Non-contact Coculture Reveals a Comprehensive Response of Chondrocytes Induced by Mesenchymal Stem Cells Through Trophic Secretion

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Received: 17 July 2017 / Revised: 4 September 2017 / Accepted: 7 September 2017 / Published online: 27 September 2017
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Abstract Coculture between mesenchymal stem cells (MSCs) and chondrocytes has significant implications in cartilage regeneration. However, a conclusive understanding remains elusive. Previously, we reported that rabbit bone marrow-derived MSCs (rbBMSCs) could downregulate the differentiated phenotype of rabbit articular chondrocytes (rbACs) in a non-contact coculture system for the first time. In the present study, a systemic investigation was performed to understand the biological characteristics of chondrocytes in coculture with MSCs. Firstly, cells (MSCs and chondrocytes) from different origins were cocultured in transwell system. Different chondrocytes, when cocultured with different MSCs respectively, consistently demonstrated stimulated proliferation, transformed morphology and declined glycosaminoglycan secretion of chondrocytes. Next, cell surface molecules and the global gene expression of rbACs were characterized. It was found that cocultured rbACs showed a distinct surface molecule profile and global gene expression compared to both dedifferentiated rbACs and rbBMSCs. In the end, cocultured rbACs were passaged and induced to undergo the chondrogenic redifferentiation. Better growth and chondrogenesis ability were confirmed compared with control cells without coculture. Together, chondrocytes display comprehensive changes in coculture with MSCs and the cocultured rbACs are beneficial for cartilage repair.

Keywords Cartilage regeneration · Mesenchymal stem cells · Chondrocytes · Coculture · Paracrine factors

Electronic supplementary material The online version of this article (doi:[10.1007/s13770-017-0084-8](https://doi.org/10.1007/s13770-017-0084-8)) contains supplementary material, which is available to authorized users.

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1 Introduction

Tissue engineering and cell therapy have attracted a great deal of interests with the hope to regenerate cartilage [1, 2]. However, two major cellular sources including chondrocytes and mesenchymal stem cells (MSCs) are concerned with several challenging issues. While chondrocytes are limited in availability and undergo the unfavorable dedifferentiation (loss of phenotype) during ex vivo expansion, MSCs tend to express a hypertrophic chondrocyte phenotype and subsequent calcification in current chondrogenic induction protocols, which result in unstable cartilage repair with inferior functions [2, 3].

In recent years, a novel concept of coculturing MSCs and chondrocytes has emerged to solicit cartilage regeneration [4, 5]. Articular chondrocytes (ACs) bear an

intrinsic hyaline phenotype and can secrete abundant cartilaginous extracellular matrix (ECM), and MSCs are readily available with the chondrogenic potential [6]. Ideally, in such a strategy, the requirement for a huge amount of chondrocytes can be attenuated and ACs can confer the instructive cues for MSCs to acquire a stable hyaline phenotype. For example, Hildner et al. [7] explored the possibility of partially substituting human ACs with human adipose-derived MSCs (hAMSCs) in matrix-associated autologous chondrocyte implantation (MACI); de Windt et al. [8] recently reported the first clinical trial to evaluate use of mixed allogenic human bone marrow-derived MSCs (hBMSCs) and autologous ACs for cartilage repair.

However, the interactions between MSCs and chondrocytes are more complicated than thought and conflicting findings are documented over the past few years [4, 9]. While studies have shown that the chondrogenic differentiation of MSCs is promoted by ACs and a hyaline phenotype can be potentially achieved [10–12], in other reports chondrogenesis of MSCs is absent and MSCs may exert the trophic effects on chondrocytes to simulate proliferation and/or ECM production [13, 14]. Also, the bidirectional interactions between cells are indicated [15, 16].

Previously, in a non-contact coculture, we had revealed that rabbit bone marrow-derived MSCs (rbBMSCs) could significantly downregulate the differentiated phenotype of rabbit ACs (rbACs) in growth medium without TGF- β [17], which was in agreement with the findings by Lee et al. [18]. We further demonstrated that rbBMSCs potentially secreted FGF-1, VEGF-A or PDGFbb to induce the phenotypic changes of rbACs in a paracrine manner [19]. In the present study, the major objective was to elaborate the biological characteristics of chondrocytes after coculture in order to gain an insight understanding of the changes in chondrocytes induced by MSCs; the second objective was to validate the effects of MSCs on chondrocytes by employing cells from different origins for both MSCs and chondrocytes.

2 Materials and methods

2.1 Cells

Both rbBMSCs and rbACs were isolated from New Zealand and white rabbits as described previously [19–21]. Bovine ACs (bACs) were isolated similarly from adult bovine stifle joints obtained from a local commercial vendor. hBMSCs and hAMSCs were from ATCC. Wistar rat bone marrow-derived MSCs (rBMSCs) were from Chinese Academy of Sciences (Shanghai, China). Chondrocytes were maintained in chondrocyte growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; high glucose,

Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 0.1 mM nonessential amino acids, 0.4 mM proline, 0.05 mg/mL Vitamin C, 100 units/mL penicillin and 100 units/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C, and MSCs were cultured in growth medium composed of α -minimum essential medium (α -MEM; Gibco) supplemented with 10% FBS, 100 units/mL of penicillin and 100 units/mL of streptomycin. The murine chondrogenic cell line ATDC5 was obtained from Shanghai Ruilu-Bio Ltd. and maintained in DMEM/F12 (Gibco) supplemented with 10% FBS, 1% ITS⁺ Premix (6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 μ g/mL sodium selenite, 1.25 mg/mL bovine serum albumin (BSA) and 5.35 μ g/mL linoleic acid; Sigma), 100 units/mL of penicillin and 100 units/mL of streptomycin. Primary rbACs were subcultured for six passages to obtain dedifferentiated chondrocytes [22, 23].

2.2 Cell culture

Different culture experiments were summarized in Table S1. Coculture was set up using a transwell insert (pore size 0.4 μ m; Millipore, Billerica, MA, USA) in 12-well tissue culture plates [19]. rbACs (passage 1, P1), bACs (P1) or ATDC5 were seeded at 5×10^3 cells/cm². After 24 h, rbBMSCs (P3), rBMSCs (P4), hBMSCs (P4), and hAMSCs (P6) were seeded in the inserts at 5×10^5 cells/insert, respectively, and cocultured with rbACs (P1), bACs (P1) or ATDC5 in chondrocyte growth medium or the maintenance medium of ATDC5 for 6 days. Monoculture of rbACs, bACs and ATDC5 for 7 days was set as controls.

To evaluate cell growth, rbACs after 6-days coculture or monoculture (now defined as P2) were plated in 12-well plates at 5000 cells/cm² and cultured in chondrocyte growth medium for 12 days. P3 rbBMSCs were also plated at 5000 cells/cm² in growth medium as control. Cell number was counted by using a hemocytometer. For successive passaging culture, rbACs after 6-days coculture were subcultured at 5000 cells/cm² in T-150 tissue culture flasks until P5 in chondrocyte growth medium or rbBMSCs-conditioned medium prepared as described [17]. As control, rbACs from monoculture were subcultured similarly in chondrocyte growth medium. The number of population doublings (PD) during each passage was calculated as, where N_0 is the inoculum cell number and N_i is the cell harvest number, and cumulative PD (cPD) was the sum of PD from P2 to P5 [24].

Micromass culture was applied to induce re-differentiation of cocultured rbACs in chondrogenic induction medium (CIM) composed of DMEM (high glucose), 1% ITS⁺ Premix, 1 mM sodium pyruvate, 50 μ g/mL sodium L-ascorbyl-2-phosphate, 40 μ g/mL proline, 10⁻⁷ M

dexamethasone, 10 ng/mL TGF- β 1, 100 units/mL of penicillin and 100 units/mL of streptomycin. After coculture, rbACs were resuspended in DMEM at 1.25×10^7 cells/mL and 20 μ L of cell suspension were spotted in each well in 24-well plates. After 2 h, 1 mL of CIM was slowly added and culture lasted for 14 days. For all culture experiments, medium was refreshed every other day.

2.3 F-actin staining

Cells in 24-well plates were rinsed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS and permeated with 0.25% Triton X-100 in PBS (PBST). Samples were then treated with Rhodamine-phalloidin (5 μ g/mL; Invitrogen) in PBS containing 1% BSA in dark for 20 min. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and fluorescence images were acquired using confocal laser scanning microscopy (CLSM; TI-LU4SU, Nikon).

2.4 Live/dead staining

Cell viability in micromass was assessed by live/dead staining. Samples were rinsed with PBS and incubated in DMEM containing 2 μ M calcein acetoxymethylester (CAM; Sigma) and 2 μ M propidium iodide (PI; Sigma) at 37 °C for 30 min in dark. Fluorescent images were acquired using CLSM.

2.5 Histochemical and immunofluorescence staining

Cells were fixed with 4% paraformaldehyde. For immunofluorescence detection of type II collagen, samples were treated with PBST, blocking buffer (1% BSA in PBST) and then mouse anti-rabbit type II collagen antibody (Calbiochem) at 1:100 dilution for 2 h. Specific antibody binding was detected by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:200 dilution; Invitrogen) at room temperature for 2 h. Cell nuclei were counterstained with DAPI and fluorescent images were acquired using fluorescence microscope (Eclipse Ti-S, Nikon). To detect glycosaminoglycans (GAG), samples after fixation were stained with 0.1% safranin O. Micromass samples were fixed with 4% paraformaldehyde, dehydrated and then embedded in paraffin. By using a rotary microtome (Leica RM2235), 8- μ m-thick sections were sliced. Sample sections were deparaffinized, rehydrated and then stained with H&E and safranin O.

2.6 Quantification of GAG content

Cells in each well were treated with 200 μ L of papain (16–40 U/mg; Sigma) solution (125 μ g/mL papain, 5 mM L-cysteine, 100 mM Na₂HPO₄, and 5 mM EDTA, pH 6.2) at 60 °C for 16–20 h and the supernatants were collected for measurement. DNA and GAG contents were determined as previously described [17]. GAG content was normalized to DNA content and expressed as GAG/DNA (μ g/ μ g). Each group was assayed with four replicates.

2.7 Flow cytometry

For each sample, 1×10^6 cells were resuspended in 500 μ L antibody diluent supplemented with 5 μ L of antibody stock solution for antibodies pre-labeled with fluorochrome as listed in Table S2, and incubated for 30 min at 4 °C in dark. Cells were then resuspended in 700 μ L of PBS with 0.1% BSA and kept at 4 °C before analysis. For CD166 and CD44, which are not pre-labeled with fluorochrome, after cells were incubated with primary antibody for 30 min, they were incubated with the secondary antibody solutions (goat anti-mouse IgG-FITC or goat anti-rat IgG-FITC, 1:200) at room temperature for 30 min. Analyses were performed on flow cytometer (FACS Calibur, BD) with FACSComp software. From each sample, 10,000 events were analyzed and cells were gated on forward and side scatter to exclude debris and cell aggregates. Percentage of positive cells was calculated as the percentage of cells having a measured fluorescence greater than 99% of non-stained cells.

2.8 RNA sequencing

About 3×10^6 cells per sample were harvested for RNA extraction using Trizol (Invitrogen) according to the manufacturer's instruction. Poly-A RNA was isolated with a TruSeq RNA Sample Preparation Kit (Illumina) and fragmented into 150-bp inserts to create cDNA libraries. Sequencing was performed using NextSeq 500 (Illumina) according to the manufacturer's protocol. The spliced reads were mapped using TopHat (<http://ccb.jhu.edu/software/tophat/>), and transcripts were assembled with Cufflinks (<http://cufflinks.cbcb.umd.edu/>). Gene assemblies were annotated with BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi/>) against the rabbit database. For each gene, the expression level was calculated from the base mean value, which was the sequencing depth for each transcript normalized to the library size. The HTSeq (<http://www-huber.embl.de/users/anders/HTSeq/>) and DESeq programs (were used to measure the differential gene expression. The functional categories of genes were established with the

EGGNOG (evolutionary genealogy of genes: Non-supervised orthologous groups) (<http://egglog.embl.de/>) and GO (Gene ontology) enrichment analyses were performed via <http://www.Geneontology.org/>.

2.9 Polymerase chain reaction (PCR)

To isolate total RNA, cell samples were lysed with Trizol (Invitrogen) and RNA extraction was performed according to the manufacturer's instruction. To synthesize cDNA, 1 µg of mRNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega) using oligo(dT). For genes listed in Table S3, reverse transcription PCR (RT-PCR) was carried out using PCR Master Mix (TaKaRa) on thermal cycler (TC-XP-A, Bioer, Hangzhou, China). PCR products were analyzed by using 2% agarose gel electrophoresis. For genes listed in Table S4, quantitative real-time RT-PCR (qRT-PCR) was performed with Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) on Mx3000P qPCR system (Agilent

Technologies). The relative gene expression levels were analyzed by using the $2^{-\Delta\Delta C_T}$ method using *GAPDH* as housekeeping gene [25].

2.10 Statistical analysis

Values were presented as average $\pm$ standard deviation. Two-tailed, unpaired Student's *t* test was applied to determine the significance of difference. $p < 0.05$ was considered statistically significant.

3 Results

3.1 Coculture between rbACs and rbBMSCs

As in Fig. 1A, the shape of rbACs became elongated in coculture in contrast to being round or polygonal in monoculture. F-actin staining showed the alignment of the

Fig. 1 Coculture between rbACs and rbBMSCs. Coculture was performed for 6 days and monoculture of rbACs was included as control. **A** Observation under bright field, phalloidin staining of F-actin, immunofluorescence staining of type II collagen and safranin

O staining (day 6); **B** cell counting (day 6); **C** GAG/DNA (day 6). M: rbACs in monoculture; C-rbBMSCs: rbACs in coculture with rbBMSCs; * $p < 0.05$, compared to M (n = 4)

microfilaments of rbACs in coculture, which was distinct from the radial organization in monoculture. Cell number was significantly higher in coculture (Fig. 1B). Production of cartilaginous ECM including type II collagen and GAG by rbACs was detected by using the immunofluorescence staining and safranin O staining, respectively. Much weaker staining was found in coculture for both type II collagen and GAG (Fig. 1A). Quantification of GAG content confirmed significant reduction of GAG production in coculture (Fig. 1C).

3.2 Coculture between cells from different origins

There were nine different coculture combinations. F-actin staining was applied to analyze the morphology of chondrocytes. Compared to monoculture, F-actin was stretched more in a paralleled alignment in all coculture (Fig. 2A). However, among the different chondrocytes, the least prominent stretching was noted for bACs. Coculture induced a dramatic reduction in the staining intensities for type II collagen and GAG than respective monoculture.

Fig. 2 Coculture between cells derived from different origins. Chondrocytes, including rbACs, bACs and ATDC5, were cocultured with hAMSCs, hBMSCs and rBMSCs for 6 days, which gave nine coculture combinations. **A** Phalloidin staining of F-actin (day 4), immunofluorescence staining of type II collagen (day 6) and safranin

O staining (day 6); **B** cell number (day 6). M: respective chondrocytes in monoculture; C-hAMSCs, C-hBMSCs and C-rBMSCs: chondrocytes in coculture with hAMSCs, hBMSCs and rBMSCs, respectively; * $p < 0.05$, compared to M ($n = 4$)

When rbACs were cocultured with all MSCs, cell number was significantly higher than that in monoculture (Fig. 2B).

3.3 Cell surface molecular profile

Cell surface molecules were compared among rbACs (P2) derived from coculture and monoculture, dedifferentiated rbACs (P6) and P4 rbBMSCs by flow cytometric analysis (Fig. 3; Table S5). For monocultured rbACs, CD49e and CD49f (92.3 and 96.8% respectively) were strongly expressed, CD44, CD81 and CD90 (27.5, 24.8 and 49.3%, respectively) highly expressed, and CD14, CD29, CD51/61 and CD166 (0.8, 5.5, 6.2 and 7.3%, respectively) at low levels. For rbACs from coculture, CD49e and CD81 (97.5 and 91.5%, respectively) were strongly expressed, CD49f and CD51/61 (69.3 and 16.3%, respectively) highly expressed, and CD14, CD29, CD44, CD90 and CD166 (0.6, 2.8, 10.9, 3.7 and 1.9%, respectively) at low levels. For P6 rbACs, CD14, CD49e, CD49f, CD51/61, CD81 (22.0, 56.3, 63.5, 38.2 and 67.1%, respectively) were highly expressed, CD44, CD90 and CD166 (1.3, 1.8 and 2.2%, respectively) at low levels and CD29 (0.0%) was not detected. For rbBMSCs, CD49e CD51/61 and CD81 (98.8, 75.8 and 88.4%, respectively) were strongly expressed, CD44 and CD49f (15.4 and 39.0%, respectively) highly expressed, and CD14, CD29, CD90 and CD166 (2.3, 0.5, 2.8 and 2.5%, respectively) at low levels. Compared to monocultured rbACs, cocultured rbACs showed marked downregulation in CD44, CD49f and CD90, and upregulation in CD51/61 and CD81; compared to P6 rbACs, cocultured rbACs had lower expression in CD14 and CD51/61, and higher levels of CD44, CD49e and CD81; compared to rbBMSCs, cocultured rbACs displayed marked lower level of CD51/61 and higher expression of CD49f. Moreover, compared to monocultured rbACs, P6 rbACs had upregulated CD14, CD51/61 and CD81, and downregulated CD44, CD49e, CD49f and CD90; compared to rbBMSCs, P6 rbACs showed higher levels of CD14 and CD49f and lower expression in CD44, CD49e, CD51/61 and CD81.

3.4 Global gene expression profile

The global gene expression was analyzed using RNA sequencing. The differential expression profiles among monoculture, cocultured and P6 rbACs were illustrated in the heat map based on the analysis of total 15,689 genes (Fig. 4A). In total, 1063 genes showed significant difference in expression among the three cell types. Specifically, there were 503 genes between rbACs derived from coculture and monoculture, 572 genes between cocultured and P6 rbACs, and 530 genes between P6 rbACs and monoculture.

Fig. 3 Flow cytometry analysis of cell surface markers. Cells including rbACs derived from both 6-days coculture and monoculture control, dedifferentiated P6 rbACs and P4 rbBMSCs were subjected to flow cytometry analysis for the expression of nine surface molecules. M: rbACs derived from monoculture; C: rbACs derived from coculture with rbBMSCs; P: P6 rbACs; rbBMSCs: P4 rbBMSCs

GO analysis categorized these differentially expressed genes into three major subgroups including the biological process, cellular component and molecular function. As shown in Fig. 4B, within the category of the biological process, major variation was for genes related to the anatomical structure development, cell adhesion, cell differentiation, cell motility, cell proliferation, extracellular matrix organization, immune system, locomotion, response

Fig. 4 RNA sequencing analysis of the global gene expression. Cells including rbACs derived from both 6-days coculture and monoculture control, and dedifferentiated P6 rbACs were subjected to RNA sequencing analysis. **A** The heat map of differentially expressed

genes; **B** Go classification enrichment analysis of differentially expressed genes. M: rbACs derived from monoculture; C: rbACs derived from coculture with rbBMSCs; P: P6 rbACs

to stress and signal transduction; for the cellular component, prominent difference was attributed to genes related to the extracellular region, extracellular space and proteinaceous ECM; for molecular function, genes related to ion binding varied. Interestingly, when comparing among these three types of cells, clustering of differentially expressed genes shared a great similarity. Some representative genes were: *IGF1R*, *PGF*, *EGFR*, *FGF*, *FGFR4*, *GDF5/6/7* and *PTGFR* (cytokines and receptors); *CCND2* and *HIF2A* (cell proliferation); *ITGAL*, *IBSP*, *ITGA4*, *ITGB2* and *ITGAV* (integrins); *HS3ST5*, *HS3ST1*, *EXTL1*, *CHST1*, *MMP1*, *MYH*, *PECAM1*, *NCAM*, *MMP3*, *COL1A5*, *THBS2S*, *VCAM1*, *L1CAM*, *COL9A* and *COL14A* (ECM); *NOS2*, *IL4R*, *IL6*, *IL23A*, *IL7*, *CXCL1/2/3*, *VCAM1*, *CCL2*, *IL18*, *IL17B*, *TNFSF14*, *PECAM1* and *CXCL5/6* (inflammation); *RASGRP1*, *KSR1*, *MAP3K8*, *GNG11* and *WNT5* (signal transduction) (data not shown).

To validate RNA sequencing analysis, qRT-PCR was performed on 18 selected genes (Fig. S1; Table 1). As listed in Table 1, compared to monocultured rbACs, cocultured rbACs showed upregulation in *IGF1R*, *HS3ST5*, *MMP1*, *MMP3*, *NOS2*, *IL4R*, *IL6*, *IL7*, *IL18*, *PECAM1*, *VCAM1*, *KSR1*, *MAP3K8* and *CCL2*, while *CCND2* and *SFRP5* were downregulated in cocultured rbACs. In addition, compared to dedifferentiated rbACs, the expression of *MMP1* and *MMP3* was increased and *IL6*, *IL7* and *IL18*

Table 1 Validation of RNA Sequencing using qRT-PCR

Gene	RNA Sequencing (C vs. M; C vs. P)	qRT-PCR (C vs. M; C vs. P)
<i>IGF1R</i>	C↑; C↓	C↑; C↓
<i>CCND2</i>	C↓; C↓	C↓; C↓
<i>MMP1</i>	C↑; C↓	C↑; C↓
<i>MMP3</i>	C↑; C↓	C↑; C↓
<i>HS3ST5</i>	C↑; Cn.d.	C↓*; C↑*
<i>CHST1</i>	C↓; C↑	C↓; C↑
<i>NOS2</i>	C↑; C↑	C↓*; C↑
<i>IL4R</i>	C↑; C↓	C↑; C↓
<i>IL7</i>	C↑; C↓	C↑; C↓
<i>IL8</i>	C↑; C↓	Cn.d.*; C↓
<i>IL6</i>	C↑; C↓	C↑; C↓
<i>PECAM1</i>	C↑; C↓	C↑; C↓
<i>SFRP5</i>	C↓; C↑	C↓; C↑
<i>VCAM1</i>	C↑; Cn.d.	Cn.d.*; C↑*
<i>PTGS2</i>	C↑; C↓	C↑; C↓
<i>KSR1</i>	C↑; C↓	C↑; C↑*
<i>MAP3K8</i>	C↑; C↓	C↑; C↓
<i>CCL2</i>	C↑; C↓	C↑; C↓

n.d. no difference

* There was difference between qRT-PCR and RNA Sequencing analyses

were inhibited in cells from coculture. Importantly, qRT-PCR analysis confirmed the differential expression of 13 out of 18 genes, albeit the expression of *HS3ST5*, *NOS2*, *IL18*, *VCAM1* and *KSRI* was not consistent.

3.5 Subculture and chondrogenic re-differentiation of rbACs after coculture

After coculture with rbBMSCs, rbACs were harvested and subcultured in chondrocyte growth medium. Within 12 days, cells derived from coculture maintained an elongated spindle-like shape, in contrast to the cobble-like morphology of cells from monoculture, especially on day 12 (Fig. 5A). The shape of subcultured rbACs from coculture also born resemblance to rbBMSCs. As shown in Fig. 5B. The proliferation of cells derived from coculture during subculture was greater than those from monoculture, although it was much lower than rbBMSCs. Further, cells after coculture (P2) were passaged successively till P5 in chondrocyte growth medium. The final cPD was 8.43 and 7.08 for cells from coculture and monoculture, respectively (Fig. 5C). Additionally, rbACs derived from

coculture were also passaged in rbBMSCs-conditioned medium, which showed a similar cPD (8.42) as in chondrocyte growth medium.

The chondrogenic re-differentiation of rbACs after coculture was assessed. While cells derived from coculture could form one intact pellet from the beginning, those from monoculture generated disintegrated smaller pellets, which fused with each other gradually with time (Fig. 6A). The pellets after 14 days was semi-transparent, with smooth whitish appearance, and live/dead staining confirmed that the majority of cells were alive. Safranin O staining demonstrated the production of abundant GAG by cells derived from both coculture and monoculture, with stronger staining intensity in the center of the pellets (Fig. 6B). Based on H&E staining, more basic ECM components were found in the center of the pellets for both cells, in agreement with safranin O staining. In addition, the zonal organization of cells and ECM was noted and more lacuna-like structures were found in the pellets for cells derived from coculture. As shown in Fig. 6C, cells from coculture showed slightly higher expression of *Acan*, *SOX9*, *Col10a1*, *CD14* and *CD44*, similar level of *Col2a1*, *COMP*

Fig. 5 Subculture of rbACs after coculture. After 6-days coculture, rbACs (P2) were subcultured for 12 days and monocultured rbACs and P3 rbBMSCs were included as controls. **A** Observation of cells under bright field within 12 days; **B** the growth curves within 12 days. In addition, rbACs (P2) derived from 6-days coculture and monoculture were passaged in subculture until P5. **C** cPD during

successive passaging. M: rbACs derived from monoculture in chondrocyte growth medium; rbBMSCs: P3 rbBMSCs in growth medium; C: rbACs derived from coculture in chondrocyte growth medium; CCM: rbACs derived from coculture in rbBMSCs-conditioned medium

Fig. 6 The chondrogenic re-differentiation of rbACs after coculture. To induce chondrogenic re-differentiation, rbACs derived from both 6-days coculture and monoculture were cultured in CIM as micromass for 14 days. **A** Observation of micromasses under bright field within

14 days, and gross view and live/dead staining of micromasses (day 14); **B** Safranin O and H&E staining of micromasses (day 14); **C** RT-PCR analysis of gene expression (day 14). M: rbACs derived from monoculture; C: rbACs derived from coculture with rbBMSCs

and *CD90*, and lower expression of *Colla1*, while expression of *Ver* was not detected for both cells.

4 Discussion

We had previously reported that rbBMSCs could down-regulate the differentiated phenotype of rbACs and induce significant changes in cell shape, cell proliferation and ECM production [17, 19]. The present study aimed at addressing two further issues: (1) whether both MSCs and chondrocytes derived from other origins than rabbits have similar phenomenon in coculture; (2) whether these changes represent a comprehensive response of chondrocytes to MSCs.

Cells derived from different sources concerning both species and tissue origins, were chosen for both MSCs and chondrocytes, and nine coculture combinations were set up. Changes in cell shape and ECM production that observed in coculture between rbBMSCs and rbACs were confirmed in all coculture combinations. However, differences were found for cell proliferation. While all MSCs stimulated proliferation of rbACs consistently, only hAMSCs and rBMSCs could promote the proliferation of bACs and all MSCs were not able to prime the proliferation of ATDC5 cells. Since the same coculture conditions were

applied, these differential effects of different MSCs on different chondrocytes in cell proliferation can be possibly attributed to the difference in trophic secretion of different MSCs [26]. In consistent with this, we had shown that the seeding density of rbBMSCs affected their effects on rbACs [19]. Acharya et al. [16] also noticed that hAMSCs were less supportive to the chondrogenesis than hBMSCs in coculture with human chondrocytes as mixed cell pellets. Moreover, distinct from maturely differentiated rbACs and bACs, ATDC5 cells are a chondrogenic progenitor cell line and very proliferative *in vitro* [27]. Hence, while both cell shape and cartilaginous ECM production of ATDC5 were apparently affected by MSCs, proliferation was not significantly primed. Nonetheless, the downregulation in the differentiated phenotype of chondrocytes by MSCs is conserved across MSCs from different sources in non-contact coculture in growth medium without chondrogenic induction factors. It is of note that Wu et al. [28] demonstrated that hBMSCs, hAMSCs and human synovial membrane-derived MSCs consistently promoted the proliferation and ECM production of human chondrocytes as mixed cell pellets in chondrogenic condition.

The expression of nine molecules, CD14 (lipopolysaccharide receptor), CD29 (integrin $\beta 1$), CD44 (hyaluronan receptor), CD49e (integrin $\alpha 5$), CD49f (integrin $\alpha 6$), CD51/61 (integrin $\alpha v/\beta 3$), CD81 (member of

transmembrane four superfamily), CD90 (Thy-1, miscellaneous surface marker) and CD166 (activated leukocyte cell adhesion molecule), was compared among rbACs from coculture and monoculture, dedifferentiated rbACs and rbBMSCs. These molecules can be categorized into three groups: tetraspanins, ectoenzymes, and other miscellaneous surface molecules (CD81 and CD90); cell adhesion molecules including integrins (CD29, CD44, CD49e, CD49f and CD166); and receptors for growth factors or other ligands (CD14) [29]. These four types of cells displayed distinct surface molecular profiles. Notably, coculture induced downregulation in CD44, CD49f and CD90, and upregulation in CD51/61 and CD81, suggesting a comprehensive phenotypic change. Cocultured rbACs were different from dedifferentiated cells, with lower levels of CD14 and CD51/61 and higher levels of CD44, CD49e and CD81. Diaz-Romero et al. [29, 30] had compared primary human ACs, dedifferentiated ones and hBMSCs, and revealed the distinct patterns of surface molecules. Nonetheless, the changes in surface molecules of rbACs upon coculture with rbBMSCs might be linked to their changes in ECM production and cell proliferation [31, 32]. Interestingly, albeit having lower CD51/61 and higher CD49f, cocultured cells shared a striking similarity with rbBMSCs, in contrast to monocultured and P6 rbACs. Previously, dedifferentiated ACs had been suggested as a bona fide cell population, being distinct from MSCs [30].

RNA sequencing was applied to analyze the global gene expression of rbACs from coculture and monoculture and P6 rbACs. Among these cell populations, 503 genes showed significantly differential expression between rbACs derived from coculture and monoculture, 572 genes between cocultured and P6 rbACs, and 530 genes between P6 rbACs and monoculture, indicating the profound changes in gene expression. Moreover, the gene expression of 13 out of 18 selected genes, including *IGF1R*, *CCND2*, *MMP1*, *MMP3*, *HS3ST5*, *CHST1*, *NOS2*, *IL4R*, *IL6*, *IL7*, *IL18*, *PECAM1*, *SFRP5*, *VCAM1*, *PTGS2*, *KSR1*, *MAP3K8* and *CCL2*, showed consistent variations between qRT-PCR and RNA sequencing analyses, confirming the reliability of RNA sequencing data. In general, major variations were found for genes related to cellular processes, ECM and signaling transduction, suggesting a comprehensive response of cocultured chondrocytes. For example, cocultured rbACs expressed higher levels of *IGF1R*, *PGF*, *EGFR*, *FGF*, *FGFR4*, *GDF5/6/7* and *PTGFR*. This is consistent with our previous findings that growth factors such as FGF-1, VEGF-A and PDGFbb are potential paracrine factors secreted by rbBMSCs to modulate rbACs [19]. In addition, a large panel of integrin genes including *ITGAL*, *ITGA4*, *ITGB2* and *ITGAV* showed the differential expression between cocultured rbACs and monoculture control and can be very significant since their expression

pattern might be associated with the pathological conditions such as osteoarthritis [32]. Variations in ECM related genes such as *HS3ST5*, *HS3ST1*, *CHST1*, *PECAM1*, *NCAM*, *COL1A5*, *THBS2S*, *VCAM1*, *L1CAM*, *COL9A* and *COL14A* implicated the changes in the differentiated phenotype of cocultured chondrocytes. Further, cocultured cells showed upregulated expression of *MMP1*, *MMP3*, *IL6*, *IL7* and *IL18*, suggesting the stimulated inflammatory response of rbACs in coculture. It has been recognized that MSCs can instigate the inflammatory effect in a non-inflammation environment as in the present study, while inhibiting the inflammation response in an inflammatory environment [33].

Subculture of cocultured rbACs demonstrated that both cell shape and proliferation were imprinted characteristics. However, successive passaging cocultured cells in rbBMSCs-conditioned medium did not further stimulate cell proliferation, suggesting that a transient coculture (6-days coculture) might be sufficient to induce the profound changes of rbACs. Cocultured cells displayed slightly better chondrogenesis than monocultured control. In one study, supplementing growth factor combination of FGF-2/TGFβ1 in growth medium could stimulate proliferation of human ACs and the primed cells showed better chondrogenic potential [34]. Taking into account stimulated cell proliferation, similar surface molecules with MSCs and chondrogenic potential, it may suggest that rbBMSCs secrete the paracrine factors, which induce the loss of differentiated phenotype and turn the differentiated chondrocytes into the progenitor-like cells, so we believe that the cocultured rbACs are beneficial to cartilage tissue regeneration. In fact, osteoarthritic chondrocytes are considered to share some similarity with MSCs, showing even superior chondrogenic potential [35]. These *in vitro* findings may lead to new therapeutic routes for cartilage regeneration [8]. Albeit it is not investigated in this work, either expanded chondrocytes in the non-contact coculture with MSCs or chondrocytes mixed with MSCs can be subsequently tested to repair cartilage defects in animal models.

Taken together, in a non-contact transwell system, the consistent changes in the differentiated phenotype of chondrocytes subjected to coculture with MSCs are demonstrated across cells derived from diverse origins. After coculture, rbACs undergo a comprehensive response and bear both distinct surface molecule patterns and global gene expression profiles. However, how MSCs remains to be further explored. We had previously demonstrated that FGF-1, VEGF-A and PDGFbb were potentially involved in the molecular mechanism [19]. A detailed protein analysis in the culture system is warranted. Moreover, MSCs-conditioned chondrocytes display greater potential of chondrogenesis. These findings may lay solid foundation for

cartilage tissue regeneration by applying the chondrocytes that cocultured with MSCs.

Acknowledgements This work was supported by National Natural Science Foundation of China (81671841), National Special Fund for State Key Laboratory of Bioreactor Engineering (2060204) and Natural Science Foundation of Shanghai (16ZR1408700).

Compliance with ethical standards

Conflict of interest All authors would like to indicate that there is no conflict of interest with regard to the work.

Ethical statement All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Shanghai Laboratory Animal Center (Shanghai) and the protocols were approved by the institutional animal care and use committee of Shanghai Laboratory Animal Center (SYXK2017-0008).

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