amplified and sequenced. Specific alleles for the donors were found. A set of D10S2325, MYCL, and SE33 or D8S1132, D10S2325, and MYCL was used to discriminate between ACs and MSCs and a set of D8S1132, D10S2325, and SE33 or D10S2325, D11S554, and MYCL to discriminate between ACs and DFs.²³ The amount of DNA present for each donor was calculated from the areas of the electropherogram, from which the ratio between two cell types could be calculated.

Fluorescent dye transfer

To assess cellular communication through gap junctions, fluorescent dye transfer was applied as previously described.^{24,25} Briefly, 5 μ M Vybrant CM-DiI (Molecular Probes) and 1 μ M calcein-AM (Molecular Probes) were diluted in PBS and incubated for 1 h at 37°C with either ACs or MSCs. Subsequently, cells were washed and cocultured with unlabeled recipient cells (MSCs or ACs, respectively). The cocultures were performed for 24 h in the three study conditions (alginate, fibrin, and pellets) and monolayer culture using a 50:50 AC:MSC ratio. The presence of dye transfer through gap junctions was assessed after 24 h by confocal microscopy (Leiss LSM 510 Meta).

Statistical analysis

Data are expressed as mean \pm standard deviation. Data were analyzed statistically using SPSS version 22.0.0. Normal distribution of the gene expression data was verified by plotting a frequency distribution histogram. Differences were tested by a repeated measures analysis of variance.

Results

Chondrocytes proliferate while MSCs reduce in numbers

STR analysis revealed that both ACs and MSCs were present after the 4-week culture. Starting with an AC-MSC

ratio of 10:90, the percentages of ACs increased up to ~20% for alginate beads and pellets and 60% in fibrin glue scaffolds. Both MSCs and DFs reduced 10% in volume during the culture for alginate beads and pellets and 30% in fibrin glue (Fig. 2). No changes in overall DNA content were found in any of the conditions, indicating that the total amount of cells did not change over time.

Expression of chondrogenic genes

In alginate, the *COL2A1* gene expression levels of both direct and indirect AC-MSC cocultures were lower compared with AC monocultures ($p=0.035$ and $p=0.046$, respectively). In contrast, in both fibrin and pellet cultures, there was no difference in *COL2A1* gene expression between direct AC-MSC cocultures and AC monocultures,

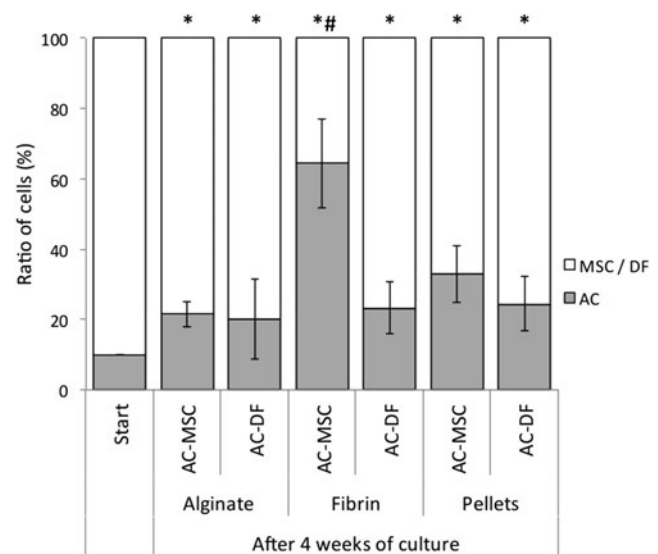


FIG. 2. Short tandem repeat analysis was performed on genomic DNA of cocultures to determine the ratios of AC:MSC and AC:DF detected after 4 weeks. Data are shown as mean \pm SD. * $p < 0.05$ compared with start; # $p < 0.05$ to all other groups.

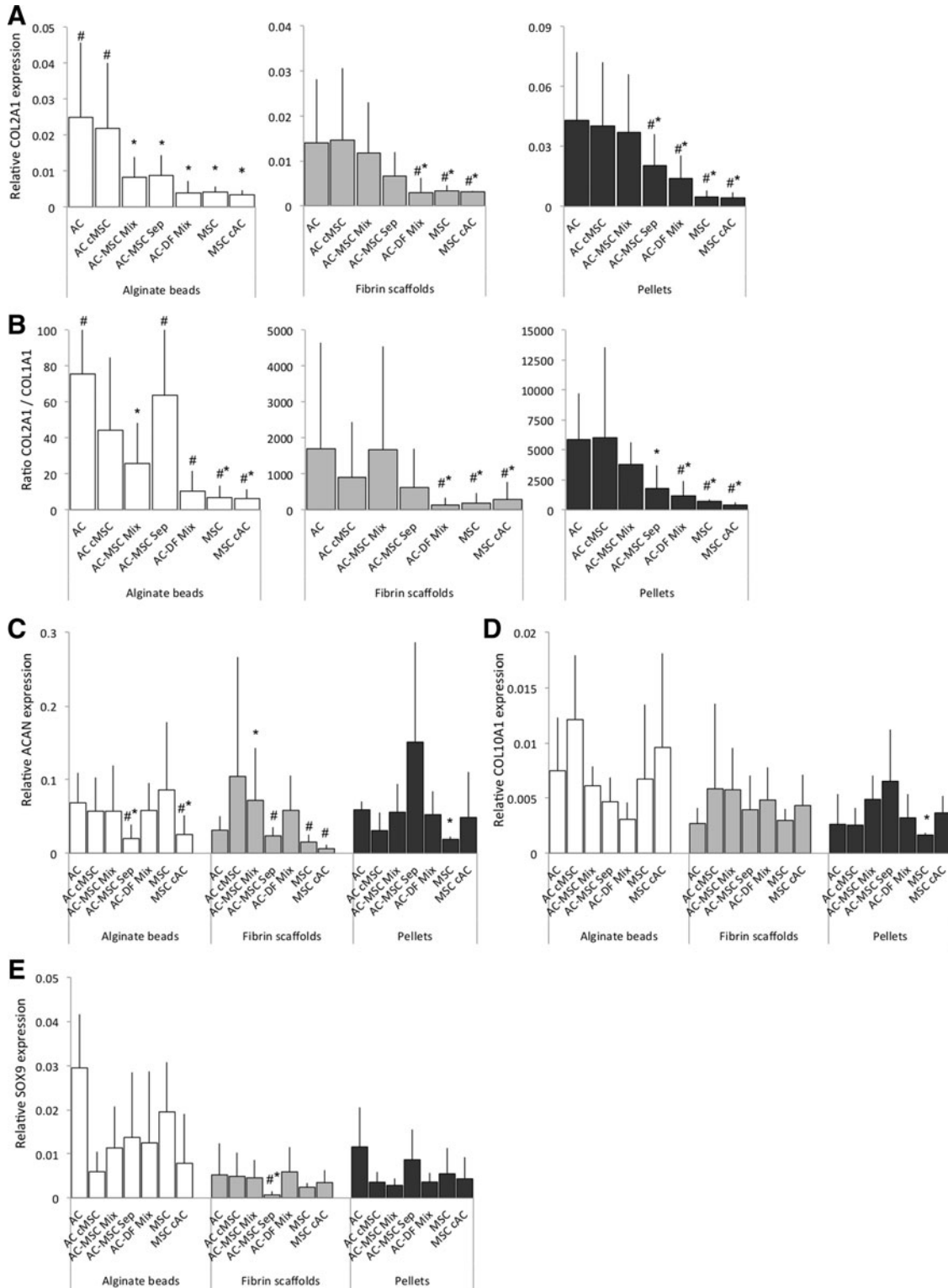


FIG. 3. Gene expression levels of type II collagen (*COL2A1*, **A**), aggrecan (*ACAN*, **C**), type X collagen (*COL10A1*, **D**), SRY (sex-determining region Y)-box 9 (*SOX9*, **E**), and the ratio of type II collagen/type I collagen (*COL2A1/COL1A1*, **B**) in cultures of AC alone with and without conditioned medium from MSCs (cMSC), of ACs and MSCs mixed (mix) together or kept separately (sep) in a 10:90 ratio, ACs and DFs in direct coculture, and MSCs alone with and without conditioned medium from ACs (cAC). Data are shown as mean \pm SD. * $p < 0.05$ compared with AC alone, # $p < 0.05$ compared with AC-MSC mixed.

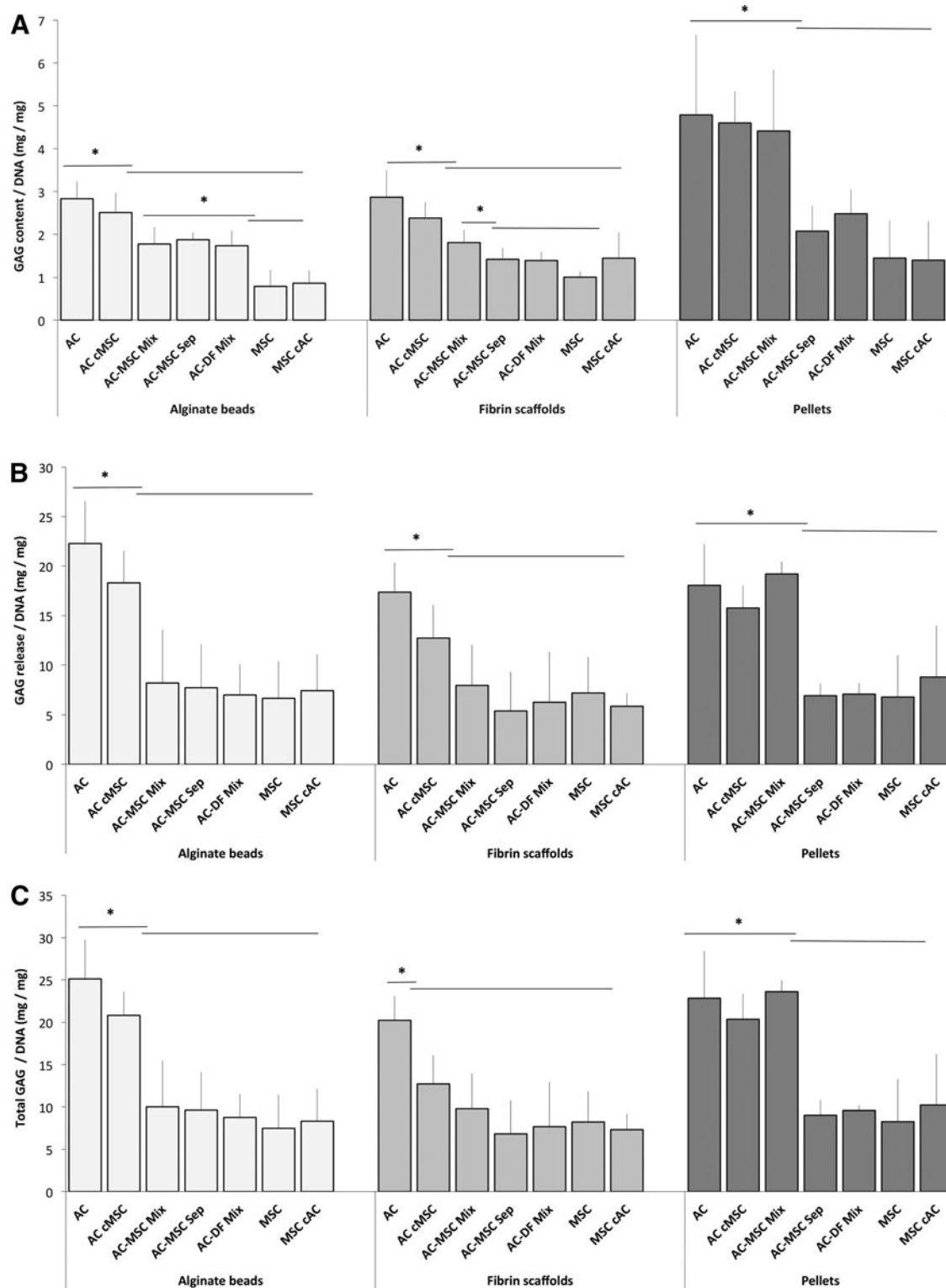


FIG. 4. Glycosaminoglycan (GAG) content (A), release (B), and total production (C) normalized to DNA content of AC monocultures with and without conditioned medium from MSCs (cMSC) of direct AC and MSC cocultures or indirect cocultures kept separately (sep) in a 10:90 ratio, AC and DF cocultures, and MSC monocultures with and without conditioned medium from ACs (cAC). Data are shown as mean \pm SD. * $p < 0.05$.

while the indirect cocultures showed lower *COL2A1* gene expression levels when cultured in pellets ($p=0.048$ and $p=0.045$) (Fig. 3). When cultured in fibrin gel and pellets (direct cocultures) of articular chondrocytes and dermal fibroblasts (AC-DF), *COL2A1* gene expression levels were lower compared with both direct AC-MSC cocultures and AC monocultures. Gene expression levels of *COL2A1* were low in MSC monocultures.

The addition of conditioned medium from AC cultures to MSCs had no additive effect on *COL2A1* gene expression levels.

For all culture systems, the ratio of *COL2A1*/*COL1A1* was lowest in the AC-DF and MSC monocultures. In alginate and fibrin, no differences were observed in the *COL2A1*/*COL1A1* ratios between AC, AC with MSC-conditioned medium, and between direct and indirect cocultures of AC-MSCs. In pellet cultures, the *COL2A1*/*COL1A1* ratio of the indirect AC-MSC coculture was lower compared with AC monocultures and ACs with MSC-conditioned medium.

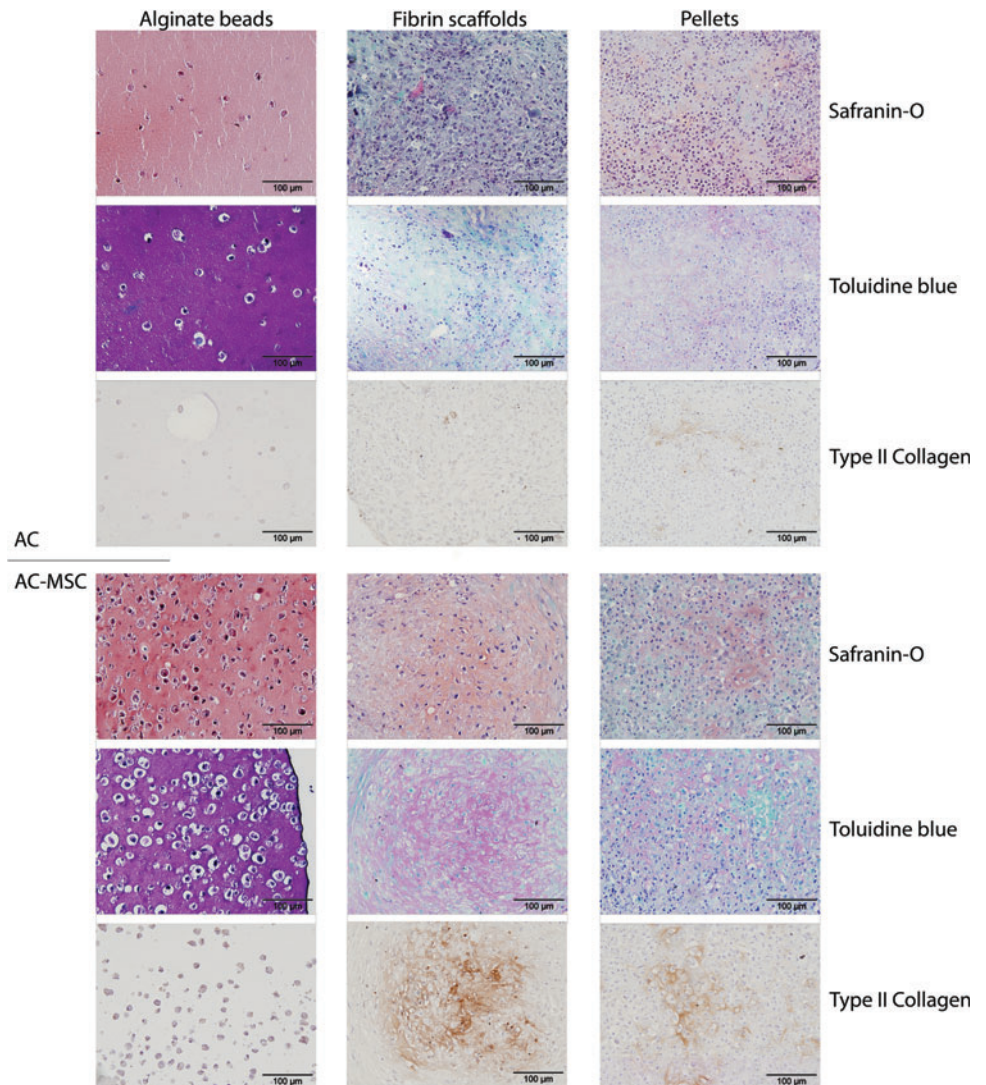
For *ACAN*, a lower gene expression was found for the indirect AC-MSC cocultures in alginate and MSC cultures in conditioned medium compared with the other cultures in this carrier. In fibrin, the direct cocultures of AC-MSCs

showed the highest *ACAN* gene expression, although not significant compared with AC-DF cocultures and ACs with MSC-conditioned medium. In pellet cultures, no differences in *ACAN* gene expression could be detected between the groups, except for MSC monocultures, in which a lower expression was observed compared with AC monocultures. MSC monocultures showed lower *COL10A1* levels in pellets compared with the other scaffolds. In fibrin glue, *SOX9* expression was lower in indirect cocultures of AC and MSCs compared with direct cocultures, whereas no difference was observed in alginate or pellet cultures.

Direct cell-cell contact stimulates cartilage production in cocultures

Pellets exhibited the highest GAG production per cell and showed that chondrocytes alone produced amounts of GAG/DNA that were comparable with those of direct cocultures of ACs and MSCs (Fig. 4A). Fibrin glue samples showed a pattern similar to that of pellets, although with a lower overall GAG/DNA content. For alginate, no statistically significant differences in GAG/DNA content were found between direct AC-MSC cocultures or indirect cocultures.

FIG. 5. Cartilage production as determined by staining proteoglycans by safranin-O, toluidine blue, and an immunostaining for type II collagen of AC monocultures and AC and MSC cocultures in alginate beads, fibrin glue, or pellets after 28 days. Color images available online at www.liebertpub.com/tea



In both fibrin glue and pellet cocultures, in which cell-cell contact could be confirmed by histology and immunohistochemistry (next section), a higher GAG/DNA content was found in direct cocultures compared with indirect cocultures, which could not be mimicked when MSCs were replaced by DFs or by adding conditioned medium from AC monocultures to MSCs. These findings were further supported by an equivalent GAG release into the medium (Fig. 4B) and total GAG production (Fig. 4C).

Histological analysis (Fig. 5) confirmed the importance of cell-cell contact as type II collagen, safranin-O, and toluidine blue staining indicated more cartilage-like tissue formation for both fibrin and pellet cultures when compared with alginate cultures. In this study, it should be noted that the alginate itself is stained intensively with safranin-O or toluidine blue, but limited staining was found in the matrix surrounding the cells. Direct AC-MSC cocultures in fibrin showed a higher GAG production compared with both AC monocultures and cocultures in alginate and pellets. Fibrin showed a characteristic behavior during culture with slow degradation of the gel in weeks, formation of aggregates, and finally pellet-like histology. The alginate beads stayed intact with single cells homogeneously distributed throughout the beads. This was confirmed by histological scoring using the adapted Bern score by two independent observers (Table 2); fibrin and pellet cultures showed a higher adapted Bern score compared with alginate cultures, and direct AC-MSC cocul-

tures in fibrin showed the highest adapted Bern score compared with the other cultures.

Direct cellular communication through gap junctions

When either MSCs or chondrocytes were stained with both DiI and calcein and mixed with the other nonlabeled cell type, dye transfer was confirmed based on the presence of calcein-positive cells without DiI staining (Fig. 6A). In alginate beads, few cells were positive for calcein without DiI staining. In fibrin glue, the number of calcein-positive and DiI-negative cells was limited. In pellet cocultures, visual evaluation revealed significantly more calcein-positive-DiI-negative cells. Moreover, cocultures of calcein-labeled ACs and DiI-labeled MSCs and calcein-labeled MSCs and DiI-labeled ACs resulted in cells positive for both calcein and DiI (data not shown). The transfer of calcein was observed both from MSCs to ACs and from ACs to MSCs (data not shown).

Expression of the gap junction protein, connexin 43, confirmed the presence of gap junctions (Fig. 6B). In alginate beads, no connexin 43 was observed in indirect and direct cocultures of ACs and MSCs. In fibrin glue containing only ACs, connexin 43 staining was limited to the outer region of the construct, while scaffolds containing both ACs and MSCs were intensively stained for connexin 43 throughout the scaffold. Pellets containing ACs were also only stained in the outer region. However, connexin 43 was predominantly expressed in the center of pellets containing ACs and MSCs.

Discussion

In this study, we have attempted to elucidate the role of cell-cell contact in cocultures to find answers as to which conditions are best supportive of chondrogenesis and why. Cocultures of ACs and MSCs have been extensively studied and have shown equal or superior chondrogenesis compared with AC monocultures.⁸ In this study, we focused on the cellular behavior of these cells in different controlled environments. We confirmed that those culture systems that allowed direct contact between cells (pellets and fibrin glue) showed better matrix production compared with an environment where cells have limited cell-cell contact (alginate) or no cell-cell contact (indirect cocultures). Histology indicated that the matrix produced by cells in alginate (distributed through the gel without direct contact) showed limited safranin-O, toluidine blue, and type II collagen staining. The lack of coculture-induced chondrogenesis in alginate was confirmed by the quantitative biochemical assay, which showed no difference for normalized GAG production between direct and indirect cocultures. In fibrin, the degradation of the scaffold was accompanied by the formation of aggregates and pellet-like structures, which likely explains the similar behavior of the cells in the fibrin and pellet conditions. In all cocultures, cellular morphology showed no signs of chondrocyte hypertrophy, which corresponded to the low *COL10A1* gene expression. The inhibitory effect on type X collagen expression in AC-MSC cocultures compared with monocultures has been shown to be highly reproducible.^{11,26-32}

In this study, we investigated whether coculture-induced cartilage tissue formation was dependent on the cell types used. This research question was mainly based on the

TABLE 2. ADAPTED BERN SCORING OF HISTOLOGICAL SLIDES

	A: Staining intensity		B: Cell distance		C: Morphology		Total Score	
	a	b	a	B	a	b	a	b
AC A								
1	1	1	2	2	0	0	3	3
2	1	1	2	2	0	0	3	3
3	1	1	2	2	0	0	3	3
AC F								
1	1	1	1	1	2	2	4	4
2	1	1	1	1	2	2	4	4
3	1	1	1	1	2	2	4	4
AC P								
1	2	2	1	1	2	2	5	5
2	2	2	1	1	2	2	5	5
3	2	2	1	1	2	2	5	5
AC-MSC A								
1	1	0	2	2	1	1	4	3
2	1	0	2	2	1	1	4	3
3	1	0	2	2	1	1	4	3
AC-MSC F								
1	2	2	2	2	2	2	6	6
2	2	2	2	2	2	2	6	6
3	2	2	2	2	2	2	6	6
AC-MSC P								
1	1	1	1	1	2	2	4	4
2	0	0	1	1	2	2	3	3
3	1	1	1	1	2	2	4	4

A, alginate beads; ACs, articular chondrocytes; F, fibrin glue constructs; MSCs, multipotent mesenchymal stromal cells; P, pellet culture.

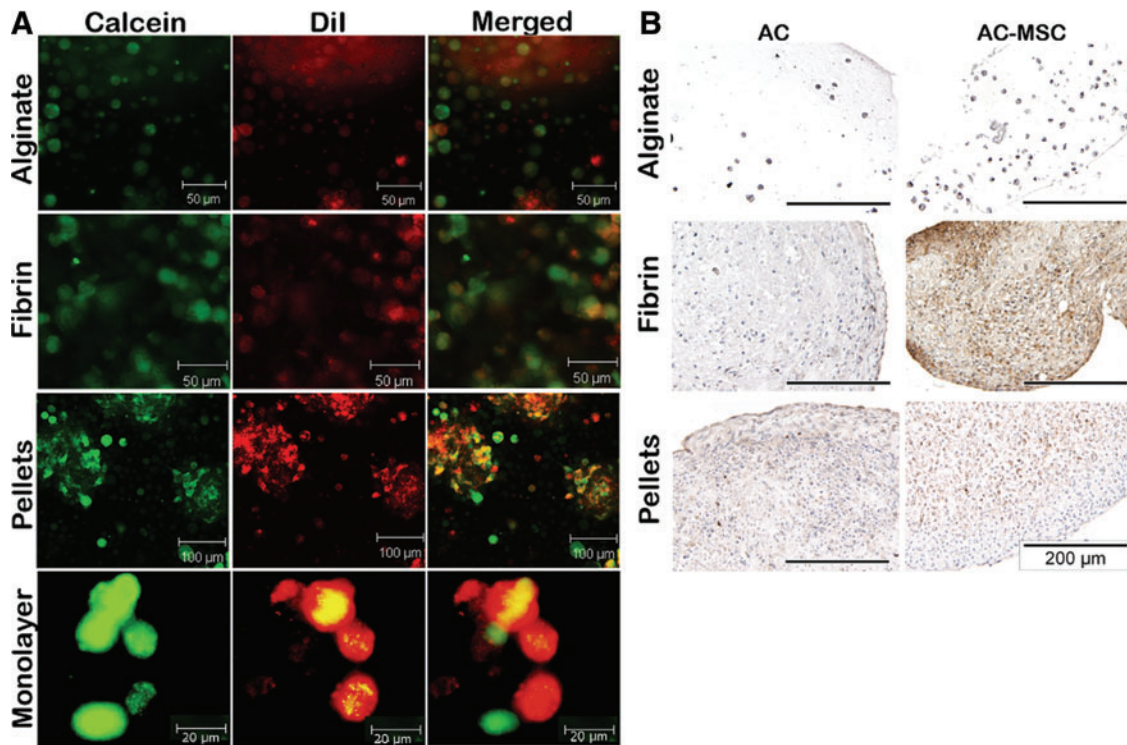


FIG. 6. Cytosolic dye transfer (A) DiI (red), calcein (green), and an overlay of DiI and calcein (merged) of DiI-calcein double-labeled donor cells (ACs) and initially unlabeled recipient MSCs showing cytosolic calcein transfer indicative of functional gap junctions; immunostaining for connexin 43 (B) in AC monocultures and AC-MSC cocultures in alginate beads, fibrin glue, or pellets after 28 days. Color images available online at www.liebertpub.com/tea

conflicting results regarding the capability of DFs to adequately replace MSCs in AC-MSC cocultures.⁸ One study found no difference in histological and quantitative GAG analysis when MSCs were replaced by DFs in cocultures of chondrocytes and MSCs,³³ while others have shown a dramatic reduction of type II collagen expression and GAG production when chondrocytes were replaced by fibroblasts in indirect cocultures with MSCs.³⁴ In the present study, we also found DFs to be inferior to MSCs in the coculture conditions. Thus, it seems likely that MSCs are crucial for cartilage regeneration in cocultures. It could be that unlike DFs, MSCs secrete specific growth factors that are unique to this cell combination. In fact, one study found different proteins such as extracellular histones, matrix proteins, and growth factors in cocultures of chondrocytes and MSCs compared with monocultures.²⁷ In addition, MSCs seem to stimulate chondrocyte proliferation specifically through fibroblast growth factor-1 expression as indicated by pericellular staining in AC-MSC cocultures.³⁵

This study has shown that ACs and MSCs that are in close proximity communicate through gap junctions. Gap junctions induce communication by direct contact between neighboring cells, allowing inorganic ions and water-soluble factors to pass from one cell to the other.³⁶ Most cells in normal tissue communicate through these junctions. Intercellular connections between chondrocytes have been shown to contain gap junctions and play a key role in cell-cell communication by exchange of nutrients such as glucose and amino acids.³⁷ In this study, for the first time, these connections are shown between ACs and MSCs by cytosolic

dye transfer in combination with connexin 43 expression. The inferior matrix production of MSCs with AC-conditioned medium also underlines the importance of direct cell-cell contact. Conditioned medium, although capable of stimulating chondrogenic differentiation, has been found to be inferior to the direct coculture system.³⁰ In this study, the effect of conditioned medium may have been compromised as we have diluted the medium. One of the biological factors involved in the paracrine action of MSCs is the extracellular vesicle, including exosomes. It has been shown in several studies that the addition of MSC-derived exosomes can have the same effects as the addition of MSCs.³⁸ However, exosomes are usually used at a concentration between 1 and 10 mg/L. At the lower MSC numbers used in this study, it is unlikely that exosomes were significant contributors to the chondrogenesis shown.

One key question in coculture-induced chondrogenesis is how the cell ratios develop over time. Using STR analysis, we found an increase in the AC:MSC ratio during the culture. This effect was the largest for the fibrin glue scaffold. The chondroinductive role for MSCs has recently been shown to play an important role in coculture-induced chondrogenesis.^{9,10} This theory stands in contrast to the suggestion that chondrocytes stimulate the chondrogenic differentiation of MSCs. Future analysis on the spatial distribution of the cells in coculture would be of interest to better understand the neocartilage formation, although stable cell surface markers capable of distinguishing chondrocytes from MSCs during several weeks of culture remain elusive. Strong evidence showing MSC differentiation into

chondrocytes in cocultures is also lacking.⁸ For example, Yang *et al.* recently showed agarose cocultures of juvenile bovine chondrocytes and bone marrow-derived MSCs to form stable neocartilage *in vitro*.³⁹ In this study, MSCs were suggested to differentiate into chondrocytes, partly based on an increase in MSC surface markers such as CD44 and CD166. Similar to our study, it could be that cartilage regeneration without cell-cell contact can be achieved in a 3D coculture environment. However, the lack of a control group with direct cell-cell contact and an analysis of the (DNA-based) ratio of cells in the generated constructs limit the comparability to our work. Besides, their culture medium contained dexamethasone, while the culture medium in the present study did not contain any inducers or stimulators of chondrogenic differentiation of MSCs. Indeed, as suggested by others, it may well be that cell-cell contact and trophic induction are synergistic in cartilage regeneration.⁴⁰ The use of clinical-grade MSCs and an environment such as fibrin glue where migration of cells is allowed during the degradation process may explain the increased survival of MSCs in our study compared with those shown by Wu *et al.*^{9,10} Indeed, cells tend to show an improved chondrogenic capacity when suspended in a degradable hydrogel. For example, poly(ethylene glycol) hydrogels experience an increase in swelling ratio upon degradation, allowing initial mechanical support that is transferred to the cartilage matrix over time.⁴¹ Cell density may also influence cellular behavior as higher cell densities in alginate, agarose, and peptide gels have shown an increase in extracellular matrix production.⁴² In this study, we used a cell density of 2×10^6 /mL cells as this concentration has been used in preclinical and clinical single-stage coimplantation studies (13). Nevertheless, investigating the role of cell densities on coculture-induced chondrogenesis in different hydrogels with mechanical loading may further optimize conditions for cell implantation.⁴³

A preclinical model that found chondrons and MSCs mixed at a 10:90 ratio in fibrin glue, capable of regenerating cartilage in both a small and large animal model, has supported the initiation of a clinical trial.¹³ This trial (NCT02037204) (www.youtube.com/watch?v=S3rIBJA03AA), using the same ratio of chondrons and MSCs and the same concentration in fibrin glue as the present study to treat focal cartilage defects in the knee, has recently completed its inclusion. An STR analysis on biopsies taken 1 year after surgery may elucidate the chondroinductive role and the fate of allogeneic MSCs in the human knee.⁴⁴ Although it seems that MSCs cannot be replaced by conditioned medium or soluble factors, novel insights in the mechanisms of cellular interactions may open new doors for single-stage cartilage repair, especially since a coimplantation strategy has feasibility-associated challenges. Indeed, it could be worthwhile to search for a single-stage MSC strategy applied in an environment that mimics the cellular interaction with chondrocytes. This is highlighted by studies showing a similar cartilage-like tissue production in direct and indirect cocultures.^{12,27} However, as *in vitro* differentiated MSCs have shown hypertrophic characteristics and limited capacity to form stable cartilage *in vivo*,^{45,46} it seems that a coimplantation technique with the use of a scaffold that allows direct cellular communication holds promise for clinical translation.

In conclusion, this is the first study to provide a comprehensive evaluation of different cellular environments during AC and MSC cocultures, which shows cell-cell contact to be essential for optimal cartilage regeneration. It is the first study to show that gap junctions are formed between ACs and MSCs. Chondroinduction with chondrocyte proliferation and MSC disappearance could be confirmed, although with a better MSC survival than previously shown. This suggests a dynamic interplay between cells in coculture with a chondroinductive effect of MSCs.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Lucienne A. Vonk, PhD

Department of Orthopaedics

University Medical Center Utrecht

Heidelberglaan 100

PO Box 85090

3508 AB Utrecht

The Netherlands

E-mail: l.a.vonk@umcutrecht.nl

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