Comparison between High Cell-Density Culture Systems for Chondrogenic Differentiation and Articular Cartilage Reconstruction of Human Mesenchymal Stem Cells: A Literature Review

Hossein Mahboudi [a*], Bahram Kazemi [a], Masoud Soleimani [b], Hana Hanaee-Ahvaz [c], Abdolreza Ardeshirylajimi [d], Mohamad Eftekhary [a], Seyed Ehsan Enderami [e]

[a] Department of medical biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [b] Hematology department, Faculty of Medical Sciences, Tarbiat Modares University , Tehran, Iran; [c] Department of Stem Cell Biology, Stem Cell Technology Research Center, Tehran, Iran; [d] Department of Tissue engineering and Regenerative Medicine, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [e] Department of Medical Biotechnology and Nanotechnology, Faculty of Medical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran

Corresponding author: Hossein Mahboudi: Department of medical biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ***E-mail*:** hoseinmahboudi@sbmu.ac.ir; ***Tel:*** +98-21 44480873.

Submitted: 2016-09-12; **Accepted:** 2016-11-24

High-cell density culture is based on the chondrogenic differentiation of human mesenchymal stem cells (hMSCs), and because the high density of cell and reduced oxygen tension are effective in chondrogenic differentiation. In the present paper, there will be a review about the methods of chondrogenic differentiation of hMSCs that utilized in in vitro and in vivo chondrogenic differentiation of stem cells for treatment of osteoarthritis. There are three High-cell density culture systems; micromass, pellet culture, and alginate culture have been used to induce chondrogenic differentiation of hMSCs. Transplanted naive MSCs can cause problems such as heterogeneous populations. To overcome this problem, new strategies for inducing differentiation of MSCs are needed. One possibility is a cell culture system. Collagen II and aggrecan are critical protein in chondrogenic differentiation. In all different methods, real time RT-PCR analysis demonstrates that collagen II and aggrecan mRNA are up regulated while collagen X and collagen I mRNA are down regulated. So these three high-density cell culture systems have been approved for chondrogenic differentiation. On the other hand, In micromass method, the induced-cartilage tissues are larger, more homogenous and rich in cartilage specific collagen II, but collagen I, collagen X and hypertrophic chondrocyte features are markedly decreased compared to other culture system. Thus, the micromass culture system is the best tool for *in vitro* chondrogenic differentiation studies.

Keywords: Chondrogenesis; Mesenchymal stem cells; High-cell density culture

Introduction

Osteoarthritis (OA) is a progressive, chronic and irreversible degenerative joint disease. Conventional osteoarthritis treatments often lead to significant complications, such as pain and limited activity. Transplantation of mesenchymal stem cells (MSCs) has several beneficial effects such as paracrine effects, anti-inflammatory activity and immunomodulatory capacity. Transplantation of MSCs is also a suggested therapeutic approach for osteoarthritis treatment (1).

Human MSCs are a promising alternative cell source for cartilage regeneration (2). Recently, MSCs have been applied for treatment of OA in clinical trials (3). MSCs are easily found in various tissues, including bone marrow, adipose tissue, spleen, synovial fluid, and lungs. These cells can differentiate into several cell types including adipocytes, chondrocytes, cardiomyocytes, endothelial cells and osteocytes (3, 4). Several studies suggested that therapeutic effects for treatment of OA were showed by transplantation of chondrogenic differentiated MSCs (5, 6). Thus, differentiation of MSCs into specific chondrogenic cells via their modulation for transplant has the potential for treatment of OA (Figure 1) (7). Although, MSCs have several beneficial effects, but there are three essential factors for considering in using undifferentiated cells (7): efficiency of direct differentiation of stem cells into specific cell types (8); the survival rate of transplanted cells (9); and host environment when the cells are transplanted because not all differentiated pathways have been discovered yet (10, 11).

These approaches have limitations (7): undefined conditions leading to heterogeneous populations of cells (8), and unexpected risk of virus-mediated genetic modifications (10). To overcome this issue, new strategies for inducing differentiation of MSCs are required. One possibility is a cell culture system. Several cell culture methods have been developed to create suitable environments for chondrogenic differentiation of MSCs, including monolayer culture, high-density cell culture (micromass culture, pellet culture, and alginate culture), hanging

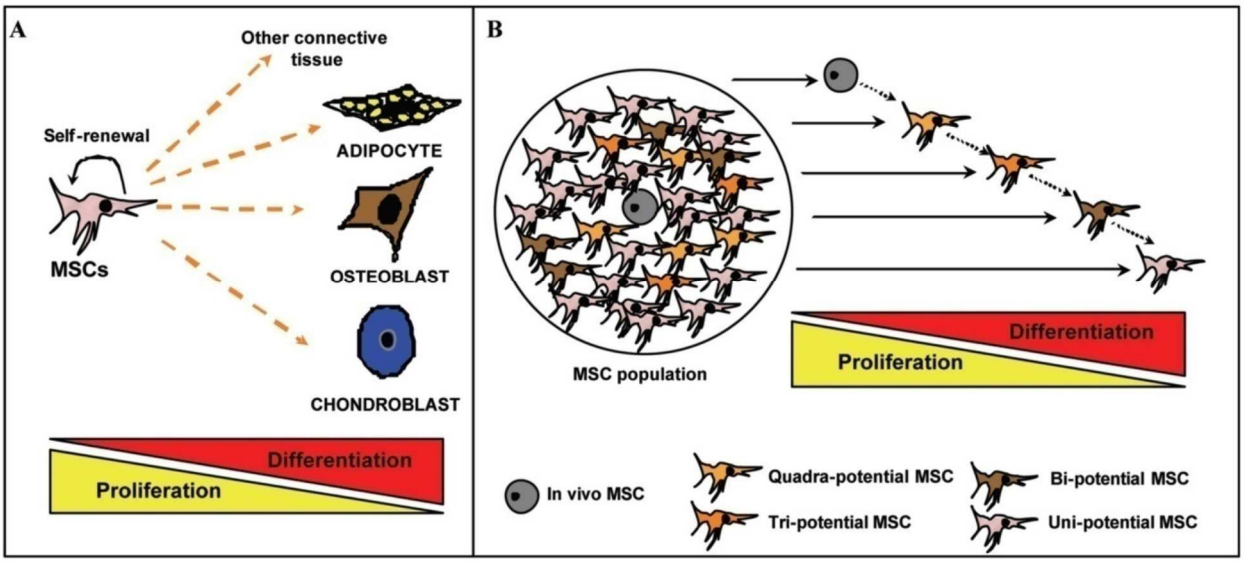

Figure 1. Models of MSC differentiation. (A) In this theoretical model, MSCs in the bone marrow constitute a primitive stem cell population (multipotent MSCs), having the potential for self-renewal and proliferation as well as the capacity to differentiate into all connective tissue cell types, when exposed to a defined environment. (B) An alternative model illustrating MSCs in vivo as a population of cells with different differentiation potentials (i.e., quadra-, tri-, bi- and uni-potential). During in vitro culture, this heterogeneous mixture of stem and committed progenitor cells is limited in his multilineage differentiation [7]. Permissions obtained from the publisher

drop and biomaterial-based scaffold culture. In the present review, high cell-density culture methods in the chondrogenic differentiation of human MSCs will be discussed.

One of these methods is alginate culture. Alginate beads are considered as a classic method for culturing chondrocytes. The original method of chondrocyte culture in the alginate beads was developed in the late eighties by Guo *et al.* (12) and was then employed and improved by other researchers. Chondrocytes are released from cartilage matrix. Beads are easy to handle and biological applications are compatible with this model of culture (13). Culture in alginate beads is also useful to re-differentiate chondrocytes that have dedifferentiated because of expansion in bidimensional culture (14).

Morphology of the three-dimensional (3D) constructs varies depending on whether the culture is a solid suspension such as alginate or agarose, where the MSCs form small groups and clusters interspersed throughout the support, or a the cells are condensed such as various sized pellet cultures that cells are more tightly packed in a tissue-like structure (Figure 2).

Pellet culture method is a standard method for the chondrogenesis of MSCs. This system provides a three-dimensional environment that allows cell–cell interactions similar to those observed in pre-cartilage condensations found during embryonic development. MSCs are capable of chondrogenic differentiation in pellet culture using serum-free medium containing glucocorticoids and transforming growth factor (TGFβ1) family (15). Therefore, pellet culture is widely used to evaluate the chondrogenic potential of MSCs and to study the signal pathways involved in chondrogenesis (16). Micromass culture system, first used to study endochondral skeletal development with embryonic chicken limb bud MSCs (17), has recently been applied to induce chondrogenesis in MSCs (18). Several studies have utilized micromass method for cartilage differentiation; because this method was designed based on generating cartilage from MSCs in the embryonic period. In micromass culture, transforming growth factor β1 (TGFβ1) treatment significantly increases the synthesis of the cartilage phenotypic markers (*SOX9*, *COL2A1*, *and ACAN*) and down-regulate the catabolic (*MMP13* and *ADAMTS5*) gene products in extracellular matrix (19).

Applied Methodology

Bone marrow harvest, cell isolation and expansion

In most studies, bone marrow samples were obtained from healthy volunteers and MSCs were isolated and purified by density gradient centrifugation combined with an attachment culture method (20, 21). Briefly, the bone marrow samples (8–10 ml) are diluted with 20 ml phosphate-buffered saline (PBS), then the cells fractionated on a Lymphoprep density gradient by centrifugation at 500g for 20 minutes. The interfacial mononuclear cells are collected and washed with low-glucose.

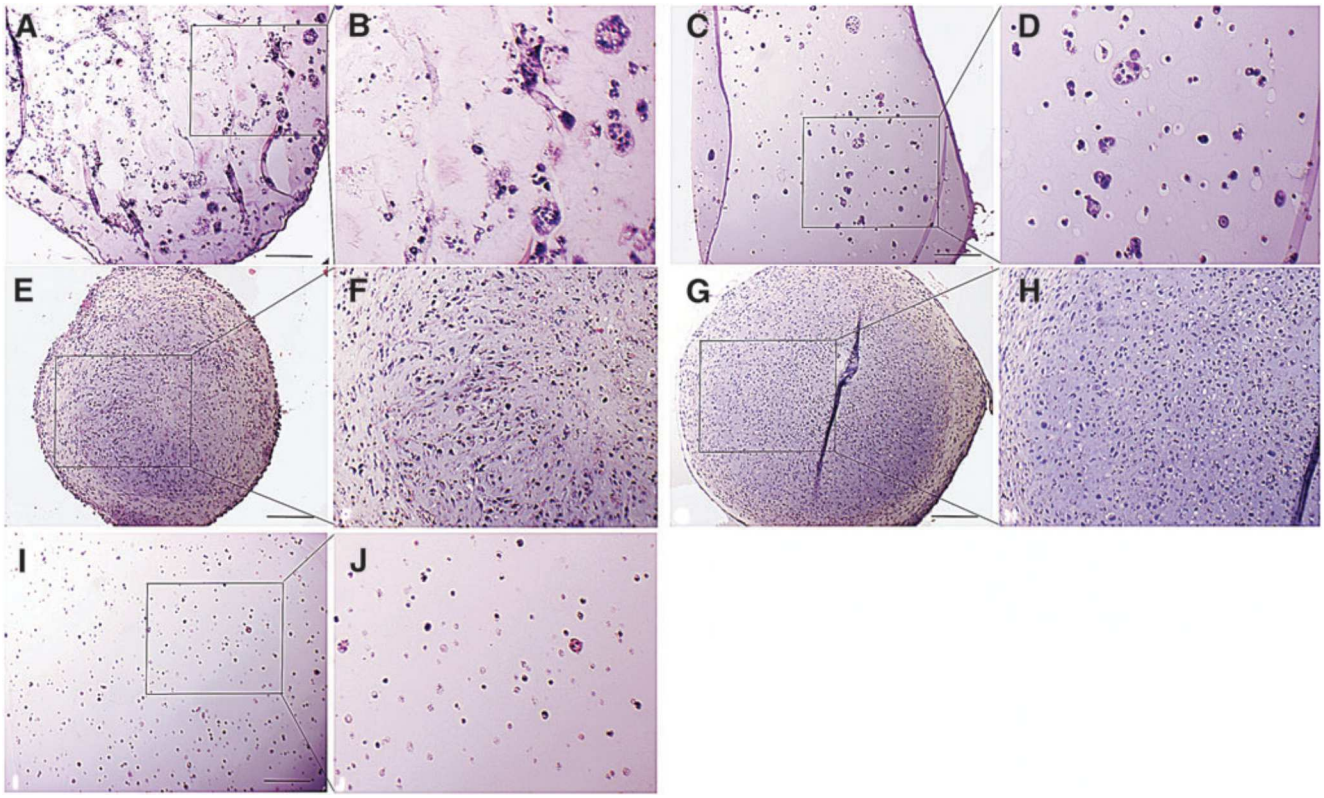

Figure 2. Photomicrographs of MSC 3D cultures collected at 28 days and stained with hematoxylin and eosin. (A, B) FA, (C, D) alginate, (E, F) 2.5 · 105 pellet, (G, H) 5 · 105 pellet, and (I, J) agarose. 200 · magnification. Images were taken at 100 · magnification. Black box represents area for magnified image adjacent to original. Scale bar = 200 mm [14]. Permissions obtained from the publisher

Dulbecco's modified Eagle medium (LG-DMEM) supplemented with 10% (v/v) FBS. The MSC primary cultures are seeded at a density of $10^6/cm^2$ in LG-DMEM with 10% FBS in 25 cm^2 flasks. The cells are incubated at 37°C under 5% CO2. Non-adherent cells are removed from the flasks after 48 h by changing the medium. Thereafter, the medium is changed every 3 days. Typically, cultures reach 80–90% confluency in day 14. In this stage, cells are trypsinized from the culture dishes using 0.25% trypsin containing 0.53 mM EDTA, counted and plated again. Cells from the 3rd passage are usually used for experiments. In the first passage (P0), cells show the typical fibroblast-like morphology of primary MSCs. During culture expansion up to P3, cells are slightly flattened but still have fibroblast-like morphology. In P3, MSC verification is performed by flow cytometry analysis from surface marker profile. As expected, MSC are uniformly positive for CD44, CD73, CD90, CD105, and CD166 and negative for the hematopoietic markers CD14, CD34, and CD45. Several studies have shown the multilineage differentiation potential of MSCs while here we are only focusing on their chondrogenic differentiation potential (Figure 3).

Chondrogenic differentiation of MScs by High density cell culture methods

Alginate culture

In alginate culture method MSCs or chondrocytes are released from cartilage by collagenase/dispase digestion. Then, they are mixed with a solution of 1.25% of alginic acid to obtain homogenous suspension. The suspension is drawn into a syringe and push gently through a needle, so that drops fall into a solution of calcium chloride. Beads form instantaneously and further polymerize after 5 min in the calcium chloride solution. MSCs or chondrocytes from any species, including human OA chondrocytes, can be cultured by using this technique (22). Encapsulated chondrocytes are still able to respond to growth factors and cytokines (23). Under these conditions, chondrocytes maintain a high degree of differentiation. Beads can be dissolved by chelation of calcium with EDTA. Almost all molecular and biochemical techniques, as well as a number of biological assays, are compatible with the culture of chondrocytes in alginate (22, 23).

Alginate beads are 3D scaffolds that closely resemble the cartilage matrix (24). In many studies, changings in chondrogenic genes were characterized and developed a stage for *in vitro*

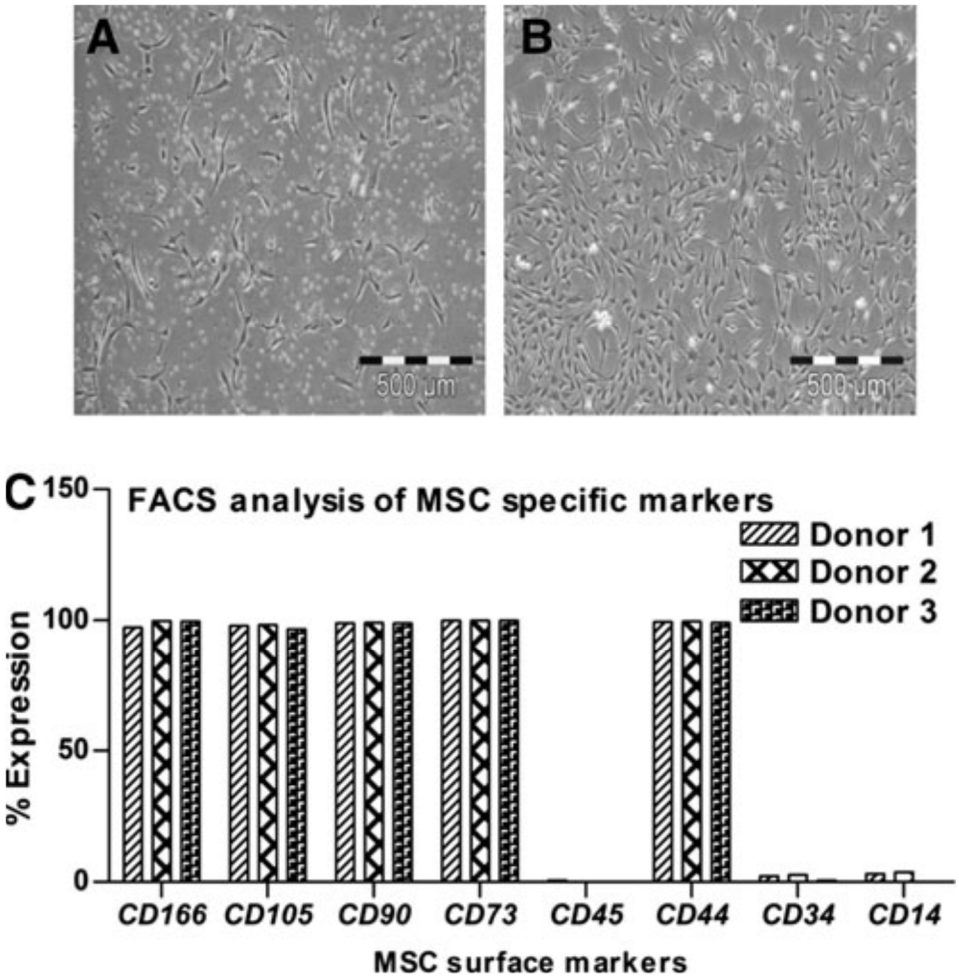

Figure 3. (A) During isolation, human bone marrow mesenchymal stem cells (MSCs) appear as single cells in passage 0 (P0), (B) then they showed uniform growth and fibroblast like morphology in P3. (C) On FACS analysis the cells were positive for typical MSC antigens like CD166, CD105, CD90, CD73, and CD44, while negative for hematopoietic lineage-specific antigens like CD45, CD34, and CD14. Bar: 500 lm. [21]. Permissions obtained from the publisher

chondrogenic differentiation of human MSCs in 3D alginate gels. A time-dependent accumulation of glycosaminoglycans, aggrecan, and type II collagen is observed in chondrogenic but not in basal constructs over 24 days. In qRT-PCR, a largely characteristic pattern of chondrogenic markers is demonstrated, that provide a basis for staging the cellular phenotype into four stages. Stage I (days 0–6) is defined by collagen types I and VI, Sox 4, and BMP-2 showing peak expression levels. In stage II (days 6–12), gene expression of cartilage oligomeric matrix protein, HAPLN1, collagen type XI, and Sox 9 reaches the peak levels, while gene expression of matrilin 3, Ihh, Homeobox 7, chondroadherin, and WNT 11 is peaked at stage III (days 12–18). Finally, cells in stage IV (days 18–24) demonstrate peak levels of aggrecan; collagen IX, II, and X; osteocalcin; fibromodulin; PTHrP; and alkaline phosphatase. Gene profiles at stages III and IV are analogous to those in juvenile articular and adult nucleus pulposus chondrocytes. These data provide comprehensive insights on chondrogenesis of human MSCs in 3D gels (25).

In another study, the differentiation of human MSCs was considered into chondrocytes under defined conditions in 3D alginate gels *in vitro* as one of the high-cell density culture, and also, that temporal change in gene expression in this system largely paralleled the spatial pattern of *in vivo* cartilage maturation in many important aspects (25).

Specifically, the early gene expression profiles are similar to that in precursor cells, with the cells showing progressive increase in markers of chondroprogenitors followed by that of mature chondrocytes and then by hypertrophic chondrocytes defined by robust upregulation of collagen type X, alkaline phosphatase, and osteocalcin (25, 26).

These stage-specific gene profiles show similarities at the earlier time point to those in fibroblast-like annulus fibrosus cells and in the later stages to juvenile chondrocytes and adult nucleus pulposus chondrocytic cells. The findings also provide insights into the stage-specific gene profiles of human MSCs undergoing chondrogenic differentiation *in vitro* that may be a useful to study differences in human MSC responses to various stimuli, such as mechanical load (27).

Biomaterial-based scaffolds extensively have been used in the field of tissue engineering. They may be better than micromass culture systems and pellet culture systems in the control of diffusion of nutrients and oxygen tension, because, scaffold properties such as material biocompatibility, porosity, mechanical strength, and incorporation of signaling molecules can be optimized to address various physiological requirements of an engineered cartilage tissue (28). Additionally, biomaterial-based scaffold cultures can provide a larger size cartilage tissue with stronger mechanical properties comparing to micromass culture systems and pellet culture systems (28).

Pellet culture system

In this method MSCs are centrifuged (150 g, 5 min) in a 15-mL polypropylene tube to form a pellet for chondrogenic differentiation in the high-cell density pellet cultures. The pellets are treated for 28 days with defined serum-free chondrogenic medium, which consist of DMEM (4.5 g/L glucose), ITS + 1 supplement, 100 mM dexamethasone, 0.17 mM L-ascorbic acid-2-phosphate, 1mM sodium pyruvate, 0.35mM L-prolin and 10 ng/mL TGFβ3. Control pellets are cultured in the same medium without TGFβ3. The medium is changed every 3 days and pellets are harvested on days 7, 14 and 21 (21).

MSC are stimulated with TGFβ3 in the standard pellet culture assay for chondrogenic differentiation. After initial centrifugation in 15 mL polypropylene tubes, MSCs settle and form loose and spherical pellets. During the first week, pellets start to increase in size and have a thinner central zone as compared to a thicker circumferential border. After 28 days, pellets are converted into firm, mechanically strong cultures. At that point, alcian blue staining of cartilage proteoglycans and antibody staining of cartilage-specific collagen type II reveal the formation of a cartilaginous extracellular matrix (ECM). Both alcian blue and collagen type II staining are negative in unstimulated pellet cultures. On the gene expression level, chondrogenesis is measured by significantly increased expression of the cartilage marker genes SOX9 and COL2A1 from day 0 to day 28. Both genes are also much more expressed in stimulated cultures than in unstimulated cultures (21).

The 3D high-cell density pellet culture is the standard assay for chondrogenesis *in vitro*. Spherical MSCs pellet is chondrogenically induced by the addition of the standard stimulus, TGFβ, leading to a firm, spherical MSC pellet resembling hyaline cartilage and consisting of differentiated MSC and their ECM. The ECM mostly consists of different proteoglycans and collagens, which are usually cross-linked and provide a protective cage in which the TGFβ-stimulated MSCs are entrapped. So, the isolation of these cells from their ECM is a challenging task, but is the prerequisite to deliver viable, chondrogenically differentiated MSCs (21).

In one study, Mujib Ullah and et al. designed a protocol for isolation of chondrogenic differentiated human MSCs from high-cell density pellet cultures. First, MSC were stimulated with TGFβ3 for 28 days to generate high-density pellet cultures. The chondrogenic nature of these pellets was verified by histochemical examination of cartilage proteoglycan, antibody staining of cartilage collagen type II, and qPCR of COL2A1 and SOX9. Then, to start pellet digestion, trypsin was applied because it is broadly accepted as the enzyme for release of cells from culture surfaces and diverse native tissues. Initially trypsin did not release any cells from intact whole pellets, and very few cells were released from small pieces of knife-scraped pellets. In line with these results, trypsin was previously found to be insufficient to isolate chondrocytes from cartilage. Since cartilage and chondrogenic pellet cultures contain a huge amount of collagen, collagenases are important digestion enzymes. Some studies had used a mixture of collagenase II and collagenase P to isolate chondrocytes from normal and osteoarthritic cartilage. Therefore, comparison of these two enzymes alone or in combination demonstrated a mixture of 300U of collagenase II and 20U of collagenase P optimum at 90 and 120 min of incubation for maximum release of viable cells. But most cells, either died in subsequent culturing or otherwise showed a low proliferation rate. However, the viable, chondrogenic cells showed different morphologies. One reason may be a non-uniform nutrient supply to single cells or cell aggregates inside high-density pellet cultures. It also seems possible that the chondrogenic capacity of the primary MSCs varied. based on cartilage proteoglycan, collagen type II expression and genes play a substantial role in providing purified chondrogenic cells and ECM for future regenerative application (21).

In this context the high-cell density pellet culture represents a model system to provide a large amount of chondrogenic MSCs, especially when such cells should be applied as a suspension for regenerative application (15). However, inside the intact pellet culture, the cells and their secreted ECM components enclose and fix each other, hindering the release of chondrogenic MSCs (29, 30). This emphasizes the need for a successful protocol to isolate cells from pellets, despite an array of published protocols for chondrocyte isolation from native cartilage. Unfortunately, such protocols are not applicable to

isolating viable cells from pellets, and we believe that appropriate pellet digestion represented the key step to achieve this protocol, but to our knowledge, no successful procedure for the isolation of such cells from pellets has been published (31, 32).

Micromass culture system

In typical cell therapy strategies, the implanted cells are terminally differentiated cells that are intrinsic to the healing site. Therefore, one important step in preparing MSCs for clinical use is providing the condition in which the cells can differentiate into mature cartilage before being implanted. This will guarantee the transplantation of only chondrocytes and therefore avoid the undesirable bone formation in the case of undifferentiated cell implantation. The micromass culture system is the routine *in vitro* preparation for MSCs to undergo cartilage differentiation; in this system, the cells are cultivated as a condensed aggregate. Manning et al. first described it for cultivation of human articular chondrocyte (33). Johnstone et al. later reported the differentiation of MSCs into cartilage in this system (34). Numerous investigations have already shown the effectiveness of micromass culture system for cartilage differentiation of MSCs from human and a variety of species (35, 36). In this technique, MSCs are first condensed into a pellet and then exposed to chondrogenic medium for three weeks. Using micromass culture system, MSCs could easily differentiate into cartilage with the characteristic metachromatic matrix accumulated among the cells. Morphologic events have already been investigated during *in vitro* chondrogenesis using the micromass culture system (37), but the exact final structural differentiation of MSCs into cartilage tissue, the subject of the present study, has not yet been reported. Such investigations would help to understand the potentials of micromass culture system in producing fully-matured cartilage tissue that is suitable for transplantation purposes.

A micromass culture system of primary immature chondrocytes for functional analysis of soluble factors involved in the maturation step of cartilage was developed. For micromass cultures, the protocol was described by De Bari et al. Briefly, confluent monolayer cultures of chondrocyte cell lines are released by trypsin-EDTA, are tested for viability by trypan blue exclusion, and re-suspended in growth medium at a density of 2.5×10^7 viable cells/mL. Micromasses are obtained by pipetting 20 µL of cell suspension into individual wells of 24-well plates. Following a 3-h attachment period without medium, the growth medium is gently added and cultures left resting for a further 24 h. The medium is then changed to serum-free and phenol red-free medium (Gibco BRL) for 24 h. Differentiation is promoted by serum starvation and ITS supplementation. On day 3 of the culture, fresh differentiation medium is added. After 48 h, some of the micromasses are harvested for Alcian blue matrix staining and others for qRT-PCR gene expression analysis of gene markers for chondrocytes: type II collagen alpha-1 (COL2A1), aggrecan (ACAN), sex determining region (SRY)-box 9 (SOX9), matrix metalloproteinase-1 and -13 (MMP1, MMP13), and a disintegrin and metalloproteinase with a thrombospondin type 1 motif 5 (ADAMTS5) (19).

Since the introduction of micromass culture system by Johnston et al., scientists have utilized the system for different purposes. Some researchers used it to study the role of macromolecules such as bone morphogenetic protein-6 (BMP6) family in promoting chondrogenic differentiation (36, 38). Others have cultivated MSCs in micromass culture system to investigate molecular events during in vitro chondrogenesis that resulte in defining the expression pattern of cartilage specific molecules such as collagen II, X, and aggrecan (18, 27, 28). Still other scientists have used this culture system to evaluate the chondrogenic potential of the cells isolated by different methods (39, 40).

Ichinso *et al.* (2005) cultured human MSCs in a micromass culture system for 21 days in order to investigate the morphologic changes during in vitro chondrogenesis. They concluded that *in vitro* chondrogenesis is very similar to that of *in vivo* (37).

Differentiation Analysis

Real-time PCR

In reverse transcription and real-time PCR analysis total RNA is isolated from pellets and micromasses using Trizol_ reagent according to the manufacturer's protocol. Total RNA (1 µg) is then converted to cDNA. All real-time qPCR are performed on a real-time PCR System in 20 µl reaction volumes containing 10 µl of SYBR Green I Master Mix, 0.6 µl 10 mM sense or antisense primer and 7.8 µl RNAse free water. The expressions of the following genes are examined: collagen type I (COL1A1), collagen type II (COL2A1), collagen type X (COL10A1), aggrecan and SOX-9. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a housekeeping gene. Primer sequences are listed in Table 1. The PCR reaction is performed for 1 min at 95°C, follow by 39 amplification cycles (15 s at 95°C, 15 s at 60°C, 20 s at 72°C). After the last cycle, a melt-curve is generated. Each PCR is processed in triplicate. The Ct value of GAPDH is subtracted from the Ct value of the interest gene (ΔCT). The average ΔCT value of the triplicates is taken. MSCs culture in the pellet culture system is used as controls (ΔΔCT). Relative expression levels for each primer set are expressed as fold changes by the $2^{-\Delta\Delta CT}$ method (41, 42).

Table 1. Primers used in real time RT-PCR for chondrogenic differentiation

Gene (accession no.)	Primer sequence	Product size (in base pairs)
GAPDH (NM_002046)	5-AGAAAAACCTGCCAAATATGATGAC-3 5-TGGGTGTCGCTGTTGAAGTC-3	126
Collagen X (NM_000493)	5-CAAGGCACCATCTCCAGGAA-3 5-AAAGGGTATTTGTGGCAGCATATT-3	83
Collagen I (NM_000088)	5-CAGCCGCTTCACCTACAGC-3 5-TTTTGTATTCAATCACTGTCTTGCC-3	79
Aggrecan (NM_013227.2)	5-TGCATTCCACGAAGCTAACCTT-3 5-GACGCCTCGCCTTCTTGAA-3	70
Collagen II (NM_033150)	5-GGCAATAGCAGGTTCACGTACA-3 5-CGATAACAGTCTTGCCCCACTT-3	84
SOX-9 (NM_000346)	5-AGCGAACGCACATCAAGAC-3 5-GCTGTAGTGTGGGAGGTTGAA-3	110

Histology and immunohistochemistry

At specified times, cell aggregates (pellets and micromasses) are fixed in 4% paraformaldehyde for 3 h, and then dehydrated with ethanol, wash with xylene and embeded in paraffin. Sections at 5 μM are cut and mounted on glass slides. Toluidine blue staining for proteoglycan and immunohistochemistry for collagen types I, II and X are performed. For toluidine blue staining, sections are deparaffinized with xylene and ethanol, and 1% toluidine blue sodium borate is applied for 30 min and wash with distilled water. For immunohistochemistry, the streptavidin–peroxidase-conjugated method is used. After deparaffinization, tissue sections are briefly treated with pepsin at 37°C for 10 min. The sections are then incubated for 10 min with a peroxidase-blocking solution and reacted with the appropriate primary antibodies (mouse anti-human collagen type II monoclonal antibody, mouse anti-human collagen type I monoclonal antibody, mouse monoclonal anti-human collagen type X antibody) diluted in PBS containing 0.2% Triton X-100 and the appropriate 2% normal serum overnight at 4°C. After rinsing with PBS, the slides are incubated for 10 min at room temperature with biotin-conjugated secondary antibodies, follow by incubation with streptavidin conjugated peroxidase working solution for 10 min. Subsequently, sections are stained for 10 min with 3, 30-diaminobenzidine tetrahydrochloride (DAB), counter stained with Mayer's hematoxylin, dehydrated and mounted. Negative controls are prepared by substituting PBS for the primary antibody (41, 42).

Transmission electron microscopy (TEM)

Day 14 cell aggregates are fixed with 2.5% glutaraldehyde in 0.1 M PBS for 2 h. The aggregates are then washed overnight at 4°C in the same buffer and post-fixed with 1% OsO4 buffer with 0.1 MPBS for 2 h. Aggregates are then dehydrated in a graded series of ethanol and embeded in epoxy resin. Ultrathin sections are double-stained with uranyl acetate and lead citrate, followed by examination with a transmission electron microscope (41, 42).

Discussion

The 3D high-cell density culture is considered as a standard culture model for its entrapped chondrogenically differentiated cells. High-cell density culture environment is pivotal for the chondrogenic differentiation of hMSCs. High-cell density pellet culture systems formed by centrifugation are widely used to evaluate the chondrogenic capacity of MSCs and to study the signal pathways involved in chondrogenesis. The presented literature demonstrated that the micromass culture system, another high-cell density culture system formed by high-cell density suspension, is superior to the other high-cell density culture. In the micromass cultures, the induced cartilage tissues are more homogenous and rich in cartilage-specific collagen II, but comparing to the pellet cultures and alginate cultures there are markedly decreased fibrocartilage-like features, collagen I, hypertrophic chondrocyte features, collagen X, indicating that the micromass culture system is a promising tool for in vitro chondrogenic studies.

Bone marrow-derived MSCs are heterogeneous with different mesenchymal lineage potentials (20), but the induced-cartilage is more homogeneous in micromass cultures than that in other high-cell density cultures. We speculate that this phenomenon is due, in part, to the culture conditions. Although, high-cell density and low oxygen tension seem to enhance MSC chondrogenesis (43, 44), the optimal cell density and oxygen tension remain unclear (45, 46). Micromass culture, is formed by high-cell density suspensions, may do better in nutrient supplementation. Pellet culture is prepared by centrifugation, and may create a low oxygen tension environment with extremely high-cell density, resulting in poor nutrient diffusion and eventually MSC apoptosis in the central region. Thus, there

should be a balance between high cell density and the diffusion of nutrients in the process of MSC chondrogenesis. Micromass cultures may provide an optimal cell density environment allowing cell-cell contact and diffusion of nutrients.

On the other hand, it is more convenient to use micromass cultures than biomaterial-based scaffold cultures (alginate culture method) to investigate the molecular mechanisms regulating chondrogenesis, such as the relationship between various signaling molecules and MSC chondrogenesis, thus validating the importance of this model.

The distinct difference between the micromass and other high density cell cultures is the efficiency of MSC chondrogenic differentiation. The same initial number of MSCs is induced into a much larger cartilaginous tissue in the micromass cultures. Histochemical results demonstrate a greater deposition of cartilage specific extracellular matrix, proteoglycans and collagen II in the micromass cultures. Furthermore, COL2A1 and aggrecan mRNA levels exhibit a 1.97 and 2.12-fold increase, respectively, in micromass cultures comparing to that in other culture systems. However, stronger matrix production in micromass cultures is not accompanied by the up-regulation of SOX-9, which is an important transcription factor involved in chondrogenesis (47). It is likely that the expression of SOX-9 at low levels is already sufficient to support matrix production, suggesting that other factors may contribute to the higher efficiency of chondrogenesis in micromass cultures. However, a 3D culture environment alone cannot maintain prolong chondrogenesis. Previous studies have reported the expression of hypertrophic-associated genes, such as collagen X, and fibrocartilage-like features as well as collagen I in pellet cultures (19, 48). In another study, expressions of collagen X and collagen I were significantly down-regulated in micromass cultures compared to that in pellet cultures, suggesting that the induced-cartilage in micromass cultures is more similar to hyaline cartilage. So the micromass culture system is more efficient than the standard pellet culture system and alginate culture in MSC chondrogenesis. Furthermore, the expressions of collagen X and collagen Iare down-regulate in MSCs differentiated in the micromass cultures indicating that the induced-cartilage is more similar to hyaline cartilage.

Conclusion

In this study, we considered a comparative review of chondrogenic differentiation of hMSCs cultured in the micromass culture, pellet culture and alginate culture. The literature suggested that the micromass culture system is superior to the pellet culture system and alginate culture. It indicates that the micromass culture system is a superior approach to study the chondrogenic potential of human MSCs.

Conflict of Interest: 'None declared'.

References

1. Ham O, Lee CY, Kim R, Lee J, Oh S, Lee MY, et al. Therapeutic Potential of Differentiated Mesenchymal Stem Cells for Treatment of Osteoarthritis. Int J Mol Sci. 2015;16(7):14961-78.
2. Bulte JW, Kraitchman DL, Mackay AM, Pittenger MF. Chondrogenic differentiation of mesenchymal stem cells is inhibited after magnetic labeling with ferumoxides. Blood. 2004;104(10):3410-2; author reply 2-3.
3. Lai RC, Yeo RW, Lim SK. Mesenchymal stem cell exosomes. Semin Cell Dev Biol. 2015;40:82-8.
4. Ghorbani FM, Kaffashi B, Shokrollahi P, Seyedjafari E, Ardeshirylajimi A. PCL/chitosan/Zn-doped nHA electrospun nanocomposite scaffold promotes adipose derived stem cells adhesion and proliferation. Carbohydr Polym. 2015;118:133-42.
5. Pedram MS, Dehghan MM, Soleimani M, Sharifi D, Marjanmehr SH, Nasiri Z. Transplantation of a combination of autologous neural differentiated and undifferentiated mesenchymal stem cells into injured spinal cord of rats. Spinal Cord. 2010;48(6):457-63.
6. Lam J, Lu S, Lee EJ, Trachtenberg JE, Meretoja VV, Dahlin RL, et al. Osteochondral defect repair using bilayered hydrogels encapsulating both chondrogenically and osteogenically pre-differentiated mesenchymal stem cells in a rabbit model. Osteoarthritis Cartilage. 2014;22(9):1291-300.
7. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med. 2004;8(3):301-16.
8. Qi Y, Feng G, Yan W. Mesenchymal stem cell-based treatment for cartilage defects in osteoarthritis. Mol Biol Rep. 2012;39(5):5683-9.
9. Kristjansson B, Honsawek S. Current perspectives in mesenchymal stem cell therapies for osteoarthritis. Stem Cells Int. 2014;2014:194318.
10. Song H, Chang W, Song BW, Hwang KC. Specific differentiation of mesenchymal stem cells by small molecules. Am J Stem Cells. 2012;1(1):22-30.
11. Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. Exp Hematol. 2000;28(6):707-15.
12. Guo JF, Jourdian GW, MacCallum DK. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tissue Res. 1989;19(2-4):277-97.
13. Liu H, Lee YW, Dean MF. Re-expression of differentiated proteoglycan phenotype by dedifferentiated human chondrocytes during culture in alginate beads. Biochim Biophys Acta. 1998;1425(3):505-15.
14. Watts AE, Ackerman-Yost JC, Nixon AJ. A comparison of three-dimensional culture systems to evaluate in vitro chondrogenesis of equine bone marrow-derived mesenchymal stem cells. Tissue Eng Part A. 2013;19(19-20):2275-83.
15. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res. 1998;238(1):265-72.
16. Pelttari K, Steck E, Richter W. The use of mesenchymal stem cells for chondrogenesis. Injury. 2008;39 Suppl 1:S58-65.
17. Mello MA, Tuan RS. High density micromass cultures of embryonic limb bud mesenchymal cells: an in vitro model of endochondral

skeletal development. In Vitro Cell Dev Biol Anim. 1999;35(5):262-9.
18. Scharstuhl A, Schewe B, Benz K, Gaissmaier C, Buhring HJ, Stoop R. Chondrogenic potential of human adult mesenchymal stem cells is independent of age or osteoarthritis etiology. Stem Cells. 2007;25(12):3244-51.
19. Greco KV, Iqbal AJ, Rattazzi L, Nalesso G, Moradi-Bidhendi N, Moore AR, et al. High density micromass cultures of a human chondrocyte cell line: a reliable assay system to reveal the modulatory functions of pharmacological agents. Biochem Pharmacol. 2011;82(12):1919-29.
20. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143-7.
21. Ullah M, Hamouda H, Stich S, Sittinger M, Ringe J. A reliable protocol for the isolation of viable, chondrogenically differentiated human mesenchymal stem cells from high-density pellet cultures. Biores Open Access. 2012;1(6):297-305.
22. De Ceuninck F, Lesur C, Pastoureau P, Caliez A, Sabatini M. Culture of chondrocytes in alginate beads. Methods Mol Med. 2004;100:15-22.
23. Loeser RF, Todd MD, Seely BL. Prolonged treatment of human osteoarthritic chondrocytes with insulin-like growth factor-I stimulates proteoglycan synthesis but not proteoglycan matrix accumulation in alginate cultures. J Rheumatol. 2003;30(7):1565-70.
24. Hauselmann HJ, Masuda K, Hunziker EB, Neidhart M, Mok SS, Michel BA, et al. Adult human chondrocytes cultured in alginate form a matrix similar to native human articular cartilage. Am J Physiol. 1996;271(3 Pt 1):C742-52.
25. Xu J, Wang W, Ludeman M, Cheng K, Hayami T, Lotz JC, et al. Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. Tissue Eng Part A. 2008;14(5):667-80.
26. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A. 2002;99(7):4397-402.
27. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res. 2001;268(2):189-200.
28. Sundelacruz S, Kaplan DL. Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine. Semin Cell Dev Biol. 2009;20(6):646-55.
29. Pearle AD, Warren RF, Rodeo SA. Basic science of articular cartilage and osteoarthritis. Clin Sports Med. 2005;24(1):1-12.
30. Bhosale AM, Richardson JB. Articular cartilage: structure, injuries and review of management. Br Med Bull. 2008;87:77-95.
31. Hayman DM, Blumberg TJ, Scott CC, Athanasiou KA. The effects of isolation on chondrocyte gene expression. Tissue Eng. 2006;12(9):2573-81.
32. Wolf F, Candrian C, Wendt D, Farhadi J, Heberer M, Martin I, et al. Cartilage tissue engineering using pre-aggregated human articular chondrocytes. Eur Cell Mater. 2008;16:92-9.
33. Manning WK, Bonner WM, Jr. Isolation and culture of chondrocytes from human adult articular cartilage. Arthritis Rheum. 1967;10(3):235-9.
34. Johnstone B. Mesenchymal stem cells and chondrogenesis. Europ Cell Mat. 2002;4:27.
35. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng. 1998;4(4):415-28.
36. Sekiya I, Colter DC, Prockop DJ. BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. Biochem Biophys Res Commun. 2001;284(2):411-8.
37. Ichinose S, Tagami M, Muneta T, Sekiya I. Morphological examination during in vitro cartilage formation by human mesenchymal stem cells. Cell Tissue Res. 2005;322(2):217-26.
38. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ. Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res. 2005;320(2):269-76.
39. Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood. 2004;103(5):1662-8.
40. Eslaminejad MB, Nikmahzar A, Taghiyar L, Nadri S, Massumi M. Murine mesenchymal stem cells isolated by low density primary culture system. Dev Growth Differ. 2006;48(6):361-70.
41. Lee Y, Stott N, Jiang T, Widelitz R, Chuong C. Early events during precartilage condensation in limb bud micromass cultures. Cells and Materials. 1998;8:19-32.
42. Zhang L, Su P, Xu C, Yang J, Yu W, Huang D. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. Biotechnol Lett. 2010;32(9):1339-46.
43. Kanichai M, Ferguson D, Prendergast PJ, Campbell VA. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. J Cell Physiol. 2008;216(3):708-15.
44. Takagi M, Umetsu Y, Fujiwara M, Wakitani S. High inoculation cell density could accelerate the differentiation of human bone marrow mesenchymal stem cells to chondrocyte cells. J Biosci Bioeng. 2007;103(1):98-100.
45. Fehrer C, Brunauer R, Laschober G, Unterluggauer H, Reitinger S, Kloss F, et al. Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. Aging Cell. 2007;6(6):745-57.
46. Malladi P, Xu Y, Chiou M, Giaccia AJ, Longaker MT. Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. Am J Physiol Cell Physiol. 2006;290(4):C1139-46.
47. Murdoch AD, Grady LM, Ablett MP, Katopodi T, Meadows RS, Hardingham TE. Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: generation of scaffold-free cartilage. Stem Cells. 2007;25(11):2786-96.
48. Mueller MB, Tuan RS. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. Arthritis Rheum. 2008;58(5):1377-88.

Please cite this paper as: Mahboudi H, Kazemi B, Soleimani M, Hanaee-Ahvaz H, Ardeshirylajimi A, Eftekhary M, Enderami SE. Comparison between High Cell-Density Culture Systems for Chondrogenic Differentiation and Articular Cartilage Reconstruction of Human Mesenchymal Stem Cells: A Literature Review. Regen Reconstr Restor. 2017;2(1):7-15.