Original Article

Co-Culture of Mesenchymal Stem Cells with Mature Chondrocytes: Producing Cartilage Construct for Application in Cartilage Regeneration

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Abstract

Background: Cell-based treatment approach using differentiated mesenchymal stem cells (MSCs) and mature chondrocytes has been considered as an advanced treatment for cartilage repair. We investigated the differentiated level of these two cell types that is crucial for their repair capacity for cartilage defect at a co-culture micro mass system.

Methods: Passaged-2 MSCs isolated from the mouse bone marrow and the primary-cultured chondrocytes obtained from rat costal cartilage were mixed at different ratios including 1:1, 1:2, and 2:1, and cultivated in the micro mass culture systems (experimental groups). Both the MSCs and chondrocytes alone in micro mass cultures were considered as the controls. After 21 days, the cultures were sectioned and examined by toluidine blue staining. Furthermore, the cells at different groups were analyzed by semiquantitative reverse transcription-polymerase chain reaction using the specific primers designed to detect the expression of both mouse and rat cartilage-specific genes.

Results: According to the toluidine blue staining, metachromatic stain appeared to be more intense at 1:2 ratios than the other groups. Based on semiquantitative analysis, all co-cultures possessed significantly more cartilage-specific gene expression than the controls (P<0.01). While mouse aggrecan and collagen II genes had significantly more expression at 1:2 ratio, rat collagen II gene was expressed in higher rate at co-culture with 2:1 ratio (P<0.01).

Conclusion: Co-culture of MSCs with mature chondrocytes seemed to provide an appropriate microenvironment whereby the two cell types exhibit higher differentiated phenotype than when they were cultured alone, and sufficient to be used as the cellular material for repair of cartilage defects.
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Keywords ● Mesenchymal stem cells ● chondrocytes ● co-culture ● cartilage differentiation

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Introduction

Recent advances in biological sciences have generated great interest in use of cell-based treatment of tissue defects. It is particularly true for cartilage damages

because the cartilage possess a limited repair capacity mainly due to its avascular nature and low mitotic potential of chondrocytes in vivo.[1-4] To date, several attempts have been made to reconstruct cartilage lesions by chondrocyte transplantation alone or chondrocyte/scaffold combination.[5,6]

Several limitations, however, are associated with the use of chondrocytes in cartilage damages, including difficulties in obtaining sufficient number of chondrocytes for auto transplantation. Furthermore, chondrocytes being expanded in culture gradually undergo differentiation loosing their morphological features as well as specialized function, hence no longer appropriate for transplantation purpose.[7-9]

Mesenchymal stem cells (MSCs) are the other cell source for cartilage repair. Nowadays, these cells have been considered as more appropriate cellular material for cell therapy because they display some valuable properties including long-term self-renewal potential and multilineage differentiation capacity into specialized cell types as bone cartilage and adipose cells. Moreover, MSCs could easily be accessed from multiple sources such as bone marrow aspirates.[10-13]

One method for chondrogenesis of MSCs is using a co-culture system in which cell differentiation could be improved by enhanced signal transduction and direct cell-to-cell contact.[14] It has been indicated that MSCs chondrogenesis could be improved in co-culture with the synovial membrane macrophages as well as the embryonic rat calvarial cells.[15,16] A disadvantage associated with such systems is that differentiated MSCs could not be purified from the co-culture cells.

To solve this problem, chondrocyte/MSCs co-culture system can be considered as a suitable setting. This is because in such system the co-cultured cells, i.e. chondrocytes, are cartilage native cells; therefore, no longer cell purification is needed. Co-culture of MSCs and chondrocyte exhibit two potential advantages. Firstly, it obviates the needs for in vitro multiplication of chondrocytes; because their low division frequency can be compensated by MSCs presence. And secondly, the presence of chondrocytes in a co-culture system provides strong chondrogenic effects on MSCs differentiation. In this system, two potential candidates of cell therapy including chondrocytes and MSCs are present.

The aim of the present study was to investigate the expression levels of some cartilage-specific genes as the outcome of the interactions occurred between different ratios of these two cell types and to find out the chondrogenic effects of the co-culture.

Materials and Methods

Murine MSCs Isolation

The present study was approved by the Ethics Committee of Royan Institute. MSCs were isolated and expanded according to our previously published article.[12] In brief, the mononuclear cell fractions from marrow of 6-8-week-old mice (NMRI strain) were collected, and counted with trypan blue in a Neubauer chamber. The cells were then diluted to an approximately 500 viable cells and plated in the wells of 24-well plates using DMEM (Dulbeco Modified Eagle Medium; Gibco, Germany) supplemented with 100 IU/ml penicillin (Sigma, USA), 100 mg/ml streptomycin (Sigma, USA) and 10% fetal calf serum (FCS, Gibco). The plates were kept in a humidified incubator at an atmosphere of 5% CO_2 at 37°C for two weeks during which several colonies of fibroblastic cells emerged. These cells were trypsinized and pooled together before they were distributed in 6-well plates (Falcon, Gibco, UK). These multiclonal cultures were kept in an incubator for 7 days at the end of which 70-80% confluence was attained. At this point, the next passages were initiated, and the cells were pooled and cultured in 75-cm^2 plastic flasks (Falcon; Gibco, UK) as passage-2 cells.

Chondrocyte Culture

Ten Wistar rats aged 8 weeks and 200-250 gr weight were euthanased by cervical dislocation. Their costal cartilage were dissected under sterile condition and placed in DMEM medium containing antibiotics including 100 IU/ml penicillin and 100 mg/ml streptomycin. The cartilage tissue were then minced into pieces of 2-3 mm dimensions, subjected to enzymatic digestion using 0.2% collagenase I for 24 h at 37°C. At the end of this period, cartilage pieces were placed in a 75-cm^2 flask in a 15 ml DMEM containing 15% fetal bovine serum (FBS) as an explants culture system.

MSCs-Chondrocyte Co-Culture System

4×10^5 cells including chondrocytes from primary culture and MSCs from passage 2 were combined. The co-culture was established in a micro mass culture system by palletizing the cells with centrifugation at 1200 g for 5 minutes. The following mixtures were then made: 1:1 ratio (one part of MSCs/one part of chondrocytes), 1:2 ratio (one part of MSCs/ two parts of chondrocytes), and 2:1 ratio (two parts of MSCs/one part of chondrocytes). The cell pellets were cultivated in

DMEM medium containing 5% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin for 21 days at 37 °C in an atmosphere of 5% CO2. The pellets of pure MSCs as well as pure chondrocytes were also cultivated in a medium containing chondrogenic inducers including 10 ng/ml transforming growth factor ß3 (TGF ß3, Sigma, USA), 500 ng/ml bone morphogenetic protein-6 (BMP-6, Sigma, USA), 100 nM dexamethasone (Sigma, USA), 50 µg/ml ascorbic 2-phosphate (Sigma, USA), 50 µg/ml Insulin-Transferrin-Selenium (ITS, Sigma, USA) and 1.25 mg/ml bovine serum albumin (BSA, Sigma, USA) as the control groups.

Evaluation of Cartilage Differentiation
   Toluidine Blue staining: To examine whether cartilage metachromatic matrix were deposited among the cells, the pellets were fixed with 4% paraformaldehyde for 2 hours, dehydrated by ascending grades of ethanol, and embedded in paraffin. Five micrometer sections were then cut and stained with toluidine blue solution. Since matrix could be secreted by either cell types, i.e. rat chondrocytes and differentiating MSCs, semiquantitative polymerase chain reaction (PCR) with specific primers for either cell types was used to accurately determine the share of each cell in matrix production.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis
   Relative RT-PCR was performed to assess the expression of a set of chondrocyte-related genes in both cell types, i.e. mouse MSCs and rat chondrocytes. Total RNA was isolated from the cells at varying ratios of co-culture and the control groups using the RNX-Plus™ kit (RN7713C; CinnaGen Inc., Tehran, Iran).
   Before reverse transcription, a sample of the isolated RNA was treated with 1U/µl of RNase-free DNaseI (EN0521, Fermentas Life Science, Lithuania) per 1 µg of RNA in order to eliminate residual DNA in the presence of 40

U/µl of ribonuclease inhibitor (E00311, Fermentas Life Science, Lithuania) and 1× reaction buffer with $MgCl_2$ for 30 min at 37 °C. To inactivate the DNaseI, 1 µl of 25 mM EDTA was added and incubated at 65°C for 10 min. Standard RT reactions were performed with 2 µg total RNA using random hexamer as a primer and a RevertAid™ First Strand cDNA Synthesis Kit (K1622, Fermentas Life Science, Lithuania) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without RevertAidTMM-MuLV reverse transcriptase (RT reaction) to provide a negative control in the subsequent PCR. To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Reaction mixtures for PCR included 2 µl cDNA, 1×PCR buffer (AMS™; CinnaGen Co., Iran), 200 µM dNTPs, 0.5 µM of each antisense and sense primer, and 1U Taq DNA polymerase.
   The PCR primers were designed specifically for each strain. For this purpose, 3' end of primers was specific for each strain so one pair of primer for a rats' gene did not amplify the same gene from mouse. The PCR primers and the annealing temperatures of the amplified products are shown in table 1. Nucleotides in red show specific 3' end for each forward primer.
   PCR reactions were performed on a Master cycler gradient machine (Eppendorf, Germany). Amplification conditions consisted of initial denaturation, 94°C for 5 min followed by 35 cycles (25 cycles for B-Tubulin) of denaturation at 94°C for 45 sec, annealing for 45 sec, extension at 72°C for 30 sec, and a final polymerization at 72°C for 10 min. Each PCR was performed under linear conditions with B-tubulin used as an internal standard. Products were electrophoresed on 1.7% agarose gel. The gels were stained with ethidium bromide (0.5 mg/ml) and photographed on a UV transilluminator (Uvidoc, U.K.). Gel images were analyzed using the UVI band map program

Table 1: Primers used in the PCR analysis

	Accession number	Gene name	Primer sequence	Annealing Temperature (°C)
1	gi|70980519|ref|NM_031163.2	Mouse Col2a1	F: GGTGTGAAGGGTGAGAG**T** R: TTACCCCGAGCACCAGC	62
2	gi|6978676|ref|NM_012929.1|	Rat Col2a1	F: GGTGTGAAGGGTGAGAG**C** R: CCAGCAGCACCAGCAGG	58
3	gi|116875857|ref|NM_007424.2|	Mouse Aggrecan	F: AAGTTCCAGGGTCACTGTTA**C** R:TCCTCTCCGGTGGCAAAGAAG	60
4	gi|532343|gb|J03485.1|	Rat Aggrecan	F: CCAAGTTCCAGGG**C**CACTGTTA**T** R:TCCTCTCCAGTGGCAAAGAAA	66
5	gi|27465534|ref|NM_173102.1|	Rat ß-tub	F: CTCACTGTGCCTGAACT**C** R: GGAACATTGCCGTAAACTGC	58
6	gi|142374674|ref|NM_011655.4	Mouse ß-tub	F: CTCACTGTGCCTGAACT**T** R: GGAACATAGCCGTAAACTGC	56

(Uvitec, Cambridge, U.K.). For semi-quantitative determination of mRNA levels of the candidate genes, transcript levels were normalized to the corresponding B-tubulin.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the values for mRNA levels of the candidate genes using SPSS 13.0 for windows. A P value of <0.05 was considered to be statistically significant.

Results

MSCs Culture

By days 7-9, some fibroblastic colonies consisting of a few spindly-shaped cells were appeared in some wells and became larger by the end of week 2 (figure 1A). These cells were tripsinized, pooled together and sub cultured in a 25-cm^2 flask (figure 1B). Sufficient number of the cells enough to conduct the next phase of the experiment was achieved upon second subculture.

Chondrocyte Culture

A few days after culture initiation, chondrocytes migrated out of cartilage explants, adhered on the culture plate surfaces and started to proliferate (figure 1C). The cells first appeared as elongated in morphology. Two weeks after culture initiation, when the culture achieved confluence, the cells tended to be polygonal in appearance (figure 1D).

Toluidine Blue Staining

At the culture with 1:2 ratio, where the number of chondrocytes was twice as MSCs, intensive homogenous metachromatic matrix was produced compared with other ratios as well as the control cultures (figure 2A-E).
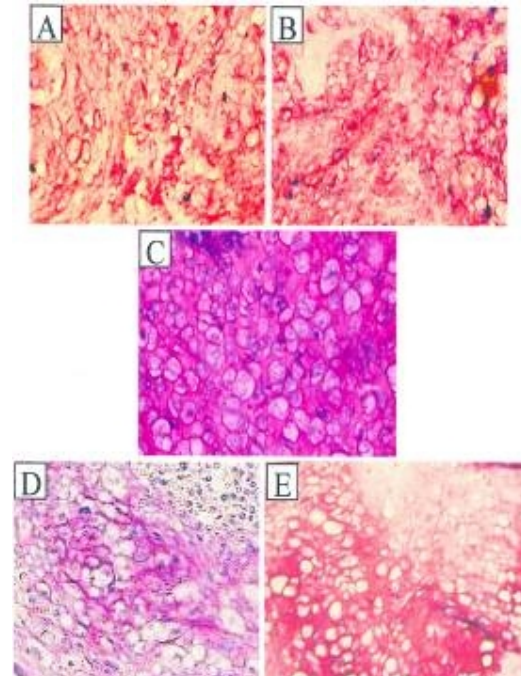

Figure 2: Toluidine blue staining for metachromatic cartilage matrix detection; A) 1:1 ratio (one part of mouse mesenchymal stem cells and one part of rat chondrocytes), B) 2:1 ratio (two parts of mouse mesenchymal stem cells and one part of rat chondrocytes), C) 1:2 ratio (one part of mouse mesenchymal stem cells and two parts of rat chondrocytes), D) Pure mouse mesenchymal stem cells, and E) Pure rat chondrocytes.
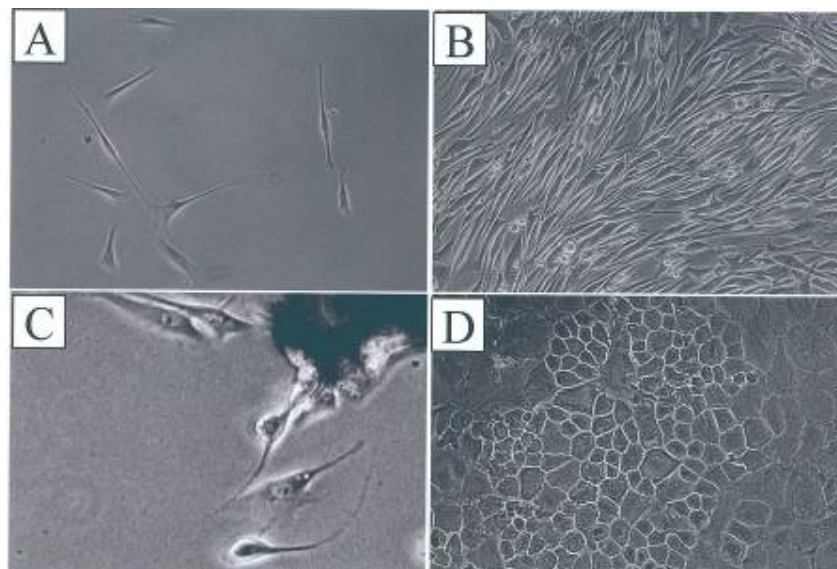

Figure 1: Cell culture; A) Mouse bone marrow cells in low-density culture, B) Purified mouse mesenchymal stem cells, C) Cartilage explant culture, and D) Confluent chondrocyte in primary culture.

Semi Quantitative RT-PCR Analysis

Mouse cartilage-related gene expression: Relative gene expression results of studied groups were in accordance with the findings obtained from toluidine blue staining. Mouse aggrecan and collagen II mRNA at co-culture with 1:2 ratio tended to be produced more than those at 1:1, 2:1 ratios and pure MSCs cultures ($P<0.01$). The cartilage-specific genes at co-cultures with 1:1 and 2:1 ratios appeared to be expressed in higher levels than those at pure MSCs micro mass culture ($P<0.005$; figure 3A-C).
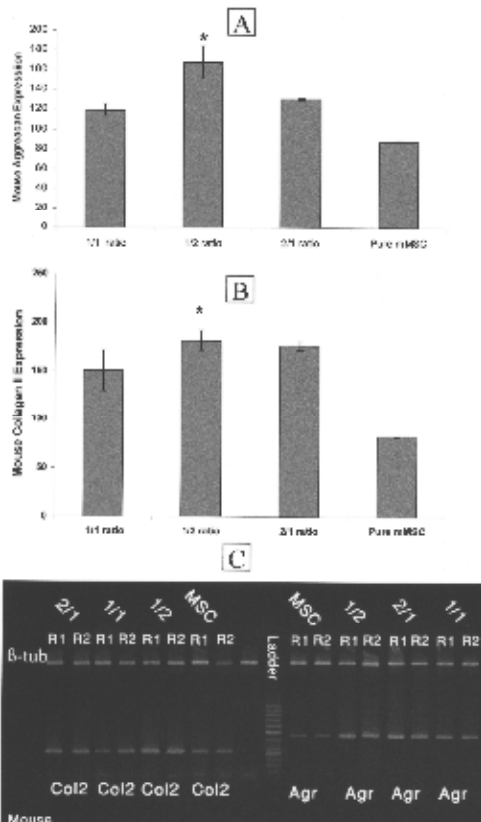

Figure 3: Semiquantitative RT-PCR analysis of mouse cartilage-relate gene expression; A) Aggrecan gene expression, B) Collagen II gene expression. At 1/2 ratio, relatively more cartilage-related genes were expressed, and C) Representative PCR gel image. R: repeat; Col: Collagen; Agr: Aggrecan ; MSC: mesenchymal stem cell.

Rat cartilage-related gene expression: The expression level of rat aggrecan and collagen II genes at different co-culture ratios and pure chondrocytes micro mass culture were in a pattern similar to those in mouse MSCs cultures. However, 2:1 co-culture ratio showed higher chondrogenic differentiation (figure 4A-C).

Discussion

In the present study, micro mass co-culture

system of rat mature chondrocytes with mouse marrow-derived MSCs was established and cartilage differentiation of the MSCs as well as the cartilaginous phenotypes of the chondrocytes were investigated at co-culture conditions.
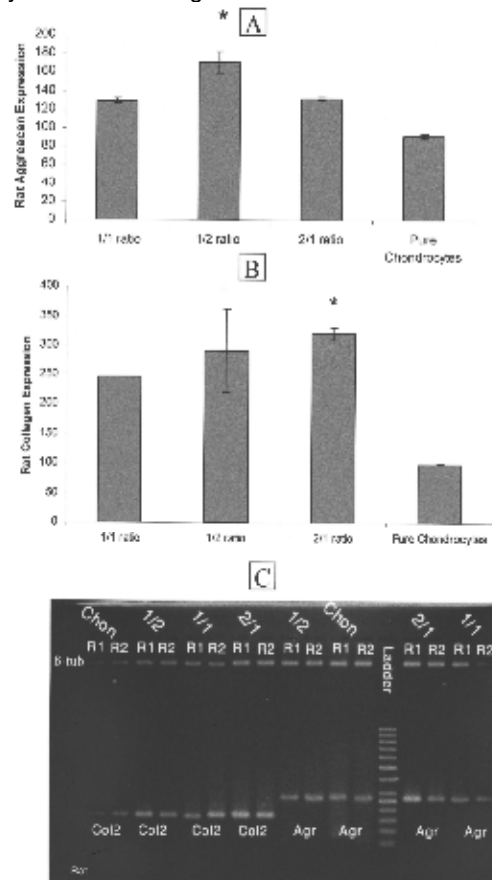

Figure 4: Semiquantitative RT-PCR analysis of rat cartilage-relate gene expression; A) Aggrecan gene expression, B) Collagen II gene expression. At 1/2 ratio relatively more collagen II gene was expressed, And C) Representative PCR gel image. R: repeat, Col: Collagen, Agr: Aggrecan , Chon: chondrocytes.

Our results showed that chondrocytes exhibited strong chondrogenic effects on MSCs even more than conventional chondrogenic medium used at micro mass culture system.

In vitro differentiation of MSCs could be of great clinical significance. In general, there are two strategies in MSCs-mediated tissue regeneration. The first approach uses the cells in undifferentiated state, allowing them to be differentiated in *in vivo* microenvironment. The disadvantage of this approach is unwanted cell differentiation- other than the desired cells- in transplantation site. For instance, if the cells are to be regenerate cartilage tissue, other cells such as bone cells may produce in the site by unwanted differentiation. Therefore, the alternative approach is to fully differentiate

MSCs into the desired cells prior to their transplantation. Such strategy could indeed guarantee the administration of the differentiated cells into the repair site. Therefore, the importance of the in vitro differentiation of MSCs into cartilage tissue becomes obvious.[17,18]

Our experimental setup indicated that co-culture of MSCs with mature chondrocytes could be used for differentiation of MSCs into cartilage. MSCs differentiated in this system do not require purification from co-culture cells since mature chondrocytes are cartilage native cells.

MSCs have traditionally been known to be able to differentiate into three mesenchymal lineages of bone, cartilage, and adipose cells. The ability of MSCs to differentiate into the cells other than those of skeletal lineage, i.e. differentiation into cardiomyocytes or hepatocytes, has been referred to trans differentiation.[19,20]

Previous studies have shown that MSCs trans differentiation can be occurred following their fusion with the mature cells in co-culture systems and after transplantations.[21,22] In the present study MSCs were co-cultured with mature chondrocytes and our study indicated an increased expression of cartilage specific genes in the co-culture system. The mechanism of differentiation was not examined in the present study. MSCs are ancestor of mesenchymal cell lineages including cartilage cells and they would truly be differentiated into these cells in proper culture conditions. The cell fusion mechanism that has been proposed by some authors would be considered as an explanation of MSCs trans differentiation into non-skeletal cell lineages.

Our study is not the only investigation carried out in this context. Kohei and co-workers studied human MSCs co-culture with bovine articular chondrocytes. Their results showed no differentiation of MSCs into cartilage cells and no chondrogenic effect of mature chondrocytes on MSCs differentiation.[1] The discrepancy may be explained by the cell type being used in the two studies (human and bovine versus mouse and rat cells). The other point is that we established the co-culture system with DMEM medium without chondrogenic supplement while Kohei and colleagues cultured the cells in chondrogenic medium. Chondrogenic medium contains inducing substances for MSCs differentiation. Applying the chondrogenic medium in co-culture interferes with MSCs differentiation into cartilage. In a chondrogenic medium, mature chondrocytes may produce some inducing substances that alter the concentration of the medium, which is not appropriate for MSCs cartilage differentiation.

In a study by Mo and co-workers human MSCs were mixed with rabbit articular cartilage chondrocytes in different ratios and the cell mixtures were encapsulated in alginate hydrogel and cultured under chondrogenic conditions.[23] In contrast to our results, Mo and colleagues observed the upregulation of cartilage specific gene expression in co-culture systems with more proportions of MSCs. This difference can be attributed to the different cell sources and different culture conditions used in the two studies.

In another study by Li and others fat-derived multi potential cells from inguinal region of rabbit were co-cultured with intervertebral disc tissue.[24] The results demonstrated that MSCs responded to soluble mediator from the disk with increase in expression of type II collagen and aggrecan genes. In the present study, we used a different cell type (costal chondrocytes) to establish the co-culture systems. Moreover, in contrast to other studies, we investigated reciprocal effect of MSCs on mature chondrocyte phenotype in the co-culture conditions.

According to the results obtained by toluidine blue staining, metachromatic matrix deposited among the cells in co-culture pellets could be resulted from secretive activity of either differentiating MSCs or the mature chondrocytes. To find out the cell type contribution in matrix deposition, we designed the specific primers for cartilage-specific genes of both cell types. Interestingly, the matrix deposition appeared to be the result of both cell type activities. Indeed, differentiated MSCs may secrete the cartilage matrix and chondrocytes also contribute to matrix deposition because they maintained their phenotypes in co-culture system.

The co-culture with 1:2 (1 part of MSCs and 2 parts of mature chondrocytes) ratio was more appropriate than the other ratios in promoting MSCs differentiation into cartilage. The synergistic effect of co-culture systems can be mediated by two mechanisms; a) direct contact of co-cultured cells with MSCs, b) soluble substances secreted by co-cultured cells.[25,26] In our study, it seems that the synergistic effect is stronger when the number of co-cultured cells are twice as MSCs. Probably, an optimal direct cell to cell contact and secretion of inducing substances were provided when 2 chondrocytes/1 MSCs mixed together in co-culture pellets.

According to previous investigations, chondrocytes lose their differentiated phenotypes at monolayer culture and regain it upon cultivation in three-dimensional cultures.[7,8,27,28] To investigate the MSCs action on rat mature chondrocyte in co-culture, we designed specific primers for rat cartilage-specific genes in

order to measure the level of differentiated state of the chondrocytes. The results indicated the higher differentiation levels of co-cultured chondrocytes that could be attributed to MSCs presence in co-culture system. In other words, at co-culture conditions, MSCs positively affected chondrocytes with a significant maintenance of differentiated state in chondrocyte population. This effect occurred more intensely at 1:2 ratio (1 part of MSCs and 2 parts of mature chondrocytes), than 1:1 or 2:1 ratios.

Conclusion

We concluded that MSCs-chondrocyte co-culture is an appropriate microenvironment for the cells interactions. This system appeared to be suitable for MSCs cartilage differentiation. For more effective differentiation, the ratio of co-cultured cells seemed to be a critical factor. MSCs induced the chondrocytes to maintain their cartilaginous phenotype.

Conflict of Interest: None declared

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