Stem Cells International

Stem Cells for Cartilage Regeneration: A Roadmap to the Clinic

Lead Guest Editor: Celeste Scotti
Guest Editors: Norimasa Nakamura, Alberto Gobbi, and Giuseppe M. Peretti

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Editorial Board

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Editorial

Stem Cells for Cartilage Regeneration: A Roadmap to the Clinic

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Cell therapies for cartilage repair date back to 1987 when the first autologous chondrocyte implantation (ACI) procedure was performed. Since then, more than 30,000 patients have been treated with these techniques, which should not be considered "experimental" anymore. Over the years, several technical improvements have been implemented, leading to incremental advances in the surgical procedure, as reviewed by Y. Nam et al. However, a predictable, standardized, and durable regeneration of hyaline cartilage tissue, capable to withstand the mechanical forces acting in the joint and to prevent joint degeneration, remains an unmet medical need [1].

In this special issue entitled "Stem Cells for Cartilage Regeneration: A Roadmap to the Clinic," the authors addressed several relevant topics, ranging from advanced in vitro and in vivo models to alternative cell sources, including induced pluripotent stem cells (iPS), and from smart materials to additional target tissues with high unmet medical need, such as the trachea or the temporomandibular joint (TMJ).

A key bottleneck to improved therapies is represented by reliable in vitro and in vivo models, capable to predict the clinical outcome. Strong advances have been made in this field, towards the development of high-throughput systems that allows testing multiple conditions with reproducible, quick, and affordable methods, and S. Lopa et al. provided a comprehensive review of microfluidics and bioprinting applications. Another important topic is quality control in cell therapies, in order to better standardize the clinical outcome. K. Shiraishi et al. reported an interesting study analysis of mRNA and miRNA correlated with in vivo cartilage repair, which may open new avenues for patient stratification and selection, beyond the mere quality control. Regarding in vivo models, a translational model capable to duplicate the challenging clinical scenarios has yet to be developed. M. Lo Monaco et al. reviewed extensively this topic, ranging from small to large animal models and providing critical insights for study planning.

The use of articular chondrocytes as a cell source has been considered a bottleneck to a more robust and reproducible regeneration of the articular surface, because of their typical age-dependency and interdonor variability in the cartilage-forming capacity [2]. For this reason, recent research focused on alternative cell sources and experimental models in order to overcome the intrinsic limitations of autologous cell therapies based on articular chondrocytes. J. N. Fisher et al. reviewed recent advances in preclinical and clinical research on a number of tissue sources of progenitor cells for cartilage repair, highlighting pros and cons of each of them, with a focus on the potential for clinical translation. K. D. Jorgenson et al. presented a suspension bioreactor incorporating microcarrier technology for the efficient culture of synovial fluid-derived MSCs, which can potentially support further research with this cell source. Infrapatellar fat pad-derived cells gained attention because of their easy accessibility and chondrogenic potential.

J. F. C. do Amaral et al. reviewed the potential of infrapatellar fat pad cells, discussing their potential for cartilage repair and the ontogeny relationship with other joint-derived cells and concluding with some perspective for translational trials using this cell source. Another cell type that showed promising preclinical data, with also a clinical trial ongoing, is synovial MSC [3]. Y. Ikeda et al. reported a successful approach to improve further the chondrogenic activity of synovial MSC, without the upregulation of hypertrophic and osteogenic genes, by enhanced IGF-1 expression. Last, human-induced pluripotent stem cells (hiPSCs) gained a lot of attention in the last decade, representing a new hope for several life-threatening and incurable diseases. Y. A. Rim et al. reported a relevant analysis of the chondrogenic potential among hiPSCs from different tissues: the finding that cord blood mononuclear cells represent a better source may support further research in this direction.

Biomaterials are a mainstay of regenerative medicine, especially for articular cartilage. However, it is still a matter of controversy whether a scaffold is strictly needed or not. In this special issue, both approaches are reported. Interestingly, F. Hached et al. reported the positive impact of a polysaccharide hydrogel on encapsulated MSCs, with respect to cell viability and ability to secrete potentially therapeutic factors. Regarding scaffold-free approaches, M. P. Stuart et al. reported a valuable method to engineer spheroids by using a micromolded nonadhesive hydrogel, without the use of growth factors.

In this special issue, the authors addressed a series of topics of relevance for the successful translation of preclinical approaches. Cell sources, biomaterials, animal models, and cell manufacturing are all critical factors for cartilage repair, which require additional work to pave the way to the next generation of regenerative therapies, possibly capable to restore durably both joint surface and function in patients in need.

Celeste Scotti
Alberto Gobbi
Norimasa Nakamura
Giuseppe M. Peretti

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Research Article

Production of Adult Human Synovial Fluid-Derived Mesenchymal Stem Cells in Stirred-Suspension Culture

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The chondrogenic potential of synovial fluid-derived mesenchymal stem cells (SF-MSCs) supports their use in cartilage regeneration strategies. However, their paucity in synovial fluid necessitates their proliferation in culture to generate clinically relevant quantities. Here it was determined that 125 mL stirred suspension bioreactors utilizing Cytodex-3 microcarrier beads represent a viable platform for the proliferation of these cells. During the inoculation phase, a bead loading of 2 g/L, an inoculation ratio of 4.5 cells/bead, and continuous agitation at 40 rpm in a medium with 5% serum resulted in high cell attachment efficiencies and a subsequent overall cell fold expansion of 5.7 over 8 days. During the subsequent growth phase, periodic addition of new microcarriers and fresh medium increased culture longevity, resulting in a 21.3 cell fold increase over 18 days in the same vessel without compromising the defining characteristics of the cells. Compared to static tissue culture flasks, a bioreactor-based bioprocess requires fewer handling steps, is more readily scalable, and for the same cell production level, has a lower operating cost as it uses approximately half the medium. Therefore, stirred suspension bioreactors incorporating microcarrier technology represent a viable and more efficient platform than tissue culture flasks for the generation of SF-MSCs in culture.

1. Introduction

Articular cartilage is a connective tissue that covers the ends of bones, providing load absorption and dissipation, and a near friction-free surface that enables bones to articulate within a joint. The avascular nature of cartilage and the low density of dispersed chondrocytes (cartilage-producing cells) greatly hinder the endogenous regenerative capacity of this tissue [1]. As such, even slight damage to cartilage can initiate the development of osteoarthritis (OA) in which cartilage degeneration is significant and results in joint swelling, chronic pain, and reduced mobility [2].

OA has traditionally been treated by administering pharmaceuticals to alleviate symptoms such as pain [3]. However, pharmaceuticals can lose their efficacy over time, result in significant undesirable side effects, and have not yet been shown to be able to maintain or regenerate cartilage [4–7]. Thus, many patients eventually have no choice but to undergo surgery [8]. In extreme cases, total joint replacement (TJR), in which the damaged joint is replaced by a prosthetic joint, is necessary. Although TJR can improve patient quality of life, patients do not completely regain normal function, and issues related to infection and joint loosening over time suggest that alternative treatments are required [7].

Newer treatment options that have been tested include transplanting plugs of cartilage isolated from non-weight-bearing areas to the defect site (mosaicplasty) [1, 5]. However, this approach can result in donor site morbidity, and methods to fix the new cartilage to the defect site, such as

sutures and pins, may actually initiate further damage [9]. A second approach has been to expand, in culture, populations of chondrocytes isolated from a cartilage biopsy for subsequent implantation into a defect site, sometimes in conjunction with biomaterials (autologous chondrocyte transplantation) [5, 6]. This approach can also result in donor site morbidity, and the use of biomaterials is not desirable [10]. Moreover, chondrocytes have limited expansion capacity in culture and tend to dedifferentiate and lose their ability to make cartilage [11]. A third method has been to drill through the subchondral bone, resulting in the release of marrow elements and the subsequent formation of a blood clot in the defect site, which, through natural healing mechanisms, is typically replaced over time by a fibrous type of cartilage [1, 6]. This fibrocartilage does not have the mechanical properties or durability of native articular cartilage [6, 12, 13].

Mesenchymal stem cells (MSCs) have recently generated considerable interest for their potential to repair cartilage. These cells can be isolated from several different sources, including bone marrow, adipose tissue, and synovial fluid. Adult human MSC populations are defined by their surface marker profile ($CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, and $CD105^+$), their capacity to attach to cell culture-grade plastic, their ability to generate colonies, and their trilineage potential to become fat, bone, or cartilage cells [14]. Despite having these characteristics in common, MSCs are influenced by the tissue microenvironment in which they reside, and thus, MSC populations from different tissues exhibit specific traits which serve to distinguish them from one another [15, 16].

MSCs isolated from within articulating joints have shown a superior capacity to contribute to cartilage repair. For example, significant efforts have been made to examine the possibility of using synovial membrane-derived mesenchymal stem cells for cartilage tissue engineering [9, 10, 17–23]. Synovial fluid-derived MSCs (SF-MSCs) are believed to originate from the synovial membrane but exist in the lubricating fluid contained within the joint cavity [24–26]. However, presumably due to local environmental influences, SF-MSCs have shown a greater capacity to generate cartilage than other evaluated MSC types, including those from synovial membrane, bone marrow, and adipose tissue [16, 27, 28]. Interestingly, during development, articular cartilage and synovial joint components are reported to be derived from progenitor interzone cells [29], and thus, adult MSCs in synovial membrane and synovial fluid may retain some of this cellular bias. This cell type has also been reported to possess robust growth potential [30]. SF-MSCs are easily harvested in a minimally invasive manner through arthrocentesis, thereby avoiding donor site morbidity [30]. Given that SF-MSCs can be derived from a very accessible source, they clearly represent a potentially valuable cell type for certain tissue engineering applications, including the repair of articular cartilage [30, 31].

Despite their accessibility, the low concentration of SF-MSCs in synovial fluid means that they cannot be isolated in sufficient numbers for the direct development of clinical repair and regeneration strategies. Moreover, if approved for therapeutic use, widespread clinical implementation of SF-MSC-based therapies will require large quantities of quality-assured cells. For these reasons, it is necessary to develop rapid and reproducible methods for the scaled-up expansion of these cells. The vast majority of stem cell research is carried out on cell populations which have been expanded in static tissue culture flasks. However, the use of tissue culture flasks for scale-up is not desirable. Due to the small culture volumes that can be accommodated in a single flask, many flasks are needed to generate clinically relevant numbers of cells making this approach for cell expansion inefficient and manually intensive. In contrast, bioreactors are scalable vessels that have been shown to be capable of supporting the expansion of a number of stem cell types [32–35] and thus represent a viable alternative to static culture flasks. A single suspension culture bioreactor can be designed to hold the same culture volume as hundreds of flasks, easily operated by a single trained individual, and computer controlled to continuously maintain an optimum and homogenous culture environment for the growth of cells. An important consideration when scaling up adherent cells in bioreactors is that they require a surface onto which they can attach to survive, grow, and proliferate, without concomitant differentiation. Microcarriers are small beads which can be introduced into a stirred bioreactor and maintained in suspension through agitation, thereby providing a surface for cell attachment and enabling the expansion of adherent cells in this dynamic environment [36, 37]. The use of microcarriers is not without its challenges, however, and studies are required to develop protocols which are customized to support both the attachment of a particular cell type to a specific microcarrier type and the proliferation of that particular cell type on the microcarrier.

In this report, studies were performed to determine the feasibility of culturing adult human SF-MSC populations in suspension bioreactors. It is shown here that microcarrier technology can be used to support the expansion of these cells without compromising their defining properties.

2. Materials and Methods

2.1. Static Culture. MSCs derived from the synovial fluid of two cadaveric male donors (donor 1 was 71 years old and donor 2 was 34 years old) showing no signs of OA were acquired within four hours of death via the Southern Alberta Tissue Transplant Program with approved ethics and consent protocols. The MSCs were isolated by standard methods [38]. Cryopreserved SF-MSCs at passage 2 were thawed and inoculated into 75 cm^2 Nunc tissue culture flasks (T-75) at a density of 5000 cells/cm^2 with Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Cat number 12-707F). DMEM was supplemented with Mesenchymal Stem Cell Growth Medium (MSCGM) SingleQuot Kit (Lonza Cat number PT-4105) which contained fetal bovine serum (FBS). This complete medium was referred to as 10% FBS DMEM. The complete medium was stored at 4°C for a maximum of 2 weeks. Cultures were incubated at 37°C and 5% CO_2, and a 50% medium change was performed after 3 days. Cultures were passaged every 5 days by harvesting the cells with 0.05% trypsin EDTA (Invitrogen Cat number

25300-120) and reinoculating them into new T-75 flasks at 5000 cells/cm^2. Cell density and viability were assessed with a haemocytometer using the trypan blue exclusion method.

2.2. Suspension Culture

2.2.1. Spinner Flask Preparation. Suspension culture expansion was carried out in 125 mL (maximum working volume) spinner flasks (NDS Technologies, NJ, USA) equipped with a suspended magnetic impeller. The inner surface of each spinner flask and its impeller were siliconized with Sigmacote (Sigma Cat number SL-2) to minimize cell and microcarrier attachment. All spinner flasks were fully assembled and autoclaved prior to use. During operation, each spinner flask was placed in a humidified incubator (37°C, 5% CO_2) on top of a Thermolyne magnetic stir plate which was used to control the stirring rate of the impeller.

2.2.2. Microcarrier Preparation. Cytodex 3 microcarriers (Sigma Cat number C3275, Lot number 030M1182V), which are dextran beads coated with denatured porcine-skin collagen on their surface, were chosen based on preliminary small-scale experiments carried out in our laboratory showing that human synovial fluid-derived MSCs can attach to these beads (data not shown). The microcarriers were prepared for use according to the manufacturer's specifications. Briefly, a known quantity of dry beads was hydrated in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) overnight in an Erlenmeyer flask and then rinsed with fresh Ca^{2+}- and Mg^{2+}-free PBS prior to being sterilized in an autoclave. Microcarriers were prepared for immediate use only.

2.2.3. Spinner Flask Inoculation. Sterilized microcarriers were rinsed with DMEM and introduced into spinner flasks with 60 mL of filtered cell culture medium. The spinner flasks were then incubated at 37°C and 5% CO_2 for approximately 18 hours before being inoculated with cells. The cells used for inoculation were harvested from static tissue culture flasks and used to generate a cell suspension that was added to the spinner flasks for a total working volume of 80 mL. After 24 hours, all spinner flasks were topped up with an additional 40 mL of cell culture medium to a final working volume of 120 mL. Great care was taken to ensure consistency in inoculation between replicate flasks.

2.2.4. Spinner Flask Sampling. At designated time points during each experiment, four representative 1000 μL samples were taken from each spinner flask with a calibrated 1000 μL pipette. Prior to each sample being taken, the flask contents were well mixed, and the sample was obtained from the centre of the culture volume. This aided in maintaining consistency between samples and in ensuring that the obtained samples were representative of the flask contents. Each sample was placed in a single, sterile 15 mL conical tube and left undisturbed in order to allow the microcarriers to settle. Once settled, the supernatant was discarded, and the microcarriers were rinsed twice with 1.0 mL of PBS (each rinse involved the addition of PBS to the microcarriers, followed by removal once the microcarriers had settled). Next, 1.0 mL of 0.1% (*w*/*v*) crystal violet in 0.1 M citric acid was added to each conical tube, and the microcarriers were incubated for 1 hour at 37°C. After 1 hour, the microcarrier suspension was agitated 10 to 15 times with a 1000 μL pipette. A 20 μL aliquot of the stained cell suspension was removed, and the released nuclei were counted with a haemocytometer as a measure of the culture cell density.

To visualize the cells on the microcarriers, a 500 μL sample from a spinner flask was placed in a well of a 6-well plate with 3.0 mL of PBS and 15 μL of 0.5% (*w*/*v*) crystal violet in methanol, and the sample was examined under a Zeiss Axiovert 200 microscope.

2.2.5. Harvesting Cells from Microcarriers. To harvest cells from Cytodex 3 microcarriers, a 10 mL sample from a spinner flask was placed in a 15 mL conical tube, and the microcarriers were rinsed twice with 4.0 mL of PBS. A volume of 1.0 mL of 0.05% trypsin-EDTA was then added to the conical tube, and the contents were gently agitated 5 times with a 1000 μL pipette. After allowing the microcarriers to settle (2 minutes), the supernatant containing the detached cells was collected with a pipette and passed through a BD Falcon 100 μm cell strainer (VWR Cat number CA21008-950) into a 50 mL conical tube. The addition of 1.0 mL of trypsin-EDTA to the microcarriers followed by gentle agitation and filtration of the supernatant into the same conical tube was repeated twice more. The filter was rinsed three times with 1.0 mL of culture medium, and the accumulated filtrate was centrifuged at 600 ×g for 5 minutes to pellet and isolate the cells.

2.3. Cell-Surface Marker Analysis.

The prevalence of MSC-specific surface antigens was determined by flow cytometry. Briefly, cells were rinsed twice with PBS and incubated in blocking solution (3% FBS in PBS) on ice in the dark for 30 minutes. Cells were centrifuged and resuspended in blocking solution at a density of 5×10^5 cells/100 μL and distributed into 100 μL aliquots in 15 mL conical tubes. Each 100 μL aliquot was stained with 5.0 μL of antibodies against human CD34, CD45, CD73 (BD Biosciences Cat number 550822, 555483, and 550257, resp.), CD90, and CD105 (Serotec Cat number MCA90F and MCA1157F, resp.). Following 30 minutes of incubation on ice in the dark, cells were rinsed three times with PBS, resuspended in blocking solution, and transferred to BD Falcon round-bottom tubes (VWR Cat number CA60819-138). Relative fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with CellQuest software.

2.4. Differentiation.

Cells were induced towards osteogenic, adipogenic, and chondrogenic fates with commercially available differentiation induction and maintenance media kits from Lonza. Differentiation protocols were carried out according to the manufacturer's instructions and are briefly described here.

2.4.1. Osteogenic Differentiation. Osteogenesis was induced in 6-well plates using an osteogenic differentiation kit (Lonza Cat number PT-3002) which included osteogenic induction medium (OIM). Cells were plated at a density of 3.0×10^4

cells/well (3.1×10^3 cells/cm^2) with 3.0 mL of 10% FBS DMEM. After 24 hours, the 10% FBS DMEM was discarded and replaced with OIM for the duration of the differentiation period. Cells were maintained in culture for 28 days and complete medium changes were performed every 3 days. Alizarin Red was used to stain for calcium deposition in osteogenic cultures as previously described [37, 39].

2.4.2. Adipogenic Differentiation. Adipogenesis was induced in 6-well plates using an adipogenic differentiation kit (Lonza Cat number PT-3004) which included adipogenic induction medium (AIM) and adipogenic maintenance medium (AMM). Cells were plated at a density of 2.0×10^5 cells/well (2.1×10^4 cells/cm^2) with 3.0 mL of 10% FBS DMEM. Complete medium changes were performed every 2-3 days with 10% FBS DMEM until the cells were 100% confluent, generally after 5 days. Once confluent, the 10% FBS DMEM was replaced with 3.0 mL of AIM for 3 days before being replaced with 2.0 mL of AMM for 2 days. This 5-day cycle with AIM and AMM was repeated two more times. At the end of the third cycle, cells were maintained in AMM for the remainder of the 28-day differentiation period with complete medium changes every 3 days.

Oil Red O (ORO) solution was used to stain for lipid droplet formation in adipogenic cultures. The stock solution of ORO was prepared by adding 0.175 g of ORO (Sigma Cat number O0625) to 50 mL of 100% isopropanol. The working solution of ORO was then prepared by adding 60% (*v*/*v*) ORO stock solution to 40% (*v*/*v*) double-distilled water. The staining procedure was carried out as previously described [37, 39].

2.4.3. Chondrogenic Differentiation. Chondrogenesis was induced using the pellet culture method with a chondrogenic differentiation kit (Lonza Cat number PT-3003) and transforming growth factor β3 (TGF-β3) (Lonza Cat number PT-4124). The chondrogenic induction medium (CIM) from the kit was referred to as incomplete CIM (iCIM) until the TGF-β3 was added at which point it was complete CIM (cCIM). Each pellet was generated using 2.5×10^5 cells in a 15 mL conical tube. Cells were centrifuged and rinsed with iCIM ($300 \times$ g for 5 minutes). The iCIM was discarded and the pellet was resuspended in cCIM. The pellets were maintained in culture using cCIM for 28 days with complete medium changes every 3 days.

To quantify the extent of chondrogenesis, the glycosaminoglycan (GAG) content was measured. The medium was discarded and the pellets were transferred to 0.7 mL Eppendorf tubes and digested in a 65°C water bath for 4 hours with 50 μL papain solution (12.5 mg of papain (Sigma Cat number P4762) and 16.32 mg of N-acetyl-2003L-cysteine (Sigma Cat number A9165) in 50 mL of 50 mM phosphate buffer). The pellets were vortexed and then centrifuged at 1000 rpm for 1 minute every 30 minutes. To ensure complete digestion, vigorous agitation with a 1000 μL pipette to completely break up the pellet was necessary prior to a final centrifugation at 3000 rpm for 5 minutes. The supernatant was isolated and evaluated for GAG content.

A standard curve was generated by serial dilution of 10 mg of chondroitin sulphate (Sigma Cat number C9819) dissolved in 1.0 mL of 50 mM phosphate buffer. The quantity of GAGs present in a given sample or standard solution was based on a color reaction with a dimethylmethylene blue (DMB) solution (8 mg of 1,9-dimethylmethylene blue (Sigma Cat number 341088) in 2.5 mL of ethanol mixed with 500 mL double-distilled water containing 1.0 g sodium formate and 1.0 mL formic acid). 10 μL of either standard solution or sample was placed in the wells of a 96-well plate with 200 μL of DMB solution and the plate was incubated at 37°C and 5% CO_2 for 30 minutes before being analyzed. All standards and samples were measured in triplicate with a plate reader at 510 nm.

2.5. Statistical Analysis. Data were statistically analyzed using one-way ANOVA. A p value less than 5% ($p < 0.05$) was considered significant.

3. Results and Discussion

Suspension bioreactors represent a scalable platform to generate large numbers of cells in culture in an efficient and reproducible manner [35, 37, 40]. However, when dealing with adherent cells and microcarrier technology, there are two distinct operating phases which need to be considered. The first is the inoculation phase during which conditions are created to encourage the inoculated cells to attach to the microcarriers. The second is the growth phase where cells attached to microcarriers are encouraged to proliferate. Conditions which are ideal for cell attachment may not necessarily be optimal to support cell proliferation [41]. Thus, to maximize cell yield, there is a need to better understand and optimize each phase of this bioprocess. Here, the results from studies concerning both phases are discussed. Cells from donor 1 were used to develop a suspension bioreactor protocol, and then cells from both donors were used to evaluate the final protocol to ensure that the methodology was not specific to cells from a single donor.

3.1. The Inoculation Phase. The inoculation phase of adherent cell types, particularly in microcarrier suspension cultures, can have a significant influence on the ultimate cell population yields [41–43]. Inoculation phase culture parameters such as agitation regimen, serum levels in the medium, microcarrier loading (g/L), and cell to bead ratio have all been shown to affect cell attachment efficiency.

Forestell et al. [41] indicated that after attachment, cells remained rounded on the microcarrier surface prior to spreading and taking on a flatter profile. These rounded cells were more susceptible to detachment than cells which had spread when exposed to the shear created by continuously stirring the medium. Yuan and colleagues [37] reported that a period of 8 hours was sufficient for attachment of bone marrow-derived mesenchymal stem cells to macroporous CultiSpher-S microcarriers. However, Hewitt et al. [44] reported that following inoculation, 24 hours was required for this same cell type to attach and then spread on microporous Cytodex 3 microcarriers. Thus, we initially defined the

inoculation phase as being the first 24 hours following the addition of human SF-MSCs to the bioreactors.

As a starting point, inoculation protocols recommended by the microcarrier manufacturer in combination with media and cell densities used in traditional static culture flasks were used. The baseline parameters were as follows: (i) continuous stirring at 40 rpm which was the minimum agitation required to maintain the microcarriers in suspension throughout the volume of medium, (ii) a 10% serum level at inoculation which mimics static culture, (iii) 4.5 cells/bead which mimics static culture at 5000 cells/cm^2, and (iv) Cytodex 3 microcarrier loading of 1 g/L. The inoculation phase was carried out in a reduced culture volume of 80 mL. After 24 hours, the cultures were topped up to a final working volume of 120 mL in preparation for the growth phase. Culture parameters used following the first 24 hours included continuous stirring at 40 rpm and 10% serum in the culture medium.

We microscopically evaluated cell attachment and spreading behaviours in response to the manipulation of several different culture parameters (agitation regimen, initial serum content, microcarrier loading, and cell to bead ratio) during the 24-hour inoculation phase. However, accurate quantification of attachment efficiency during this period was challenging due to the low cell numbers in the bioreactor. Thus, we instead assessed the impact of manipulating a particular inoculation phase parameter by measuring cell numbers that resulted in culture after 10 days (24-hour inoculation phase plus a subsequent 9-day growth phase). During the growth period, culture conditions were maintained consistent across all vessels to isolate any effects associated with the inoculation phase. This technique was deemed appropriate as higher cell attachment efficiencies during the inoculation phase have been directly correlated to higher subsequent cell yields [41–43]. Thus, measuring cell yield provided us with a means to evaluate cell attachment efficiency in response to a particular inoculation parameter.

3.1.1. Effect of Agitation Regimen. Literature reports of agitation rates employed during the inoculation of mesenchymal stem cells into suspension culture range from 0 rpm (i.e., no stirring) for the first 24 hours [43, 44] to continuous stirring at 30 rpm for the first 18 hours [36]. Others have reported using an intermittent agitation regimen consisting of alternating periods of stirring and rest [45–47]. Forestell and colleagues [41] found that cell attachment occurs less frequently at higher agitation rates. As such, they used the minimum agitation speed required to suspend the microcarriers. In those studies where intermittent agitation was reported, cultures were agitated for anywhere from 5 seconds to 30 minutes followed by 10 to 30 minutes of rest.

In the current study, cultures were stirred at 40 rpm either continuously or intermittently with cycles of 3 minutes of agitation followed by 27 minutes of rest. It has previously been shown that this regimen facilitated the attachment of bone marrow-derived MSCs to microcarriers [37]. The resulting growth curves are shown in Figure 1. The exponential growth rate was 0.0126 h^{-1} for both continuous and intermittent cultures. The maximum cell densities reached

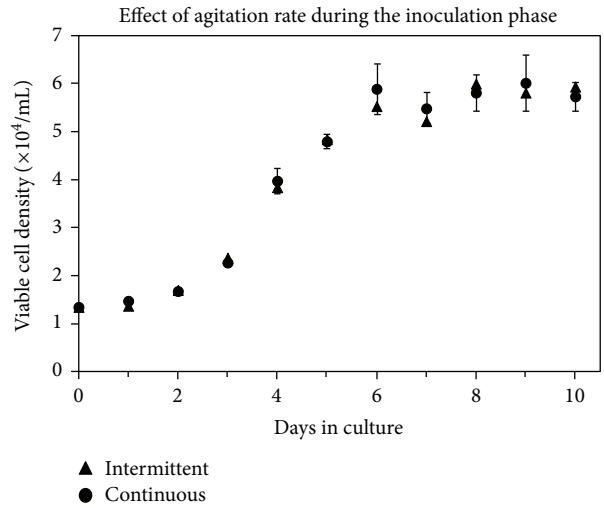

Figure 1: Effect of agitation regimen on the attachment of SF-MSCs to Cytodex 3 microcarriers during the inoculation phase. During the first 24 hours, cultures were either agitated at 40 rpm for 3 minutes every 30 minutes (Intermittent) or continuously at 40 rpm (Continuous). Data were collected in duplicate; error bars represent the range of data collected.

were 6.1×10^4 and 6.0×10^4 cells/mL for the continuous and intermittent agitation regimen, respectively.

From this study, it appeared that there was no significant difference in SF-MSC attachment efficiency to Cytodex 3 microcarriers when using continuous versus intermittent agitation. This is not an unusual result as successful attachment of human placental MSCs on Cytodex 3 microcarriers [44] and bone marrow-derived MSCs on Cytodex 1 microcarriers [36] has been reported with continuous agitation of 30 to 50 rpm during the first 18 to 24 hours. We have noted that MSCs from synovial fluid tend to attach more readily to cell culture plastic than other MSC types in the presence of serum, and this tendency may have translated to the interactions between SF-MSCs and microcarriers in suspension culture. Based on this result, all future experiments in this study incorporated continuous stirring at 40 rpm during the first 24 hours.

3.1.2. Effect of Serum Level. The effect of culture medium serum level (0%, 5%, or 10% with respect to volume) during the inoculation phase was studied as it has been shown to impact attachment efficiency of bone marrow-derived mesenchymal stem cells (BM-MSCs) to microcarriers [36]. It should be noted that all microcarriers used in this study had been exposed to culture medium containing the respective serum content for a period of 18 h prior to being placed in the spinner flask (i.e., the 5% and 10% scenarios had been precoated with serum). Moreover, after the 24-hour inoculation phase, all spinner flasks were topped up to a final working volume of 120 mL medium so that the final concentration of FBS was 10% in all cases during the growth phase. Figure 2(a) illustrates similar cell attachment regardless of serum level during the first hour after inoculation, with cells in all cases appearing rounded on the surface of the beads. However, after 24 hours, most of the cells in the 5% and

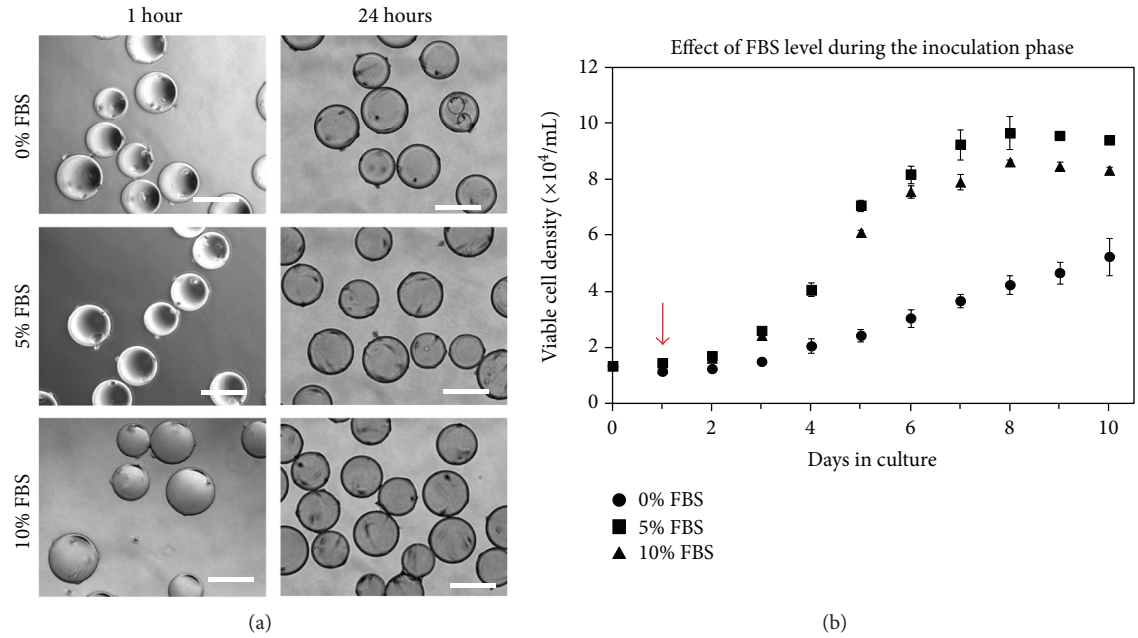

Figure 2: (a) Photomicrographs showing the effect of initial serum content on the attachment of SF-MSCs to Cytodex 3 microcarriers. Cell-microcarrier contact occurred within 1 hour of inoculation for all three serum levels evaluated. 24 hours after inoculation, many cells in 0% FBS were still rounded on the surface compared with 5% and 10% FBS where cells had spread. Photomicrographs were taken at 10x magnification. Scale bars represent 200 μm. (b) Effect of initial serum content on the attachment of SF-MSCs to Cytodex 3 microcarriers and subsequent cell population expansion. Cells were cultured in 0%, 5%, or 10% serum for the first 24 hours. After 24 hours, as indicated by the arrow, the serum levels were adjusted to 10% *v*/*v* in all cases. Data were collected in duplicate; error bars represent the range of data collected.

10% serum conditions had spread, whereas many of the cells in 0% serum were still rounded on the surface.

The results presented in Figure 2(b) show that the 0% serum condition during inoculation did not subsequently support cell growth as effectively as the 5% and 10% conditions. Not only did 0% serum at inoculation result in a prolonged lag phase, but after 10 days, the exponential growth rate of $7.75 \times 10^{-3}\,h^{-1}$ was significantly lower when compared with $0.0159\,h^{-1}$ and $0.0160\,h^{-1}$ for 5% and 10% serum, respectively. The maximum cell density obtained over the course of 10 days in the 0% serum inoculated condition was also significantly lower than that for both the 5% and 10% cases. The maximum cell density in the 5% serum was higher than the maximum cell density achieved with 10% serum. The maximum density in 5% serum was 9.66×10^4 cells/mL compared to 8.62×10^4 cells/mL in 10% serum.

Schop et al. [36] and Forestell et al. [41] both reported higher attachment efficiencies at 0% to 5% (*v*/*v*) serum levels for the attachment of mammalian cells on Cytodex 1 microcarriers compared to 10% serum levels. They speculated that serum lowers surface hydrophobicity, which could negatively impact cell attachment. Conversely, it has been reported that precoating Cytodex 2 microcarriers (cross-linked dextran matrix) with fetal bovine serum decreased the fraction of unoccupied beads 7 hours after inoculation, thereby improving attachment efficiency [48]. FBS is known to contain many different proteins, such as fibronectin and albumin. It has been suggested that when beads are exposed to FBS, albumin readily adsorbs first, and this could interfere with cell attachment, but that over time, this albumin is replaced with fibronectin, which promotes cell attachment [48]. The precoating of microcarriers with serum has also been shown to enhance the attachment of other cell types including human bone marrow-derived MSCs on CultiSpher-S beads [49]. This current result clearly illustrates that simply changing one condition during the inoculation phase can have a significant impact on subsequent cell yields. Based on the results obtained, subsequent experiments used culture medium containing 5% serum to precoat the microcarriers for 18 hours and also for the duration of the inoculation phase.

3.1.3. Effect of Microcarrier Loading and Cell to Bead Ratio. Cell to bead ratio is another important factor that affects ultimate bioreactor cell yields as it directly impacts the frequency of cell-bead interactions, a necessary prelude to cell attachment. Under ideal circumstances, the initial cell to bead ratio should be unity, if it could be ensured that each bead would only allow for the attachment of a single cell. However, during the inoculation phase, the number of cells per microcarrier has been shown to follow a Poisson distribution [41, 42, 45]. As a result, some microcarriers will have more than one cell attached to their surface, while a portion of the microcarriers may not be occupied by any cells at all. Thus, to ensure that a majority of beads are occupied at the end of the inoculation phase, it is necessary to have a cell to bead ratio greater than unity. According to the Poisson distribution, the proportion of

vacant microcarriers is theorized to be less than 2% when the cell to bead ratio equals or exceeds a value of 4.

To investigate the effect of cell to bead ratio and microcarrier loading on the attachment of SF-MSCs on Cytodex 3 microcarriers, a two-level factorial design experiment was carried out. Microcarrier loadings of either 1 g/L or 2 g/L Cytodex 3 were used, which correspond to available surface areas of 2.7 cm^2/mL and 5.4 cm^2/mL, respectively. The cell to bead ratio selected as the baseline value was 4.5 cells/bead, which is equivalent to 5000 cells/cm^2 typically used to inoculate static tissue culture flasks. A lower cell to bead ratio of 2.25 cells/bead was also evaluated. Whereas this could result in a greater proportion of unoccupied beads at the end of the inoculation phase, a low inoculation density could result in a greater overall cell fold increase. Cell to bead ratios higher than 4.5 were not evaluated since this would require a larger number of cells for inoculation and could potentially result in a lower cell fold increase as a significant proportion of the cells could fail to attach and subsequently perish, thereby wasting inoculum.

As shown in Figure 3, it was found that at both 1 g/L and 2 g/L microcarrier loading, a seeding density of 2.25 cells/bead does not reach final cell densities comparable to those achieved using a seeding density of 4.5 cells/bead. Additionally, at a seeding density of 2.25 cells/bead and a microcarrier loading of 1 g/L, a 3-day lag phase was observed. These trends are similar to those reported in the study by Hu et al. [42] where it was reported that a critical number of cells per microcarrier is required for a normal pattern of growth. The growth rates during the exponential phase for 2.25 cells/bead at 1 g/L and 2 g/L were similar at 0.0103 h^{-1} and 0.0102 h^{-1}, respectively. The exponential growth rates using 4.5 cells/bead were 0.0159 h^{-1} and 0.0118 h^{-1} for 1 g/L and 2 g/L, respectively. The decrease in growth rate at higher microcarrier loadings has previously been observed for other cell types and in those cases has been attributed to an increase in bead-to-bead collisions resulting in cell damage and death [50]. Here relatively high cell viabilities were maintained in culture, and significant quantities of cell debris were not observed during the growth phase, suggesting that the increased frequency of collisions at high bead loadings may reduce growth and proliferation without necessarily killing cells. A positive effect of such collisions, however, is the transfer of cells from one bead to another. As expected, a majority of the microcarriers had at least one cell attached when microscopically analyzed after 24 hours. However, during the growth phase, the number of empty microcarriers observed decreased suggesting that cells had transferred from a bead with cells to an empty bead, either during a bead-bead collision or through a process of detachment into the surrounding medium and then reattachment.

Based on the shortened lag phase and increased growth rates obtained when using 4.5 cells/bead, this ratio was selected for all future experiments. Despite the higher growth rate at 1 g/L, an initial microcarrier loading of 2 g/L was selected for future studies as it (i) supports cell proliferation, (ii) provides a surface area to volume ratio that is twice that of 1 g/L of beads and is similar to that used in static culture vessels (generally between 4.2 and 6.1 cm^2/mL), and (iii) supports twice the theoretical maximum volumetric cell density (cells/mL) of a 1 g/L bead loading, which is an important consideration as it means that twice as many cells have the potential to be produced within a given vessel.

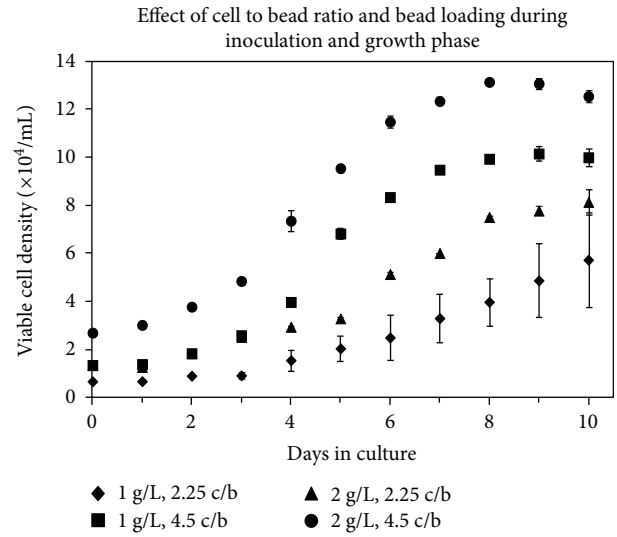

Figure 3: Effect of microcarrier loading and cell to bead ratio on the attachment of SF-MSCs to Cytodex 3 microcarriers. Spinner flasks were inoculated with either 0.12 g (1 g/L) or 0.24 g (2 g/L) of Cytodex 3 at a cell density of either 2.25 or 4.5 cells/bead (c/b). Data were collected in duplicate; error bars represent the range of data collected.

3.2. Growth Phase. Once favourable parameters had been established for the inoculation phase, variables that were hypothesized to affect cell population expansion during the growth phase were evaluated. Specifically, the effects of agitation rate and periodic medium replenishment were investigated in an effort to identify their impact on cell growth kinetics. In these experiments, the inoculation parameters selected (continuous agitation at 40 rpm, 5% serum culture medium, 4.5 cells/bead, and a microcarrier loading of 2 g/L) were applied to all cultures for the first 24 hours. At the end of the inoculation phase, various agitation rates and feeding patterns were tested throughout the growth phase.

3.2.1. Effect of Agitation Rate. Agitation in microcarrier-based suspension culture has several effects: (i) it creates a more homogeneous culture environment, (ii) it introduces shear stress into the culture environment, (iii) it provides collision energy between microcarriers, and (iv) it increases mass transfer [41]. While a more homogeneous environment and increased nutrient transport may be beneficial for the culture of cells, high shear stresses can serve to damage cells attached to surfaces, promote the detachment of cells from the surface of a microcarrier, and result in strong collisions between microcarriers leading to cell death [50]. It has been shown that a shear stress as low as 6.25 dyn/cm^2 may be sufficient to remove cells from surfaces, with 10–30 dyn/cm^2 resulting in damage to cells [51, 52]. Since shear stress and collision energy are related to bioreactor agitation rate, stirring speed was manipulated to evaluate the impact of

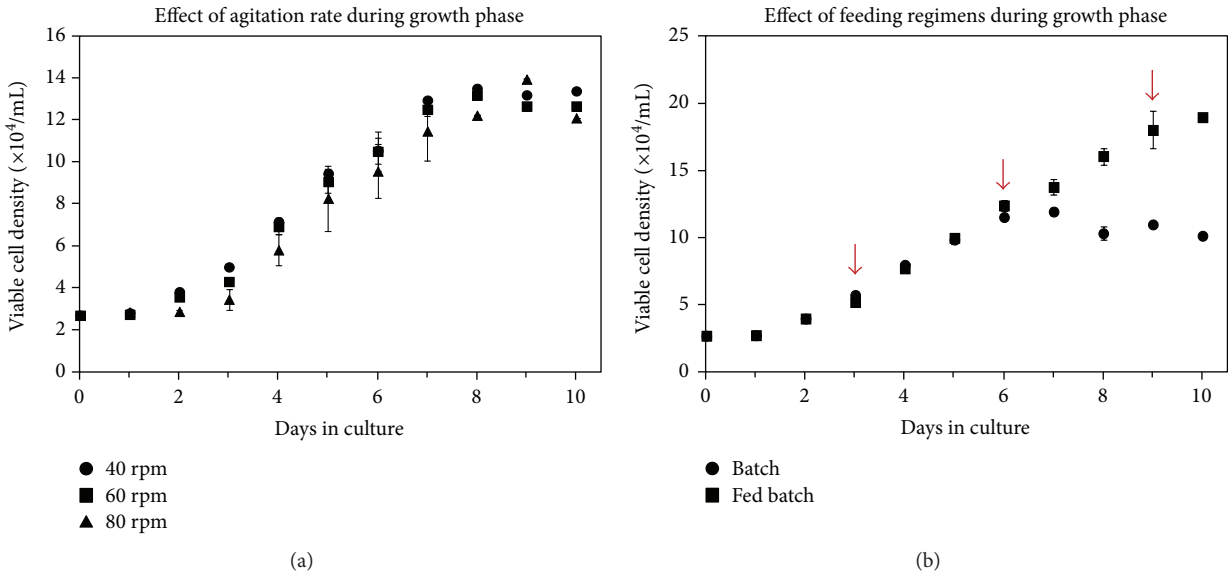

Figure 4: (a) Effect of agitation rate on the growth of SF-MSCs on Cytodex 3. Six spinner flasks were stirred continuously at 40 rpm for the first 24 hours. After 24 hours, the agitation rate in two spinner flasks was increased to 60 rpm and in another two was increased to 80 rpm. Data were collected in duplicate; error bars represent the range of data collected. (b) Effect of feeding on cell growth. In the cyclic fed batch condition, a 50% medium change was performed on days 3, 6, and 9, indicated by the arrows. No medium replenishments were made in the batch condition. Data were collected in duplicate; error bars represent the range of data collected.

shear on SF-MSC proliferation. Preliminary experiments showed that a low agitation rate of 40 rpm in the spinner flasks was sufficient to maintain Cytodex 3 microcarriers in suspension and also provide a homogenous, well mixed environment (data not shown).

It has been reported from aggregate studies with baby hamster kidney (BHK) cells that the maximum shear stress experienced by cells at the surface of an aggregate ranges from 0.8 dyn/cm^2 to 5.6 dyn/cm^2 for agitation rates of 25 rpm to 100 rpm [52]. Previous studies in our laboratory involving neural stem cell aggregates in the same spinner flask bioreactors being used in the current study found that the shear stress experienced by a single cell at the surface of an aggregate ranged from 4.9 dyn/cm^2 to 9.86 dyn/cm^2 for agitation rates from 40 rpm to 100 rpm ([53]; Gilbertson et al., 2006). Since these shear stress values are all less than those which have been reported to cause damage to a cell immobilized to an aggregate surface or static surface, agitation rates of 40 rpm, 60 rpm, and 80 rpm were evaluated in this study.

Although there was no evidence of significant differences in proliferation between any of the agitation rates tested, a slightly longer lag phase was observed at 80 rpm (Figure 4(a)). It is likely that the sudden increase from 40 rpm during the inoculation period to 80 rpm at the start of the growth phase led to some cells detaching from the beads. At 40 rpm, 60 rpm, and 80 rpm, the exponential phase growth rates were 0.0128 h^{-1}, 0.0123 h^{-1}, and 0.0126 h^{-1}, respectively, and the maximum cell densities obtained were very similar between the three agitation rates. However, the maximum cell density at 80 rpm occurred later than at the lower agitation rates, presumably because of the extended lag phase. The similarity in cell expansion at the different agitation rates indicates that shear was not a significant factor affecting proliferation rate in the range tested. In addition, there was no noticeable difference in the level of cell debris in the culture medium at the end of the experiment, suggesting that cells were not being destroyed at any of the tested agitation rates. Given that significant differences were not found, an agitation of 40 rpm was used in all subsequent studies as this could facilitate the transition from the inoculation phase to the growth phase.

3.2.2. Effect of Feeding Regimen. The rapid expansion of cells in culture can result in the depletion of key nutrients such as glucose and glutamine from the medium, while simultaneously increasing the levels of waste metabolites including lactate and ammonium ions. The absence of nutrients and the presence of wastes may negatively impact ultimate cell yields. There are numerous examples in the literature showing that regular medium changes, in which a portion of the spent culture medium is removed and replaced with an equal volume of fresh medium, can extend the productive life of a culture and increase cell yields, including those of bone marrow-derived MSC cultures [46].

To determine if medium components were limiting SF-MSC growth in suspension culture, the effect of medium replenishment was evaluated by culturing cells in either batch culture, where no medium replenishments were made, or in cyclic fed-batch cultures, where a 50% medium replenishment was performed on days 3, 6, and 9. Growth curves generated over a 10-day period for batch and cyclic fed-batch conditions are shown in Figure 4(b). Due to initial similar growth patterns, the exponential growth rates for the batch and cyclic fed-batch cultures (from day 2 to day 6) were both 0.0136 h^{-1}. However, feeding extended the life

of the culture, thereby allowing a greater overall cell yield. The maximum cell density achieved in the cyclic fed-batch culture was 1.89×10^5 cells/mL (day 10), which was significantly higher than that achieved in the batch culture, 1.19×10^5 cells/mL (day 7). This translated to a viable cell fold increase of 7.0 over 10 days under cyclic fed-batch conditions, compared to only 4.4 over the same time period under batch conditions. It is important to note that these results also showed that the microcarrier surface area was not limiting in batch culture and that the lack of cell growth beyond day 6 was likely due instead to nutrient depletion or waste accumulation in the medium.

Nutrient and metabolic waste product concentrations were monitored throughout the 10-day growth period for batch and cyclic fed-batch cultures. SF-MSC glucose and glutamine consumption rates during the growth phase in batch cultures were calculated as 3.88×10^{-10} mmol/cell·h and 2.41×10^{-10} mmol/cell·h, respectively. These rates are comparable to the values obtained in a previous study that found the average glucose consumption rate of human bone marrow MSCs to be $3.83 \pm 0.69 \times 10^{-10}$ mmol/cell·h [54]. Additionally, lactic acid and ammonium production rates during the growth phase in the batch cultures were 1.95×10^{-9} mmol/cell·h and 1.74×10^{-10} mmol/cell·h, respectively. The yield of lactic acid over glucose ($Y_{\text{Lac/Glc}}$) during the growth phase in the batch cultures was 2.94 mol/mol. A value higher than the theoretical maximum, 2 mol/mol, indicates that lactate is being produced from other sources (i.e., glutamine) [49]. $Y_{\text{Lac/Glc}}$ values ranging from 1.4 to 6.5 mol/mol have been reported previously for mesenchymal stem cells [36]. The yield of ammonia from glucose was 0.689 mol/mol. As little as 2.0 mM of ammonia has been reported to inhibit proliferation of human MSCs, whereas lactic acid concentrations upwards of 24 mM may be required before inhibitory effects become apparent [54]. Based on the concentration profiles, the concentration of ammonia in batch culture approached 2.0 mM after 6 days in culture, while the concentration of ammonia in all cyclic fed-batch cultures did not exceed 1.5 mM (data not shown). Since the nutrient profiles showed that glucose and glutamine were not depleted, it is possible that inhibitory levels of ammonia may have played a role in the reduced proliferation observed in batch culture, as the maximum cell density was also achieved on day 6. Another possibility is that the addition of fresh medium replenished other medium components, such as amino acids [55], which were depleted but not measured in this study, thereby contributing to higher cell densities in cyclic fed-batch cultures.

Based on the results of this study, a 50% medium replenishment approximately every 3 days was incorporated into the standard protocol for maintenance of human SF-MSCs in suspension culture.

3.3. Serial Subculture on Microcarriers. Whereas the feeding study clearly indicated that the lifespan of a culture could be extended by replacing spent medium with fresh medium, the yield of cells would ultimately be limited by the finite surface area provided by the microcarriers for monolayer growth. To increase cell yields beyond that point, two strategies were investigated: (i) serial passaging of microcarrier cultures and (ii) bead-to-bead transfer. In the serial passaging method, cells were harvested from microcarriers using trypsin and then reinoculated into new culture vessels with fresh microcarriers and culture medium. In this approach, the total number of cells produced would increase over time as either the cells would be inoculated into an increasing number of vessels or into larger vessels capable of supporting greater culture volumes. In the bead-to-bead transfer method, fresh microcarriers were added to an existing culture vessel and conditions manipulated to encourage cell migration from confluent beads to new, unoccupied beads within the same vessel. Successful bead-to-bead transfer has previously been reported for porcine MSCs [45], goat MSCs [46], and human MSCs [36, 47] grown on a variety of microcarriers. Assuming the success of both methods, one major advantage of bead-to-bead transfer over serial passaging of microcarrier cultures is that the cultures could be maintained in a single vessel for an extended period of time with minimal cell handling. Not only would this reduce the number of times the cells are exposed to the proteinase trypsin, but it also would lower the probability of contamination. Additionally, in the event a vessel becomes too small to support a target cell number, the entire culture volume could be transferred to a larger bioreactor along with fresh beads without the need to first trypsinize the cells from the existing beads.

3.3.1. Serial Passaging. SF-MSCs expanded to passage 5 in static tissue culture flasks were inoculated into microcarrier culture and exposed to the inoculation and growth protocols described earlier. After six days, the cells were harvested from the microcarriers by trypsinization and inoculated into new vessels containing fresh microcarriers for three consecutive passages (6 days each) under the same conditions. Figures 5(a)–5(c) show photomicrographs taken on the sixth day of culture for three consecutive passages. With each subsequent passage, fewer total cells and a larger proportion of unoccupied beads were observed. Cell densities were plotted over the course of the three passages in Figure 5(d). Although the data shows that the harvested cells were able to reattach to microcarriers, they were unable to proliferate to the same degree as cells that had been harvested from static tissue culture flasks.

Hu et al. [56] reported serial propagation of human foreskin fibroblasts on Sephadex microcarriers (dextran beads). Although the cells used in the current study were able to reattach to new microcarriers and continue to grow, the extent of proliferation was found to decrease slightly with each subsequent inoculation. Similar results were described by Forestell and colleagues [57] for the serial subculture of human fetal lung fibroblasts on Cytodex 1 microcarriers. They found significant decreases in cell growth with each transfer from vessel to vessel. Forestell et al. [57] were able to overcome diminishing proliferation by decreasing the serum content and developing a custom medium supplement. It would be prudent in the future to examine the use of defined growth media to examine how serum levels impact SF-MSC behaviour during serial subculture. Analysis of the

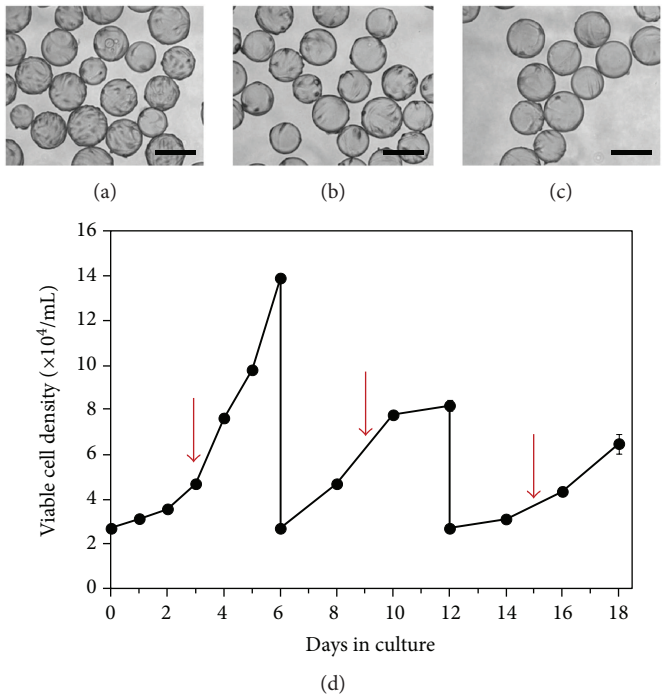

Figure 5: Serial passaging of SF-MSCs on Cytodex 3 microcarriers using the improved protocol. Cells were serially passaged 3 times. Each passage lasted 6 days. (a–c) Photomicrographs showing microcarriers from the last day of culture for three consecutive passages. There are fewer cells and a larger proportion of unoccupied beads with each subsequent passage. Photomicrographs were taken at 10x magnification. Scale bars represent 200 μm. (d) Serial passaging of SF-MSCs on Cytodex 3. Lower maximum cell densities were achieved with each subsequent passage. A 50% medium replenishment was performed on days 3, 9, and 15, as indicated by the arrows. Data were collected in duplicate; error bars represent the range of data collected.

spent culture medium over the three serial passages indicated that glucose and glutamine were not depleted at any time, and waste metabolite concentration was also maintained at low levels (data not shown). As such, the diminished growth was likely not nutrient related.

3.3.2. Bead-to-Bead Transfer. The effectiveness of bead-to-bead transfer of SF-MSCs on Cytodex 3 microcarriers during suspension culture was evaluated. Although the protocol developed in the previous sections used a microcarrier loading of 2 g/L, cultures were initiated with only 1 g/L for this particular study as microcarriers would be added periodically to the cultures, and thus, the benefit of a high surface area to volume ratio would eventually be achieved regardless of the initial bead loading. Moreover, a microcarrier loading of 1 g/L was shown to be effective earlier, and a smaller initial loading also meant that fewer cells were required as inoculum. Spinner flasks were initially inoculated with 4.5 cells/bead and a microcarrier loading of 1 g/L in 80 mL of 5% FBS DMEM and stirred continuously at 40 rpm for the first 24 hours. After 24 hours, spinner flasks were topped up with an additional 40 mL of medium and the serum levels were adjusted to 10% FBS DMEM. Every 6 days, 1 g/L of fresh microcarriers was added to the cultures, and culture volumes were reduced to 80 mL and stirred at 40 rpm intermittently for 3 hours (3 minutes on, 27 minutes off). Intermittent agitation was chosen because the work by Wang and Ouyang [58] involving Vero cells (i.e., African green monkey kidney cells) on Cytodex 3 showed that this operational mode enabled microcarriers to be in close proximity long enough for cells to effectively migrate from one bead to another. Whereas bead-to-bead transfer is also possible through collisions while in suspension, this latter approach can be traumatic and lead to cell damage and cell death. After 3 hours of intermittent agitation, cultures were stirred continuously at 40 rpm. The spinner flasks were topped up with an additional 40 mL of culture medium 24 hours after the microcarrier addition. 50% medium replenishments were performed on days 6, 9, 12, 14, and 16.

Intermittent agitation was stopped after 3 hours because photomicrographs showed that many of the new beads had acquired cells after this time (Figures 6(a)–6(d)). Twenty-four hours after the addition of fresh microcarriers, the proportion of remaining empty beads decreased further compared to 3 hours postmicrocarrier addition. Similar to the observations noted earlier during the inoculation phase studies, this again suggests that cells were also able to transfer between microcarriers during periods of continuous agitation. The transfer of human BM-MSCs on Cytodex 3 beads during agitation was also observed by Hewitt et al. [44]. Cell adhesion is weakened during cell division and it is possible that it was during mitosis that cells were able to detach from their original microcarrier and reattach to a new bead [59].

Growth curves for cells from two donors are shown in Figure 6(e). The steady increase in viable cell densities shows that the bead-to-bead transfer method together with feeding is a viable method to enable prolonged cell growth in a single vessel. Additionally, a stationary phase was not reached

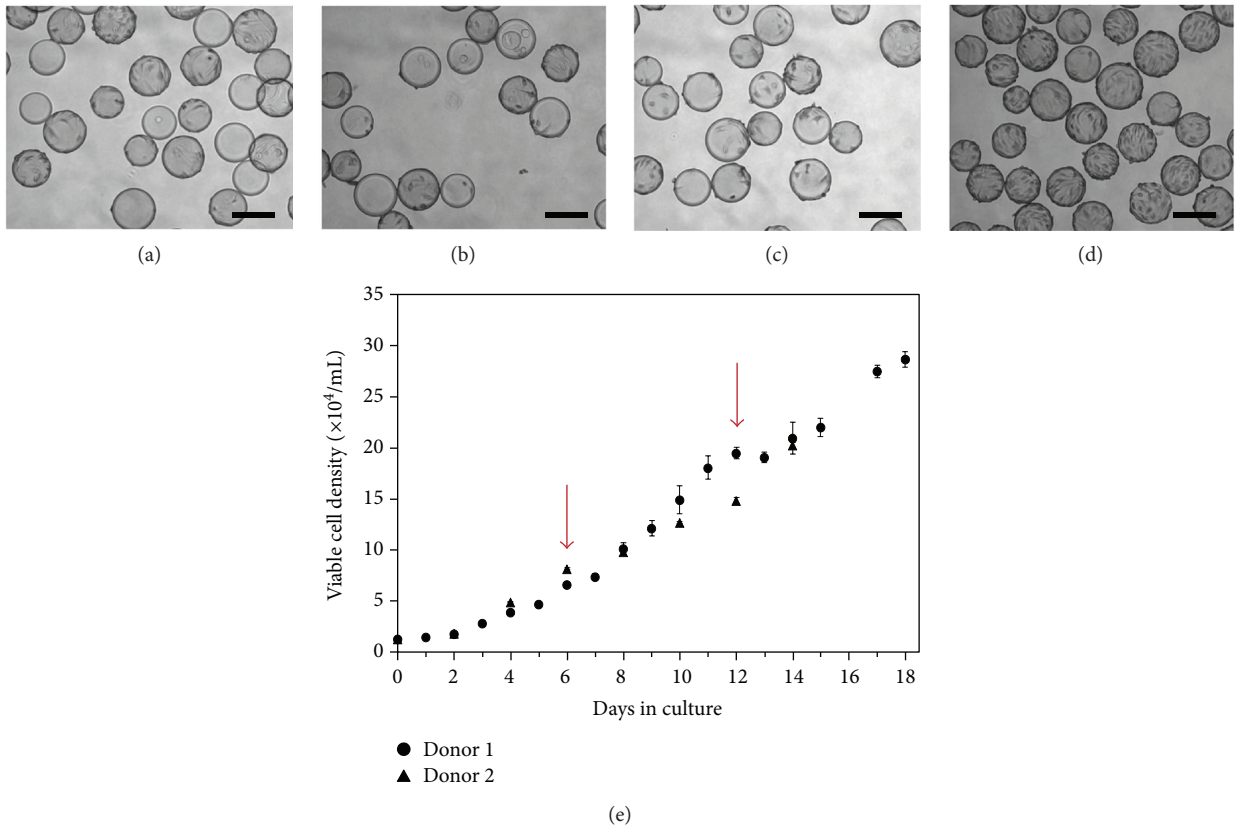

Figure 6: Bead-to-bead transfer of SF-MSCs on Cytodex 3 microcarriers. 1 g/L of fresh microcarriers was added to the culture on days 6 and 12 (shown with arrows). After addition, the cultured were stirred intermittently at 40 rpm for 3 hours (3 minutes on, 27 minutes off). After 3 hours, the culture was continuously stirred at 40 rpm. Shown are photomicrographs (a) immediately following addition of fresh microcarriers (day 6). Roughly half of the beads are empty and half are occupied and nearing confluence; (b) 3 hours after microcarrier addition on day 6. Cells had started to transfer to new microcarriers, but still appeared rounded on their surface; (c) 24 hours after microcarrier addition. More noticeable cell transfer had occurred and very few beads remained unoccupied; (d) 6 days following inoculation (day 12, prior to second addition of fresh microcarriers). Most beads were near confluence. Photomicrographs were taken at 10x magnification. Scale bars represent 200 μm. (e) Growth curve of bead-to-bead transfer of SF-MSCs on Cytodex 3 microcarriers. Shown are data for cells derived from donors 1 and 2 to show that the utility of the protocol developed was not unique for only a single set of cells. The increases in viable cell densities show that the bead-to-bead transfer method was successful. Data were collected in duplicate; error bars represent the range of data collected.

suggesting that cell yields could have increased further had the experiment been allowed to continue. After 18 days in culture, a maximum cell density of 2.87×10^5 cells/mL was achieved for donor 1, which translates to a 21.3-fold increase in the number of cells. Additionally, the volume of medium required per new cell generated was only 1.28×10^{-5} mL which was nearly half of what was required for each new cell generated in static culture (2.40×10^{-5} mL) over the course of 18 days. These values represent the total medium requirement, including medium changes. Therefore, not only was this method more cost-effective, but it also overcame the need to regularly expose cells to enzymes and significantly reduced labour requirements as frequent passaging was not necessary. Based on the studies presented thus far, it was evident that microcarrier technology could be used to effectively scale-up human SF-MSCs in suspension bioreactors. However, a series of characterization studies were needed to ensure that this mode of cell expansion had not adversely affected the defining qualities of these cells.

3.4. Cell Characterization after Expansion in Suspension Culture. SF-MSCs expanded for 18 days in suspension culture using the bead-to-bead transfer method were harvested and then characterized in terms of surface marker profile and differentiation potential to determine if they had retained their defining characteristics. Uninduced cultures, as well as cells grown under static conditions, were used as controls.

3.4.1. Cell-Surface Marker Profile. The surface marker panels analyzed were CD34, CD45, CD73, CD90, and CD105. This selection was based on the criteria outlined by Dominici et al. [14] which state that an MSC population must express CD73, CD90, and CD105 and must lack expression of CD34 and CD45. The surface marker profiles of donors 1 and 2 are presented in Figure 7 and indicate that both donors had high expression levels for positive makers and low expression levels for negative markers after being cultured using our optimized protocols.

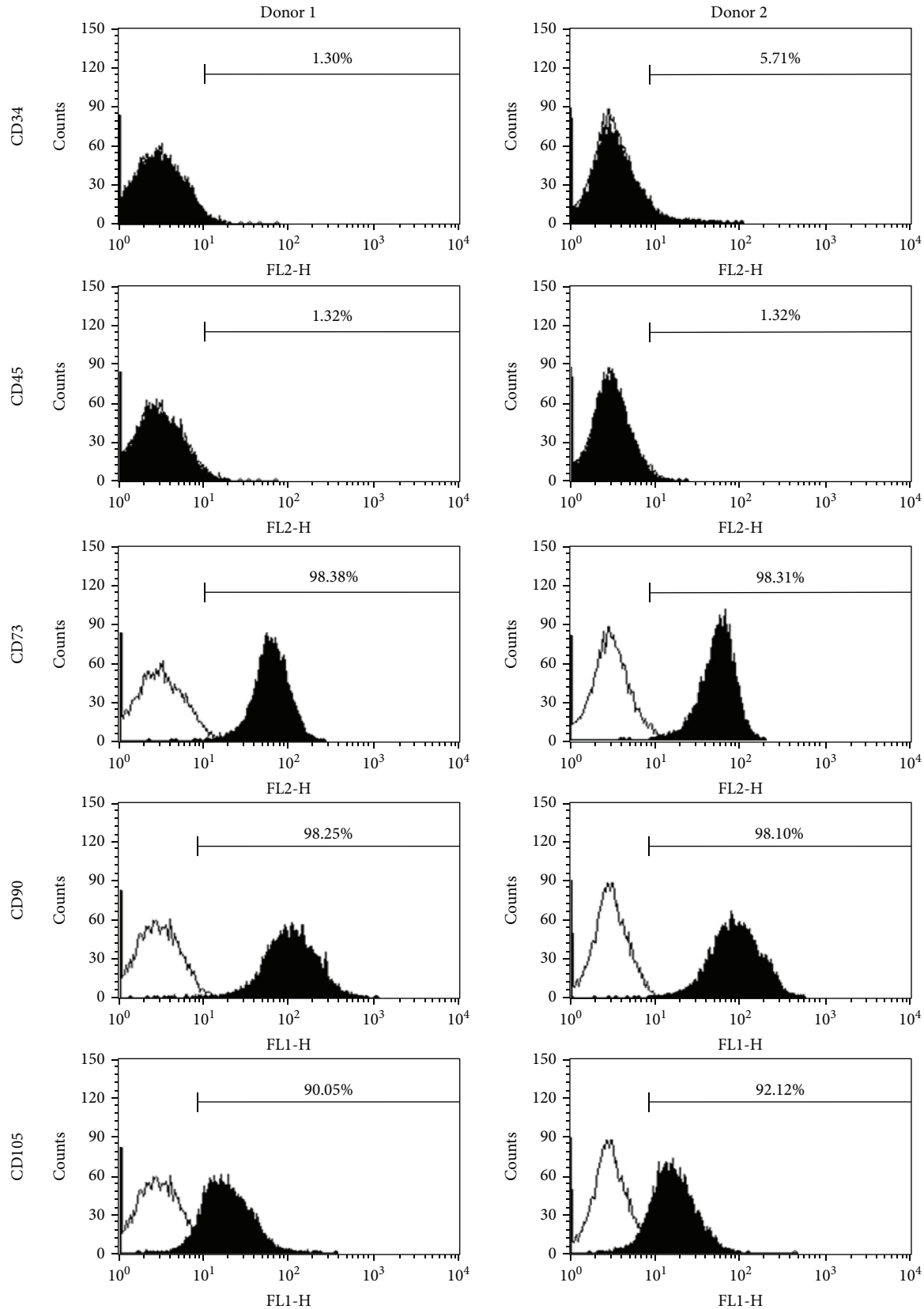

Figure 7: CellQuest histogram plots for surface markers associated with SF-MSCs. The plots show specific antibody staining (solid) versus isotype control staining (empty). Prior to analysis, cells were cultured for 18 days in spinner flasks using the bead-to-bead transfer method. A background noise level of 2% or less was accepted.

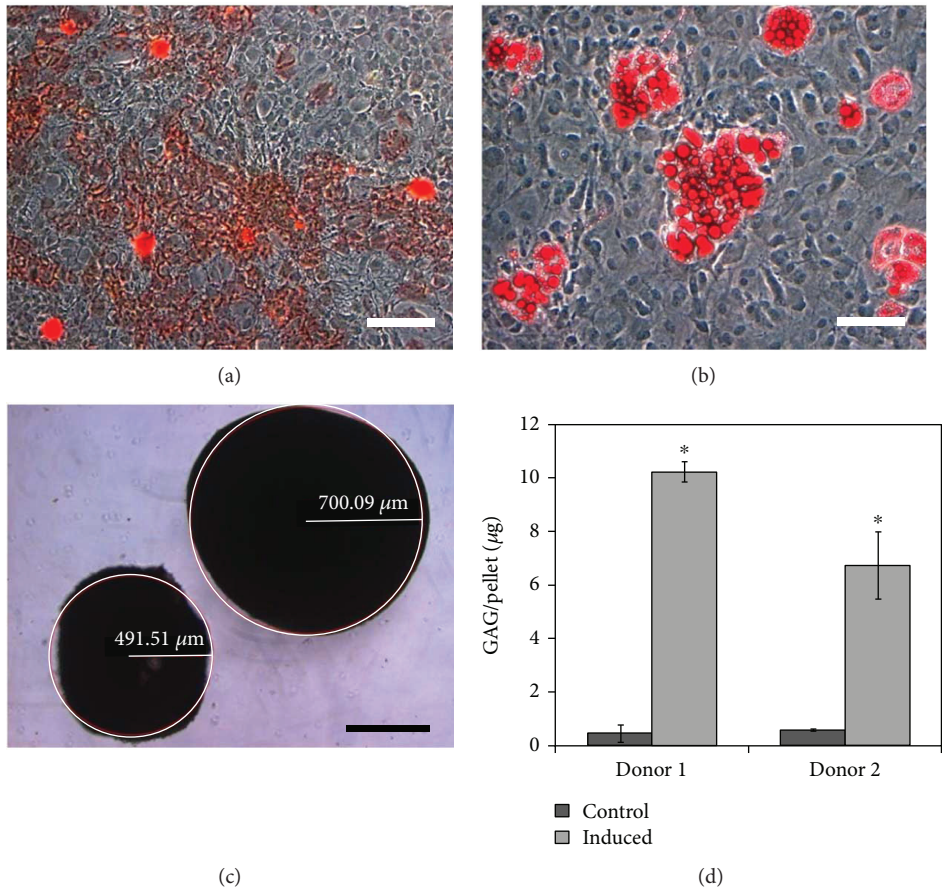

Figure 8: Multipotent differentiation potential of SF-MSCs cultured for 18 days in spinner flasks using the bead-to-bead transfer method. Multipotency was evaluated for cells isolated from both donor 1 and donor 2. Shown here are photomicrographs for donor 1, which are representative of the qualitative results obtained for donor 2. (a) Osteogenic differentiation of donor 1 SF-MSCs. Calcium deposition is an indication of osteogenesis and was detected using Alizarin Red. Photomicrograph was taken at 10x magnification. Scale bar represents 200 μm. (b) Adipogenic differentiation of donor 1 SF-MSCs. Intracellular lipid droplet formation is an indication of adipogenesis and was detected using Oil Red O. Photomicrograph was taken at 20x magnification. Scale bar represents 100 μm. (c) Chondrogenic differentiation of donor 1. Glycosaminoglycan (GAG) production is an indication of chondrogenesis and results in an increase in pellet size (right pellet was induced; left pellet was not induced (control)). Photomicrograph was taken at 5x magnification. Scale bar represents 500 μm. (d) Quantitative chondrogenic differentiation analysis of donor 1 and donor 2 using the pellet culture method. 2.5×10^5 cells/pellet were cultured in either 10% FBS DMEM (control) or chondrogenic induction medium (induced) for 28 days at 37°C and 5% CO_2. GAG production was quantified using 1,9-dimethylmethlylene blue after digesting the pellets in papain solution. Data were collected in triplicate; error bars represent the standard deviation of the data. $^*p < 0.05$ compared to the control.

3.4.2. Differentiation Potential. Multipotency assays were performed to verify that cell populations expanded on microcarriers under stirred conditions using the newly developed protocols retained their capacity to undergo trilineage differentiation. Several other researchers have previously shown that bone marrow-derived MSCs grown on CultiSpher-S and Cytodex 1 and 3 microcarriers retain their osteogenic and adipogenic differentiation potential [36, 37, 47, 49].

Osteogenesis and adipogenesis were qualitatively verified by staining-induced cultures with Alizarin Red and Oil Red O, respectively, as shown in Figures 8(a) and 8(b). Two different analyses were performed to verify chondrogenesis. The first was to perform a size analysis on cell pellets which had been exposed to chondrogenic factors normally used to differentiate MSCs towards a chondrogenic lineage. Once induced, cells tend not to proliferate, so an increase in induced pellet size compared to an uninduced pellet of cells would indicate an upregulation of extracellular matrix production. For the cells grown on microcarriers in suspension culture, it was found that the induced pellets were significantly larger than the uninduced controls with an estimated radius of 700 μm compared to 490 μm, respectively ($p < 0.05$; see Figure 8(c)). This result showed that the cells maintained an ability to undergo chondrogenic differentiation even after being placed in suspension culture. To determine if the chondrogenic capacity of the cells grown in suspension culture was altered relative to cells cultured in traditional static tissue culture flasks, MSCs expanded for 18 days in static culture alongside the suspension cultures were also pelleted and then either chondrogenically induced or left uninduced. For these static culture cells, it was found that the induced pellets were significantly larger than the uninduced

control values with an estimated radius of 690 μm compared to 400 μm, which is similar to the results found for cells from suspension culture. These results highlight that the chondrogenic capacity of the cells was not negatively impacted by being placed on microcarriers in suspension culture.

The second analysis related to chondrogenesis was to perform a GAG analysis on the pellets. For the cells grown in suspension culture, the average GAG content in the uninduced SF-MSC controls from donors 1 and 2 were $0.47 \pm 0.31\,\mu$g and $0.51 \pm 0.05\,\mu$g, respectively, whereas in the induced pellets, the GAG levels were significantly higher for both donor 1 and 2 at $10.24 \pm 0.39\,\mu$g and $6.74 \pm 1.26\,\mu$g, respectively (see Figure 8(d)). For the cells grown in static culture, the GAG levels in the induced controls were $9.94 \pm 0.04\,\mu$g and $8.89 \pm 2.63\,\mu$g for donors 1 and 2, respectively. The observed difference in differentiation potential between the two donors may be attributed to inherent donor-to-donor variability, or possibly due to the difference in donor age. Donor-to-donor variability in MSC populations is commonly reported in the literature [25, 54, 60]. In addition, it is generally believed that MSC function declines with age [61, 62], thereby contributing to any observed differences in behaviour between cells from different age donors, although the chondrogenic potential of synovium-derived MSCs has been shown to be age independent (De Bari et al., 2001).

4. Conclusions

The mesenchymal stem cells isolated from the synovial fluid of articulating joints have the potential to be used in cartilage repair strategies. However, the development of clinical applications will require large number of cells. This work clearly illustrated that microcarrier technology in scalable suspension culture bioreactors can provide the controlled environment required to expand synovial fluid-derived mesenchymal stem cells without compromising their defining characteristics, thereby providing a viable alternative to using tissue culture flasks. Moreover, this approach was found to be effective for cells from more than one donor. The ability to reproducibly support the expansion of stem cell populations from different donors is an important consideration when developing bioprocesses for clinical purposes.

Conflicts of Interest

The authors indicate no potential conflicts of interest.

Authors' Contributions

Kristen D. Jorgenson performed the research and participated in designing the experiments, analyzing the data, and writing the paper. David A. Hart participated in designing the experiments, analyzing the data, and writing the paper. Roman Krawetz was involved in acquiring and isolating in T-flasks the cell populations used in this study and in reviewing the manuscript. Arindom Sen participated in designing the experiments, analyzing the data, and writing the paper.

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Review Article

Current Therapeutic Strategies for Stem Cell-Based Cartilage Regeneration

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The process of cartilage destruction in the diarthrodial joint is progressive and irreversible. This destruction is extremely difficult to manage and frustrates researchers, clinicians, and patients. Patients often take medication to control their pain. Surgery is usually performed when pain becomes uncontrollable or joint function completely fails. There is an unmet clinical need for a regenerative strategy to treat cartilage defect without surgery due to the lack of a suitable regenerative strategy. Clinicians and scientists have tried to address this using stem cells, which have a regenerative potential in various tissues. Cartilage may be an ideal target for stem cell treatment because it has a notoriously poor regenerative potential. In this review, we describe past, present, and future strategies to regenerate cartilage in patients. Specifically, this review compares a surgical regenerative technique (microfracture) and cell therapy, cell therapy with and without a scaffold, and therapy with nonaggregated and aggregated cells. We also review the chondrogenic potential of cells according to their origin, including autologous chondrocytes, mesenchymal stem cells, and induced pluripotent stem cells.

1. Introduction

Articular cartilage is a hyaline lining on the articular surface of bone ends. It cushions external impacts and reduces friction between bones to enable smooth and painless joint motion. Chondrocytes are the only resident cell type in cartilage and comprise 1–5% of articular cartilage. These cells produce collagen, proteoglycans, and hyaluronic acid, which are components of the extracellular matrix (ECM) and underlie the mechanical properties of cartilage [1, 2].

Cartilage damage is characterized by gradual destruction of articular cartilage, an avascular connective tissue with a poor regeneration capacity. Damage of articular cartilage results in pain, swelling, and a limited range of motion due to its limited intrinsic healing ability. It can be triggered by pathologic changes caused by trauma, aging, genetic factors, and inflammation. Hypertrophy of chondrocytes and synovial membranes, cartilage degeneration, chronic arthritis, and systemic inflammation can also occur, leading to varying degrees of chondrocytosis, which is the growth of chondrocytes [3].

Several attempts have been made to regenerate articular cartilage. Treatment depends on the condition of the patient and their degree of cartilage damage. In the case of complete cartilage degeneration, total joint replacement is the only option [4]. Microfracture and autologous chondrocyte implantation (ACI) have been proposed as surgical options for partial cartilage lesions. For patients with cartilage degeneration of an intermediate severity, tissue engineering approaches are emerging as a means to restore cartilage more effectively than microfracture or ACI.

Mechanical, biological, and chemical scaffolds can mitigate the disadvantages associated with cell-based therapy, such as insufficient integration into host tissues, inaccurate cell delivery, and degeneration of healthy cartilage. A scaffold-based approach has been developed to better fill cartilage lesions with autologous chondrocytes. When chondrocytes are propagated in a 3D environment, less

dedifferentiation occurs and more hyaline cartilage forms [5]. The development of hyaline-like cartilage is improved by implantation of hyaluronic acid scaffolds containing autologous chondrocytes into defect sites [6, 7]. However, despite great efforts to mimic the in vivo environment using biological reactors, exogenous machinery, and biochemical stimulation, tissue with the same properties as healthy cartilage has not been generated [4]. Moreover, the limited number of primary cells (i.e., chondrocytes) reduces the effectiveness of this treatment. Consequently, stem cell-based methods have been developed to avoid the disadvantages associated with primary chondrocyte therapy.

Of the various types of stem cells, bone marrow-derived stem cells (BMSCs) and adipose stem cells (ASCs) have many advantages for clinical applications due to their chondrogenic potential [8–14]. It is easier to separate and proliferate BMSCs and ASCs than primary chondrocytes. These stem cells can differentiate into bone and cartilage and thereby regenerate cartilage in vitro and in vivo [14–19]. However, it is difficult to obtain large numbers of BMSCs and ASCs via in vitro culture because extensive expansion can alter their phenotypes [20–23]. In addition, the yield and differentiation capacity of BMSCs decrease with age and in pathogenic conditions [14, 24, 25]. For these reasons, a new cell source for cartilage regeneration is needed.

In this regard, induced pluripotent stem cells (iPSCs), which can proliferate indefinitely and be produced in large numbers, are of interest. Human iPSCs (hiPSCs) are pluripotent, similar to embryonic stem cells (ESCs), but have no associated ethical problems. hiPSCs can be produced without integrating genes into the genome and can differentiate into chondrocytes in vitro [14, 26]. In addition, a large number of hiPSC libraries prepared from donors, homozygous for the human leukocyte antigen (HLA), have been established. Theoretically, a relatively small number of these HLA-homozygous hiPSC lines would cover the majority of the population.

Here, we summarize the shortcomings and outcomes of various cartilage regeneration strategies and describe various attempts to treat cartilage defects. Moreover, this review discusses stem cell-based engineering to repair cartilage, focusing on hiPSCs. Finally, the future use of hiPSCs for cartilage regeneration is considered.

2. Articular Cartilage

Articular cartilage is an elastic connective tissue that covers the ends of bones in diarthrodial joints. It is generated by and composed of chondrocytes. During development, skeletal tissues (including cartilage) are derived from the mesoderm germ layer. Mesenchymal tissues derived from the mesoderm differentiate into chondrocytes. Chondrocytes produce ECM proteins that are rich in proteoglycans. The accumulated ECM proteins lubricate the surface, meaning it can transmit loads without friction [27]. Articular cartilage has a complex composition with various cellular and ECM networks. The characteristics of chondrocytes, the only cell type in articular cartilage, differ according to their location. Chondrocytes located at the surface of articular cartilage produce lubricin, a protein specific to the superficial zone that lubricates the surface [28]. Chondrocytes in the middle zone synthesize a large amount of aggrecan [29]. In deeper regions, most chondrocytes are in a resting state and synthesize proteoglycans. Most synthesized proteins are non-collagenous; therefore, the turnover rate of type II collagen is relatively low. This protein has a half-life of 117 years, unless it is damaged [30].

There are three main types of cartilage: elastic cartilage, fibrocartilage, and hyaline cartilage. Articular cartilage in knee joints is mostly composed of hyaline cartilage. The smoothness and flexibility of hyaline cartilage are intermediate between those of elastic cartilage and fibrocartilage. After a lesion is generated in hyaline cartilage, scar-like tissue (fibrocartilage) forms. It is almost impossible to repair a hyaline cartilage defect by regenerating hyaline cartilage. Moreover, articular cartilage is avascular, alymphatic, and aneural. The lack of blood vessels limits its regeneration ability. The blockade of blood vessels by the dense ECM hampers the delivery of nutrients to damaged cartilage. Chondrocytes receive nutrients by diffusing through the ECM. The low percentage of chondrocytes (1–5%) also hinders the recovery of damaged cartilage [1].

OA, which is related to aging, is the most common form of arthritis and affects millions of people worldwide. Pain is usually caused by the degeneration of articular cartilage in joints [31]. Even the smallest lesion can affect the whole cartilage tissue during the progression of damage. Such cartilage damage is caused by metabolic imbalances [32]. An imbalance between catabolic and anabolic factors leads to cartilage degradation [32]. Pathological changes include cartilage degradation, osteophyte formation, and inflammation. Cartilage degeneration is triggered by sheer stress generated by mechanical forces at the joint surface. This stimulates the proliferation of quiescent chondrocytes and increases their production of ECM proteins and ECM-degrading enzymes [33]. A disintegrin and metalloproteinase with thrombospondin motifs, collagenases, and matrix metalloproteinases degrade collagen II and proteoglycans in the cartilage ECM [2, 34]. This cascade of events degrades hyaline cartilage, which is eventually replaced by fibroblast-like cells. Consequently, hyaline cartilage is replaced by fibrocartilage, leading to stiffness and additional pain. Various strategies have been developed to treat the damaged cartilage and are discussed in this review (Figure 1). A major challenge is to prevent cartilage damage and to regenerate hyaline cartilage.

3. Current Repair Approaches for Cartilage Regeneration

3.1. Microfracture. Microfracture surgery creates small fractures in the underlying bone. These fractures induce a healing response in damaged articular cartilage by releasing BMSCs [35]. Bone marrow "clots" can firmly adhere to the rough surface of the fractured bone [36, 37]. This promotes the healing of articular cartilage with fibrous tissues or hyaline-like cartilage. Numerous studies report favorable results of microfracture [36, 38–43]. In the late 90s, Bae et al. detected

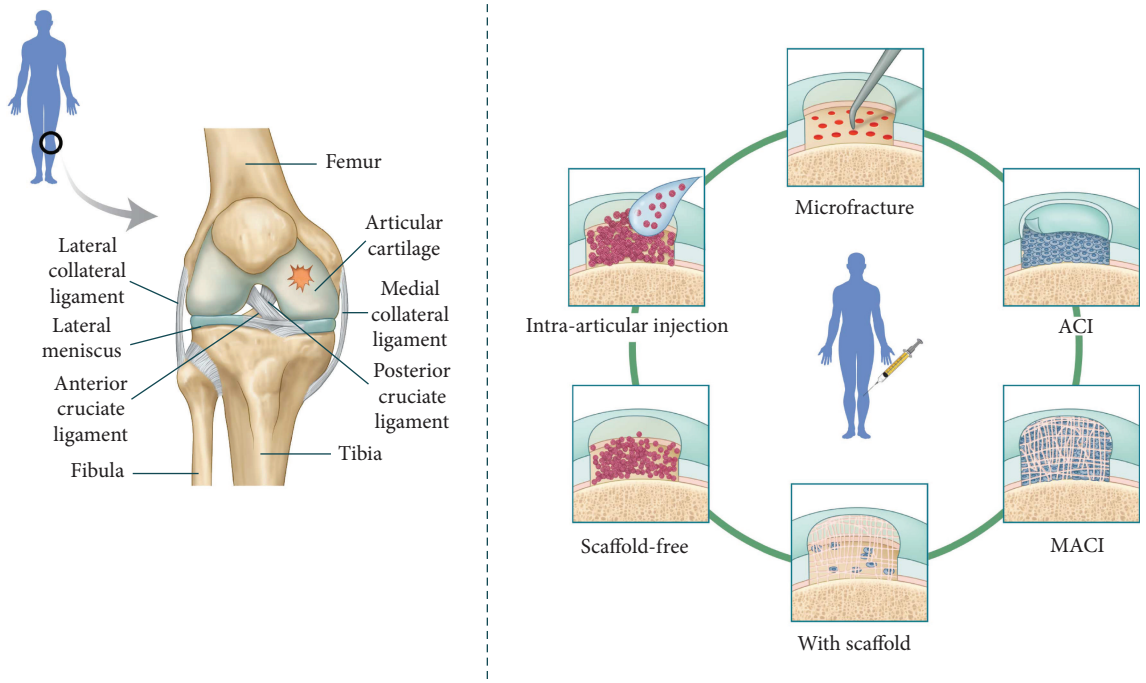

Figure 1: Techniques to regenerate cartilage. Microfracture involves penetrating the osteochondral bone at a depth of 3-4 mm, with each hole separated by 3-4 mm. MSCs migrate from bone marrow to the cartilage defect. ACI involves injecting a patient with their own chondrocytes. MACI involves placing 3D scaffolds, such as those composed of hyaluronic acid or collagen types I and III, into cartilage defects together with autologous chondrocytes. Biocompatible scaffolds have also been developed. There are also scaffold-free techniques that use chondrospheres or self-assembling processes. Smaller chondrospheres are expected to improve therapeutic access via intra-articular injection.

type II collagen at the fracture site in 46 patients with moderate OA at 1 year after surgery [44]. However, studies reported mixed results in the short-, medium-, and long term [45–47]. The quantity and quality of the patient's BMSCs are thought to influence the effectiveness of this approach. Moreover, postoperative rehabilitation is thought to be as important as the surgery itself. Tissue formed following microfracture begins to mature at 8 weeks after surgery [48]. Miller et al. reported that patients who received continuous passive motion (CPM) therapy demonstrated better recovery after microfracture. They concluded that CPM therapy should be performed for 8 weeks after this procedure [49]. Based on this study, CPM for 6–8 hours per day is recommended for patients who have undergone microfracture surgery [50]. However, random cell differentiation induced by microfracture often leads to the formation of fibrocartilage, which is biomechanically inferior to hyaline-like cartilage [44, 51]. Without the mechanical rigidity of hyaline cartilage, the regenerated tissue may deteriorate after 18–24 months and osteophytes may develop due to penetration of the subchondral bone in 25–50% of cases [4, 52]. Moreover, microfracture is less effective for the restoration of large lesions (>3 cm^2) [53]. Despite these shortcomings, the Food and Drug Administration (FDA) and many clinicians believe that microfracture is a good option for cartilage recovery [54, 55].

3.2. ACI and Matrix-Induced ACI. ACI is one of the most promising procedures for long-term cartilage regeneration [53, 56–60]. This method involves obtaining cartilage from the low-weight-bearing part of the joint via a punch biopsy. The isolated cartilage is enzymatically digested to isolate chondrocytes. These chondrocyte are expanded in vitro, transplanted into cartilage defects, and sealed with periosteal flap membranes. Unlike microfracture, ACI is effective for the treatment of large cartilage defects (>3 cm^2). ACI has yielded favorable clinical and functional results in long-term studies lasting more than 10 years [61–63]. In addition, because this process uses the patient's own cells, potential immune complications are avoided [64, 65]. Matrix-induced autologous chondrocyte implantation (MACI) is an improved version of ACI. Unlike ACI, MACI involves the culture of autologous chondrocytes on type I or type III collagen membranes prior to implantation [66, 67]. This avoids the need to close the defect with watertight sutures [66]. It also helps to maintain the characteristics of articular chondrocytes during long-term cultivation and prevents leakage of chondrocytes inside the joint [68, 69]. However, ACI and MACI both involve two invasive procedures, namely, harvesting chondrocytes and transplanting them back into the patient. Hypertrophy along the flap is also a problem [53]. Alternative membranes, such as porcine membranes composed of a mixture of collagen and hyaluronic acid scaffolds, have been used; however, they can increase the immune response [6, 70, 71]. The effectiveness of these procedures is also limited by the low number of chondrocytes in the harvested cartilage. Indeed, chondrocytes constitute less than 5% of cartilage tissue [60]. Consequently, these cells must be expanded in vitro. However, chondrocytes cultured as a monolayer readily dedifferentiate. Chondrocytes lose

their chondrogenic characteristics when grown in a monolayer and start to express fibroblast markers such as collagen type I. Therefore, tissue regenerated using such autologous chondrocytes may be fibrocartilaginous [26, 72].

4. Approaches to Improve Chondrogenesis

4.1. Scaffolds. Chondrogenesis is thought to require a three-dimensional (3D) environment. During development, chondrogenesis is a complicated process regulated by various growth factors and mechanical factors. A scaffold is commonly used to facilitate in vitro chondrogenesis for tissue engineering [73, 74]. Articular chondrocytes and mesenchymal stem cells (MSCs) are the most commonly used cells in cartilage tissue engineering [75–77]. The structural, mechanical, and biochemical properties of scaffolds can improve cell survival and differentiation. The type of scaffold (i.e., natural or synthetic) is also important [78–81]. Natural biodegradable polymers include polysaccharides, polynucleotides, and proteins, whereas synthetic biodegradable polymers include poly-lactic acid, poly-glycolic acid, and poly-lactic-co-glycolic acid (PLGA) [82]. Scaffolds should ideally be absorptive or biodegradable and support cartilage formation. When creating a scaffold, efforts should be made to ensure it facilitates cell migration. In addition, the pore architecture, elasticity, surface energy parameters, molecular mobility, chemical functionality, pH, and degradation of the scaffold should be considered, as well as any inflammatory responses it may elicit [83]. 3D scaffolds are preferable to two-dimensional (2D) scaffolds for cartilage regeneration. Indeed, 3D structures support cell aggregation, mimic the in vivo environment, and improve cell communication and ECM production [84, 85]. However, scaffolds also have disadvantages. Chondrocyte dedifferentiation, cell death, and cell leakage have been reported in scaffold-based chondrogenesis. Moreover, an inappropriate cell distribution, poor differentiation, and inadequate integration with host tissues are common problems associated with cell transplantation using scaffolds [78, 86, 87]. For example, PLGA scaffolds have been proposed to structurally support cartilage formation [88]. Although these scaffolds yielded promising results for the treatment of full thickness cartilage defects with BMSCs in vivo, their therapeutic efficacy is limited due to the hydrophobicity of PLGA. Efforts are being made to improve cell attachment, function, and differentiation, as well as the scaffold itself [89–91].

4.2. Scaffold-Free 3D Culture: Pellet and Micromass Culture. Chondrogenesis is less efficient in a 2D monolayer than in a 3D culture system [92]. Dedifferentiation of chondrocytes in conventional monolayer culture is a major issue for cartilage engineering. In such a system, chondrocytes lose their original characteristics, acquire a fibroblastic morphology, and secrete collagen type I, rather than collagen type II or aggrecan [93, 94]. However, these changes are reversed when dedifferentiated chondrocytes become confluent [95]. Before the emergence of scaffolds, scaffold-free 3D culture systems were generally used for chondrogenesis. These systems mimic precartilage condensation in the developing limb bud and allow cells to interact as they do during cartilage development [96–99]. Cell density significantly influences chondrogenic differentiation [100]. Pellet culture and micromass culture remain the most widely used method for cell-based therapy and cartilage research. Pellet culture of growth plate chondrocytes was first used as an in vitro model of cartilage mineralization [101, 102]. Subsequent studies used this system to study the effects of growth factors on chondrocyte phenotypes, properties of ECM proteins, and the bioenergetics of chondrocytes [103–106]. Early studies of chondrogenesis and hyaline cartilage engineering showed that pellet culture supports in vitro chondrogenesis using MSCs or chondrocytes in the presence of growth factors. Cells differentiated in pellet culture are similar to native articular cartilage in terms of their distribution, density, and matrix composition, without cell phenotypical changes or the assistance of a scaffold [93, 97]. Micromass culture was first performed with chicken limb bud MSCs and was recently used for chondrogenesis. Several studies claim that chondrogenesis is more efficient in micromass culture than in pellet culture. Collagen types I and X are upregulated in larger pellets of chondrogenic cells. Cells trapped in the central region are often undifferentiated and necrotic [107–109]. Although micromass culture supports efficient chondrogenesis, other studies suggest that pellet culture is more suitable for clinical use because of the limited number of chondrocytes generated via micromass culture and their tendency to dedifferentiate [110]. A higher number of cells are required to generate a cartilage-like construct without an artificial scaffold or matrix [111, 112]. Pellet culture and micromass culture are popular methods for in vitro chondrogenesis using various cell types.

5. Adult Stem Cell-Based Chondrogenesis

5.1. BMSCs. MSCs can be easily collected from various tissues; however, they are most commonly isolated from bone marrow in humans (Figure 2) [113–115]. Bone marrow stromal cells were first proposed to differentiate into mesenchymal cells, including adipocytes and osteoblasts, in the late 80s via a process called mesengenesis, and these cells were consequently named "BMSCs." This process has been studied using various in vitro assays. Moreover, BMSCs have been used to treat several diseases [116–121]. In post-traumatic OA models, injection of autologous BMSCs improves the regeneration of joint cartilage exhibiting articular degeneration and osteophyte formation. While autologous chondrocytes are terminally differentiated, BMSCs can differentiate into various cell types (e.g., fibroblasts and chondrocytes) within the joint [122]. The injected BMSCs might also elicit immunomodulatory effects. However, the multipotency of BMSCs is useful for tissue engineering and cartilage regeneration. After several trials with monolayer cultures, aggregates of BMSCs cultured in defined medium were demonstrated to undergo chondrogenic differentiation [25, 97, 123–125]. One important advantage of BMSCs over autologous chondrocytes is that they are more easily expanded in vitro. In vitro chondrogenic differentiation of BMSCs has been widely studied. Treatment with fibroblast

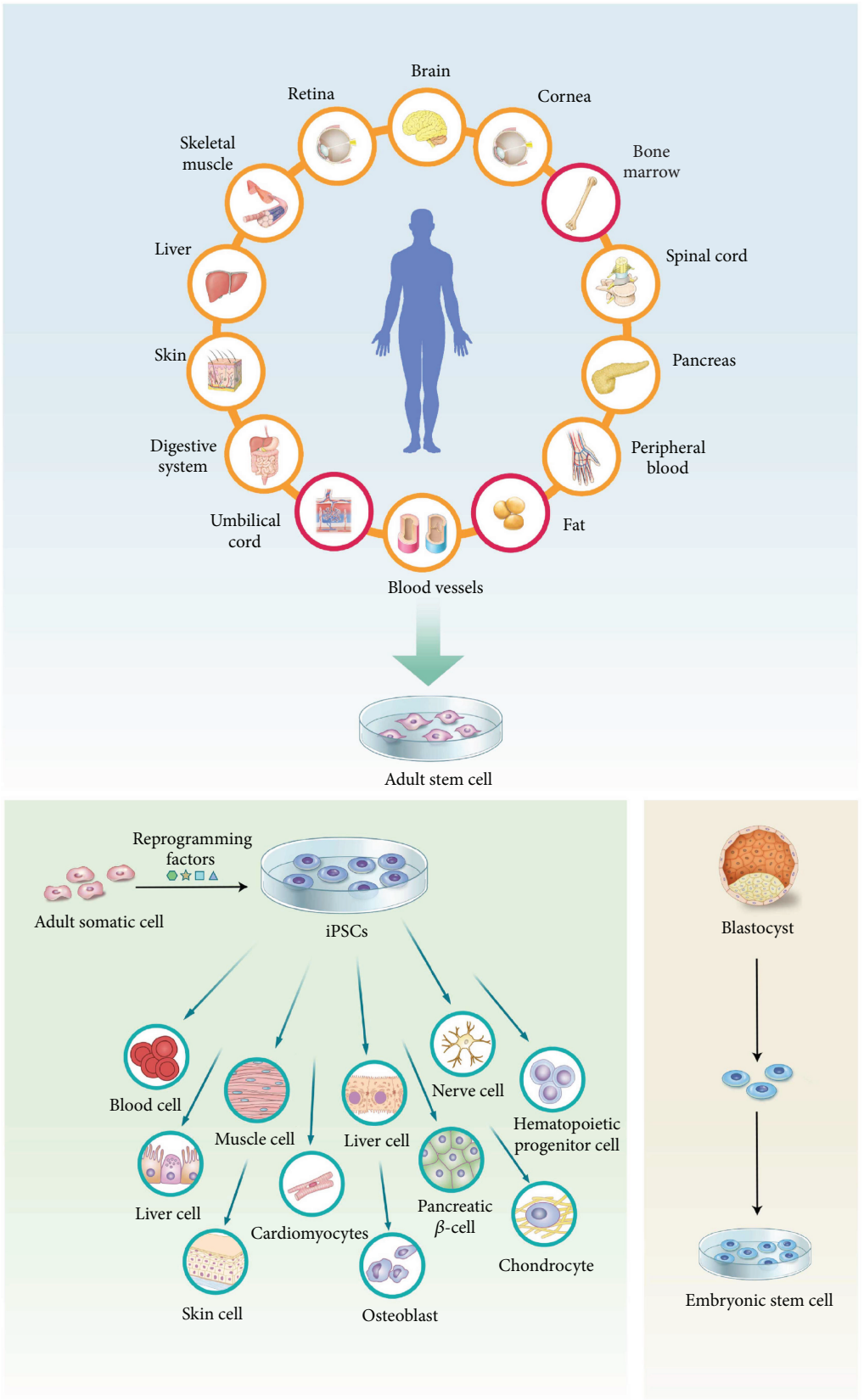

Figure 2: Somatic stem cells (adult stem cells) support healing in the body, for example, replace cells and repair defects. Adult stem cells used in culture are usually obtained from fat, umbilical cord, or bone marrow. ESCs isolated from early embryos can differentiate into various cell types. iPSCs can be artificially generated by reprogramming a patient's own cells and can also differentiate into several lineages.

growth factor 2 (FGF2) enhances the proliferation and chondrogenic potential of these cells [126, 127]. FGF2-treated BMSCs demonstrate enhanced expansion (increase of 3500-fold versus nontreated BMSCs), increased accumulation of proteoglycans, and downregulation of collagen type I expression. However, BMSCs also have several disadvantages. Patients can experience pain during bone marrow harvesting, and a small volume of bone marrow is obtained, yielding a low number of BMSCs [128]. Only ~1500–3000 fibroblast-forming colonies are obtained from 1 mL of human bone marrow, and it has been suggested that bone marrow biopsies larger than 2 mL are significantly contaminated by peripheral blood [129]. Ex vivo expansion is required to obtain a sufficient number of BMSCs for clinical use, especially in elderly patients and those expected to have few BMSCs [130]. Similar to chondrocytes, most adult stem cells (e.g., BMSCs) exhibit decreased proliferation and a reduced differentiation potential after 4–6 passages [20, 131]. Dexheimer et al. reported that faster proliferation of BMSCs correlates with the formation of larger pellets, fewer apoptotic cells, and higher expression of proteoglycans and collagen type II [132]. Despite the advantages of BMSCs, the slow proliferation rate of cultivated BMSCs and the small number of cells obtained from bone marrow must be resolved.

5.2. ASCs. ASCs are obtained by isolating the stromal vascular fraction (SVF) of fat tissue. This is the cell pellet produced when a lipoaspirate, the waste product of liposuction surgery, is digested with enzymes such as collagenase [133]. After serial passaging, adherent cells are harvested as ASCs. Both ASCs and SVFs have a therapeutic potential. The less invasive harvesting procedure and higher yield of ASCs and SVFs have led to these cells being suggested as alternatives to BMSCs. A total of 1×10^7–5×10^8 ASCs are routinely obtained from 300 mL of lipoaspirate, and their viability is >90% [134–137]. This yield is higher than that from bone marrow aspirates, and ASCs are also reportedly easier to culture, proliferate faster, and can be cultivated for longer before becoming senescent [134, 135, 138–140]. Jo et al. investigated the therapeutic effects of intra-articular injection of ASCs for cartilage regeneration in an early phase clinical trial [141]. While ASCs improved cartilage regeneration, numerous studies reported that ASCs can differentiate into cartilage [142–144]. It has been suggested that ASCs have a greater chondrogenic potential than chondrocytes. Indeed, ASCs can maintain their chondrogenic potential for more than 15 passages, longer than chondrocytes [145–147]. However, another study reported that ASCs regenerate cartilage less efficiently than BMSCs [148]. Diekman et al. confirmed that BMSCs expressed a higher level of COL2A1 than ASCs and synthesized more ECM when cultured in alginate beads and a scaffold [149]. Winter et al. showed that ASCs are less sensitive to a chondroinductive environment and that their differentiation is less complete than that of BMSCs after 2 weeks of culture [148]. Chondrogenesis of BMSCs was better than that of ASCs in a 3D system. However, gene expression of aggrecan was higher in ASCs than in BMSCs in the presence of BMP6, while expression of chondrogenic markers was higher in BMSCs than in ASCs in the presence of TGFβ. Hamid et al. suggested that ASCs should be differentiated prior to passage 4 [150]. Moreover, in that study, expression of chondrogenic markers was highly upregulated at week 1, but decreased at weeks 2 and 3. On the other hand, gene expression of collagen type X was highly unregulated at week 3, indicative of hypertrophy. Chondrogenic induction was only prominent after 1 week of differentiation, even when ASCs were used before passage 4.

5.3. Cartilage Regeneration Using Adult Stem Cells. In the surgically induced cartilage damaged animal model, intra-articular injection of labeled BMSCs promoted cartilage tissue regeneration compared to the control group. This result was possible despite the relatively low detection of the labeled BMSCs at the cartilage regeneration site [151]. In addition, when injected with BMSCs in porcine models, cartilage regeneration effect was shown as well [152].

Black et al.'s study assessed the clinical effects of locally derived MSCs in placebo-controlled trials and showed that the range of motion was significantly improved after a single injection of intra-articular adipose-derived MSCs [153].

In mono-iodoacetate-induced rat models, the use of intra-articular BMSCs allowed the animals to distribute significantly greater weight through the affected limb. Despite this functional enhancement, no statistical significant difference appeared between the treatment and the control groups. Also, cartilage and subchondral bone pathology and synovial inflammation were observed in groups treated with BMSC injections [154, 155]. Phases I and II trials using ASCs in the treatment of osteoarthritis (OA) showed MRI evidence of cartilage regrowth [141]. Histological evaluation of collagen type II revealed that hyaline cartilage was regenerated after the injection of 100 million MSCs into a single joint, followed by 6 months of follow-up.

Based on the observed positive preclinical results of using MSCs with arthroscopic techniques, Saw et al. published a randomized controlled trial that included the use of peripheral blood MSCs with arthroscopic microfracture/microdrilling of chondral lesions [156]. As a result, histological analysis and MRI evaluation showed that the quality of cartilage restoration was significantly improved in participants who received MSCs. Another study reported a randomized clinical trial evaluating the efficacy of MSCs after arthroscopic partial medical meniscectomy [157]. The study showed improved clinical outcome compared to the control group and also showed evidences of regeneration of the meniscus volume. Even though preclinical studies have shown inconsistent results, the benefits of intra-articular injection of MSCs or ASCs for improved therapy were proven through various studies.

6. iPSCs for Chondrogenesis

6.1. iPSCs. hiPSCs were first generated in 2006 by transducing mouse fibroblasts with four Yamanaka factors (Klf4, Oct3/4, c-Myc, and Sox2). In 2007, hiPSCs were successfully produced by introducing KLF4, OCT3/4, SOX2, and c-MYC or SOX2, OCT3/4, NANOG, and LIN28 into human somatic cells [158, 159]. hiPSCs have similar characteristics as human

ESCs. However, while hiPSCs can proliferate indefinitely and self-renew, they are not associated with the major ethical issues that complicate the use of ESCs. Therefore, hiPSCs were recently highlighted as an alternative cell source for regenerative medicine [14, 160].

Initially, hiPSCs were routinely generated from skin dermal fibroblasts. However, invasive surgery is required to obtain such cells. Other somatic cells currently used for reprogramming, such as blood cells, urine cells, and keratinocytes, are easier to obtain [161–166]. Cord blood cells, dental pulp stem cells, joint synoviocytes, and adult stem cells (e.g., ASCs and MSCs) have also been successfully reprogrammed [160, 167, 168]. While earlier protocols used lentiviral or retroviral transduction to facilitate integration of Yamanaka factors, hiPSCs should be produced via nonintegrating methods for clinical use. Sendai virus, episomal vectors, small molecules, proteins, and modified RNAs are commonly employed to avoid integration [169–171]. hiPSCs are now widely used for regenerative medicine, drug screening, and even "disease-in-a-dish" modeling.

A pancreas is produced by injecting rat iPSCs into mouse blastocysts that lack a pancreas due to deletion of the Pdx1 gene [172]. Theoretically, human organs can be generated by injecting hiPSCs into pig blastocysts; however, this is complicated by technical issues [26, 173]. Moreover, the blood vessels of iPSC-derived organs are formed by the host animal [26]. This is not a problem for the generation of cartilage because this tissue is avascular [26]. Reprogrammed iPSCs exhibit pluripotency when transplanted into immunodeficient mice, and they generate teratomas containing tissues of all three germ layers (endoderm, mesoderm, and ectoderm). Cartilage-like tissue is found in these teratomas, demonstrating that hiPSCs can undergo chondrogenesis. Therefore, hiPSCs are a promising cell source for chondrogenic tissue engineering.

6.2. Cartilage Regeneration Using hiPSCs. The ultimate goal of regenerative medicine with iPSCs is not only to produce cells of interest but also to create new tissues or organs [26, 172, 173]. Teratomas generated from hiPSCs contain hyaline cartilage. This indicates that hiPSCs can differentiate into human cartilage or chondrocytes [26]. Before the emergence of hiPSCs, attempts were made to induce chondrogenesis of human ESCs via three methods: coculture with articular chondrocytes, induction of MSC-like cells, and direct conversion [26, 174–182].

Protocols used to induce chondrogenic differentiation of hiPSCs were mostly derived from these methods (Table 1). The most widely used approach induces MSC-like cells from hiPSCs (Figure 3). There are several protocols to obtain these progenitor cells, which can be roughly classified into two types: (1) monolayer culture and (2) embryoid body (EB) formation. The latter is more commonly used because it involves mesodermal induction via a defined process. In an early study, Medvedev et al. attempted to induce chondrogenesis of hiPSCs derived from fetal neural stem cells isolated from human embryos [183]. Chondrocytes generated from these hiPSCs exhibited characteristics of chondrocytes. EBs were generated from these hiPSCs, and the media was replaced by chondrogenic differentiation medium. Expression of ECM proteins such as collagen type II and the early chondrogenic marker Sox9 was subsequently detected. In 2012, Zhu et al. also induced chondrogenic differentiation of EBs. Koyama et al. suggested a more defined protocol for in vitro chondrogenesis of hiPSCs in 2012 [184]. While EBs are usually used for differentiation into the three germ layer lineages, they suggested a new protocol for differentiation into the mesenchymal progenitor cell lineage. EBs were generated and grown on gelatin-coated plates for 1 week, after which mesenchymal-like cells or "outgrowth cells" sprouted from these EBs. The outgrowth cells had similar characteristics as MSCs, such as expression of CD44, CD90, CD73, and CD105. The authors reported that 1-2×10^4 cells/cm^2 was the ideal density for proliferation. Outgrowth cells were dissociated, cell clumps were removed with a strainer, and chondrogenic differentiation was induced. Chondrogenic pellets generated from hiPSC-derived mesenchymal progenitor cells had a cartilage morphology and contained lacuna. Both hiPSCs and human ESCs were successfully differentiated toward the chondrogenic lineage using this protocol. Our group also confirmed the successful chondrogenic differentiation of cord blood-derived hiPSCs via this method [14, 185]. In 2012, Diekman et al. suggested a different approach for chondrogenesis [186]. They isolated cells that expressed collagen type II tagged with green fluorescent protein (GFP) and induced chondrogenesis. Similar to the study by Koyama et al., chondrogenic differentiation was not directly induced. hiPSCs were first predifferentiated into the chondrogenic lineage by micromass culture, and then these cells were dissociated and aggregated by pellet culture. Pellets of GFP+ cells were larger and produced more glycosaminoglycan ECM proteins than those of GFP− cells. The authors concluded that this protocol enhances the chondrogenic properties of engineered tissue and could be a way to eliminate undifferentiated cells, which may prove helpful for transplantation of hiPSCs in the future. However, protocols using EBs are time-consuming and are thought to give rise to a heterogeneous population due to variations in cell number and EB size [175]. Several researchers attempted to differentiate hiPSCs directly without EBs via micromass or pellet culture using specific medium, a coated matrix, or feeder layers [187–189]. However, these methods negatively affect related signaling pathways. Therefore, a fast, inexpensive, and efficient protocol is required for future applications.

6.3. Cartilage Regeneration Using hiPSCs in Animal Models of OA. The healing ability of chondrogenic cells derived from hiPSCs was recently investigated in several animal models. In 2014, Ko et al. induced chondrogenic differentiation via EB culture and using alginate beads [16]. Cells in EBs were dissociated and transferred to chondrogenic differentiation medium for micromass culture. The generated chondrogenic pellets or alginate hydrogels were implanted into osteochondral defects created on the patellar groove of immunodeficient rats. Twelve weeks later, the defects were filled with smooth and firm tissue, while the control group had a rough surface with or without fibrous tissue. Histological analysis also demonstrated the restoration of proteoglycans in the

Table 1: Studies that attempted to induce chondrogenic differentiation of iPSCs.

Year	Reference	MSC-like cell induction method	Chondrogenesis method	Primary cells used for reprogramming	Growth factors	Animal experiments
2011	Medvedev et al. [183]	—	Direct media change of EBs	Human fetal neural stem cells	TGFβ3, BMP2	—
2012	Koyama et al. [184]	EB formation, outgrowth cell induction	Pellet culture	Human dermal fibroblasts	TGFβ3	—
2012	Diekman et al. [186]	Micromass culture for predifferentiation	Pellet culture	Mouse fibroblasts	TGFβ3	—
2013	Guzzo et al. [216]	Monolayer culture on gelatin-coated plates	Micromass culture, pellet culture	Human dermal fibroblasts	BMP2	—
2014	Guzzo et al. [216]	—	Micromass culture	Human dermal fibroblasts, human articular chondrocytes, human cord blood cells	BMP2	—
2014	Ko et al. [16]	EB culture	Micromass culture	Human dermal fibroblasts	TGFβ3	hiPSC-derived chondrocytes implanted into osteochondral defects of immunosuppressed rats as pellets or in alginate hydrogels
2015	Nejadnik et al. [131]	Monolayer culture	Pellet culture	Human adipose-derived stem cells, human dermal fibroblasts	TGFβ3	hiPSC-derived chondrocytes expressed human vimentin and integrated with host articular cartilage
2015	Yamashita et al. [191]	Monolayer culture	Monolayer culture to induce cartilaginous nodules	Human dermal fibroblasts		Chondrogenic nodules implanted into defected regions of SCID rats and mini-pigs
2016	Zhu et al. [190]	—	Direct media change of EBs and transfer to gelatin-coated plates for chondrocyte induction	Human dermal fibroblasts	TGFβ1	Single hiPSC-derived chondrocytes directly injected into the knee joints of a MIA-induced rat model of OA
2017	Nam et al. [14, 185]	EB formation, outgrowth cell induction	Pellet culture	Human cord blood mononuclear cells	TGFβ3, BMP2	—

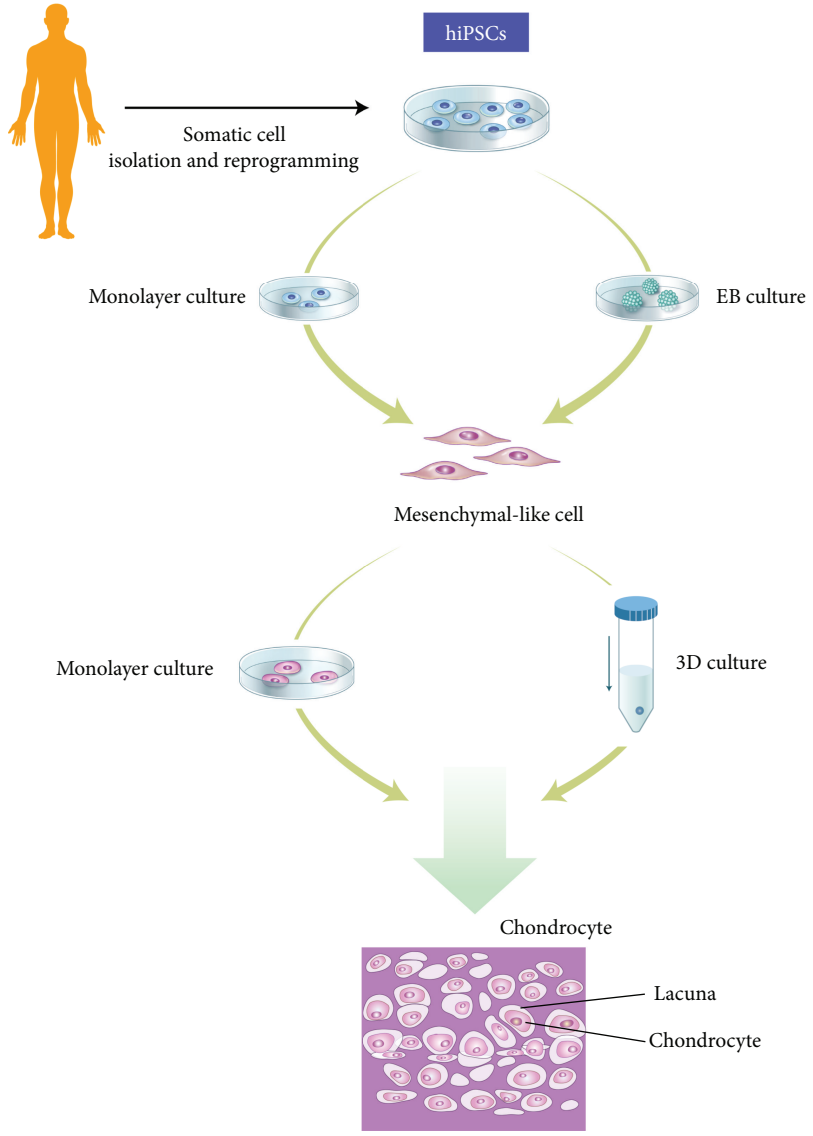

Figure 3: A simple scheme of the various methods used to differentiate iPSCs into chondrocytes.

defected areas. However, the authors reported that matrix formation was inadequate due to the implantation of allogenic iPSCs, despite the persistence of implanted hiPSCs and the use of immunodeficient rats.

In 2015, Nejadnik et al. generated chondrogenic pellets of ASC-derived hiPSCs [131]. They induced differentiation into the mesenchymal lineage via monolayer culture because they felt that EB culture gave inconsistent results. To confirm the quality of the iPSC-derived mesenchymal cells, hiPSC-derived MSCs and hiPSC-derived chondrogenic cells were differentiated for 21 days and seeded into a polyethylene glycol and chondroitin sulfate methacrylate-based scaffold. Pellets were implanted into an osteochondral defect generated in the distal femur of nude rats and evaluated via serial imaging for 6 weeks. Magnetic resonance imaging showed that the implants did not form teratomas and also detected a decreased water content and increased ECM formation, indicative of successful engraftment. Moreover, the cells expanded in vivo and the scaffold was eventually degraded. Histology confirmed the engraftment of both hiPSC-derived MSCs and chondrogenic pellets in the defect, as demonstrated by Alcian blue and collagen type II staining. Chondrogenic pellets expressed higher levels of matrix proteins; however, hiPSC-derived MSCs also promoted regeneration.

Chondrocytes derived from hiPSCs were implanted into a nonsurgical monosodium iodoacetate- (MIA-) induced cartilage damaged rat model. Zhu et al. generated chondrogenic pellets via EB culture and induction of outgrowth cells [190]. Rather than pelleting the outgrowth cells, they cultured them in chondrogenic differentiation media. Under these conditions, the sprouting outgrowth cells were thought to be chondrocytes. Thereafter, the authors injected 500 μL of the cell suspension (1×10^6 cells/mL) into the joint at 1 week after induction of damage using MIA. Fifteen weeks later, rat knee joints were imaged by microcomputed tomography and analyzed by histology. hiPSC-derived chondrocytes had a better regeneration capacity than hiPSCs. Histological

analysis demonstrated that the injected hiPSC-derived chondrocytes localized on the cartilage surface and increased the level of proteoglycans. Proliferating chondrocytes were also detected, suggesting that cartilage was being repaired. The authors concluded that joint function was improved; however, rat joints still showed dyskinesia and did not fully repaired, indicating that stem cell injection can improve joint repair, but only in early stage of destruction.

In 2015, Yamashita et al. implanted hiPSC-derived chondrogenic cells into larger animals. Cartilaginous nodules were generated from a monolayer of hiPSCs [191]. These nodules eventually separated from the bottom of the dish and were transferred to a petri dish and maintained in suspension culture for up to 42 days. The nodules did not form tumors when subcutaneously implanted into severe combined immunodeficiency (SCID) mice. Cartilaginous particles maintained for 28 days were implanted into osteochondral defects of SCID rats. Repair of the defects was confirmed at 4 weeks after transplantation, with high expression of collagen type II. Expression of ECM proteins was higher in nodules maintained for 42 days than in those maintained for 28 days. To determine if human cells migrated to other organs or lymph nodes, the authors investigated expression of human β-actin. This was not detected in any other organ, and pluripotency markers were not expressed in nodules differentiated for 21 days or longer. Finally, the nodules were implanted into cartilage defects of mini-pigs weighing 27.0–30.5 kg and treated with the immunosuppressant cyclosporine. The nodules were viable for 1 month after transplantation. Chondrogenic nodules expressed human vimentin and integrated with host articular cartilage. The authors concluded that the nodules can repair cartilage defects even under heavy-weight-bearing conditions.

Taken together, these results demonstrate the potential of hiPSC-derived cartilaginous particles for articular cartilage regeneration. Early phase animal trials are being conducted, the results of which will help to translate this procedure into future clinical applications.

7. Conclusions and Future Perspectives

Cartilage damage causes joint destruction, pain, physical disability, and morbidity. However, the avascular nature and low mitotic activity of cartilage limit its intrinsic regeneration capacity. Although biological agents may slow cartilage degradation, the optimal treatment to promote cartilage repair has not been defined.

Cell-based therapies are emerging as a means to regenerate cartilage. One of the points to consider is the stability of the cells before use. Rubio and colleagues questioned the safety of locally derived MSCs through a controversial study in 2005 [192]. When BMSCs were implanted in immunodeficient mice, spontaneous stem cell mutation and malignant tumors appeared. Later, the study was withdrawn after evidence showed that the malignant traits were associated with contamination of cell lines, but not with MSCs [192]. In a similar situation, further studies on long-term cultured BMSCs (evidence of malignant transformation) were withdrawn on the same grounds [193, 194]. Importantly, current clinical trials have shown that MSC therapy is safe. Safety has been demonstrated through a recent systematic review and meta-analysis of a total of 1012 participants who received intravascular MSC therapy for a variety of clinical symptoms, including ischemic stroke, Crohn's disease, cardiomyopathy, and ischemic heart disease [155, 195]. In the case of hiPSCs, the risk of teratoma formation is the biggest problem that cannot be overlooked. Therefore, efforts (i.e., complete differentiation or purification) to avoid this risk are necessary.

Autologous chondrocytes and adult stem cells (i.e., BMSCs and ASCs) are generally used for cartilage regeneration; however, the low numbers of these cells limit their clinical applications. By contrast, hiPSCs can proliferate indefinitely and support chondrogenesis in vitro and in vivo. Using a defined quality control process and a chondrogenic differentiation protocol, hiPSCs can become the ideal cell source for cartilage engineering.

hiPSCs have several advantages. These cells can be theoretically generated from every individual; however, this is not economically viable. It is expensive to prepare hiPSCs from a patient under good manufacturing practice (GMP) guidelines [196]. Consequently, the concept of a HLA-homozygous hiPSC bank has emerged [197]. It is estimated that 100 HLA-homozygous hiPSC lines from each race would cover the majority of the population [198]. Cartilage is considered to be an immunoprivileged tissue due to its avascular and alymphatic nature and the dense ECM that surrounds chondrocytes [26]. The use of HLA-matched hiPSCs may minimize immune rejection during allogeneic transplantation for cartilage engineering (Figure 4). HLA-A, HLA-B, and HLA-DR are closely related to rejection [199, 200]. Many researchers are currently collecting cells homozygous for these three HLA types; however, further research is required to improve allogeneic transplantation of neocartilage.

Many cartilage transplantation procedures currently involve surgery. Further damage to the knee joint might exacerbate the immune reaction and hamper recovery. Therefore, the development of an accessible and noninvasive treatment might be ideal for cartilage recovery. Various treatments involve a single injection of cells (e.g., Cartistem). Intra-articular injection of BMSCs and ASCs has been investigated as a means to treat OA [201–208]. Zhu et al. demonstrated that hiPSC-derived chondrocytes may also be clinically applicable by noninvasive procedures. However, chondrocytes demonstrate better viability and function in 3D conditions. Moreover, the properties of cells are better in spheroids than in 2D systems [209]. Specifically, cells in spheroids have a higher viability and readily polarize [210]. Furthermore, the use of scaffold-free spheroids avoids the biocompatibility issues associated with the implantation of scaffolds. This approach can improve tissue regeneration in clinical settings. Spheroids can spontaneously fuse with each other and thereby increase in size [209]. Researchers currently pellet $1–5 \times 10^5$ cells in the presence of growth factors [98, 110, 211–214]. Babur et al. termed such aggregates "macropellets" and reported that large spheroids (1-2 mm) are characterized by redifferentiation, with varying amounts

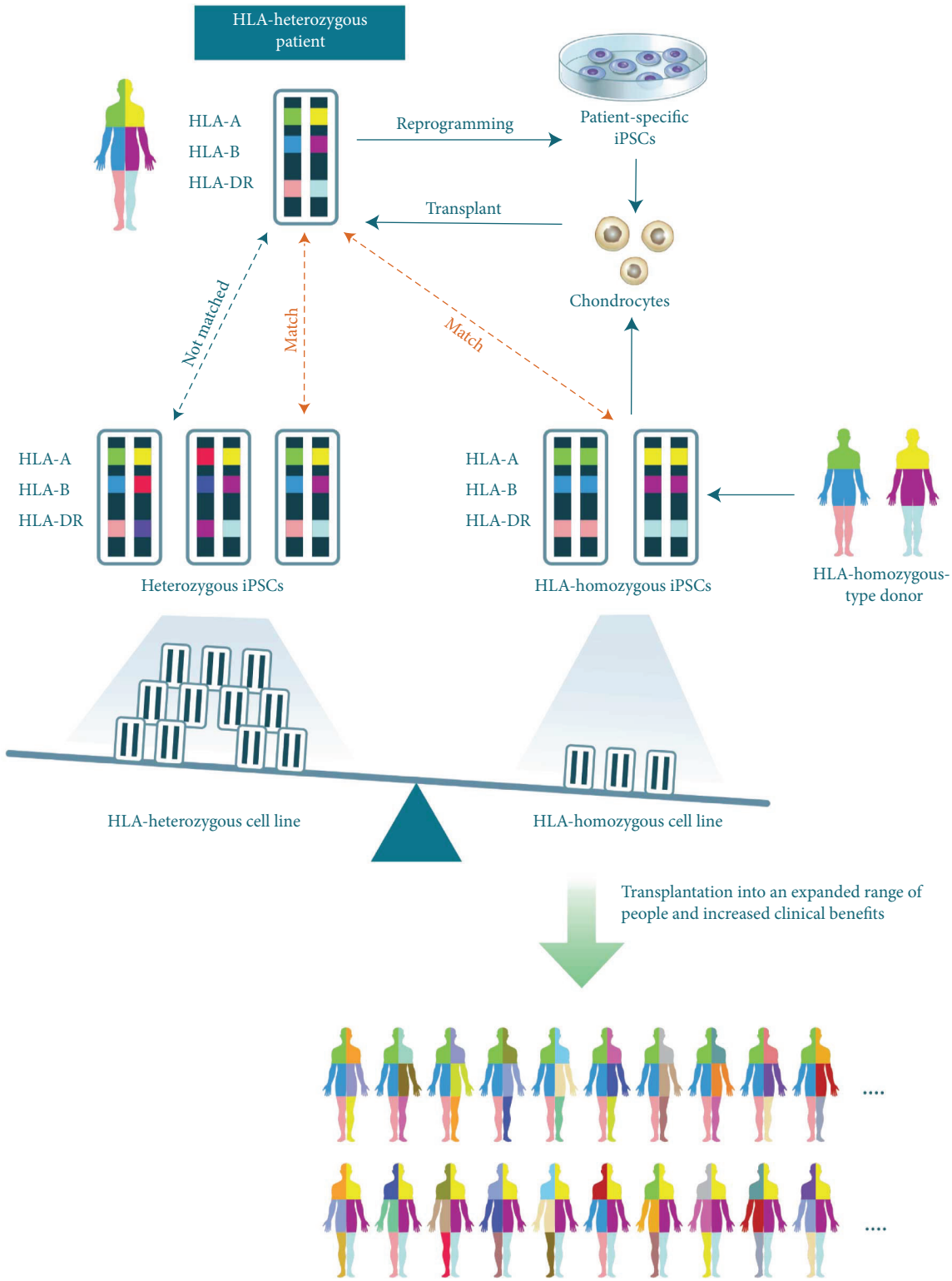

Figure 4: The clinical transplantation of hiPSCs homozygous for HLA-A, HLA-B, and HLA-DR, the three types closely related to immune rejection. Theoretically, a relatively small number of homozygous stem cell lines would cover the majority of the population.

of ECM deposited throughout the pellet [215]. Using a microwell technique, they generated "micropellets" measuring $193 \pm 20\,\mu$m. These small pellets produced higher levels of ECM proteins, which is thought to be related to the increased contact of cells with the surrounding environment. Taken together, we believe that intra-articular injection of a minimized chondrogenic pellet or spheroid is ideal to regenerate damaged cartilage (Figure 5).

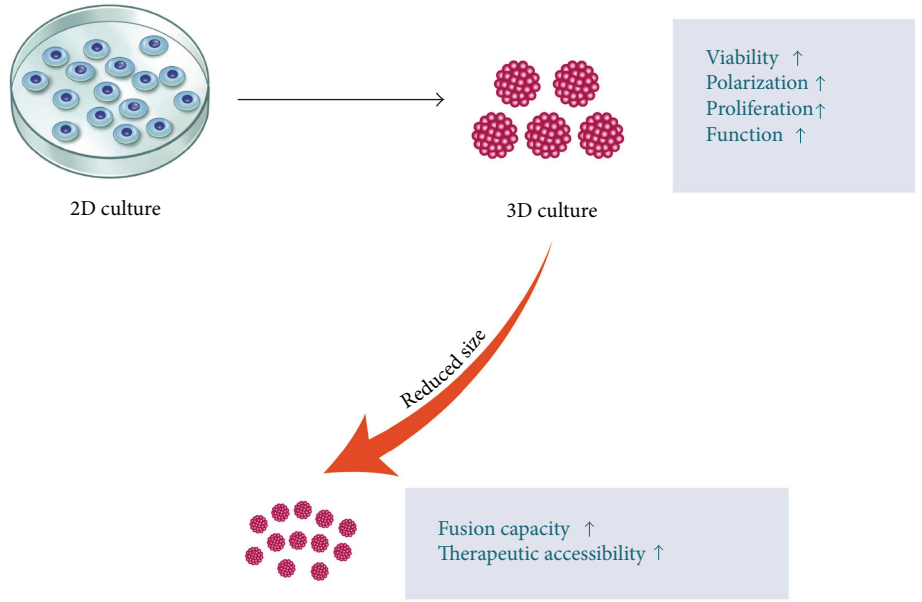

Figure 5: A 3D culture method for tissue engineering. Cells cultured in a 3D system have considerably improved biological properties and a higher regeneration potential than cells cultured in a 2D system. Sophisticated techniques for mass production of spheroids are also being developed. "Micropellet" 3D culture may also improve therapeutic accessibility by reducing the size of the product.

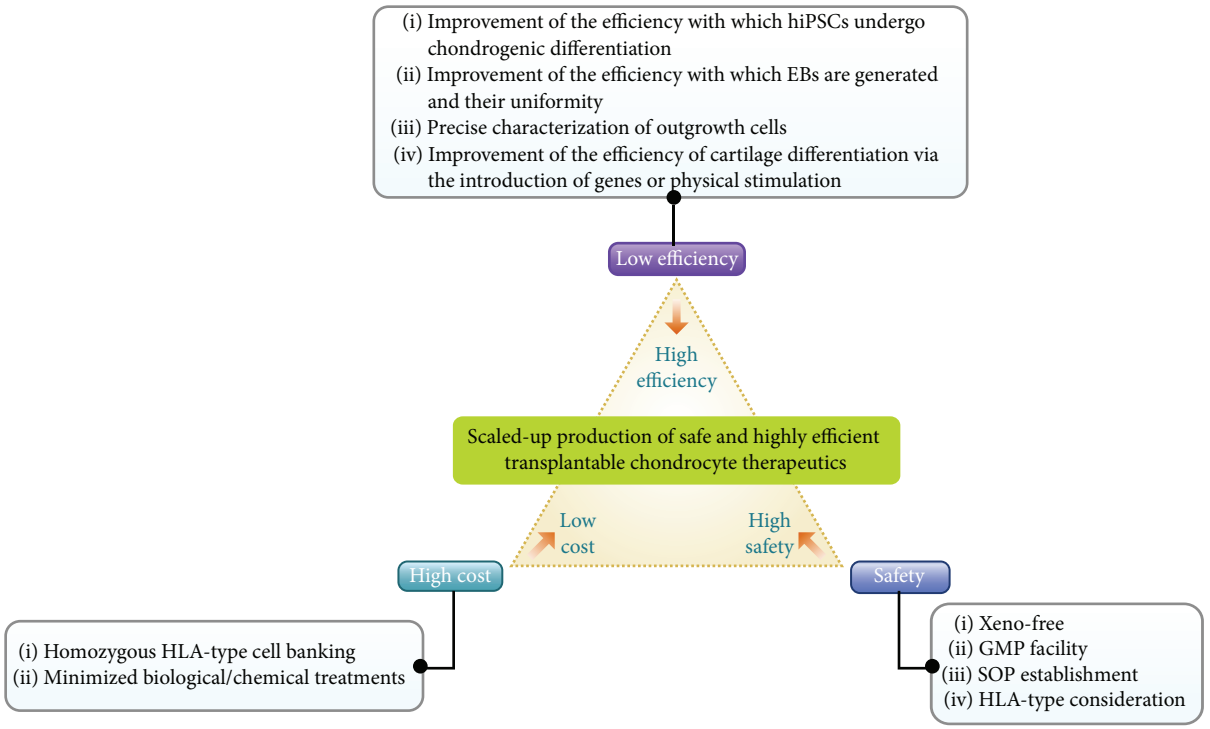

Figure 6: Commercialization strategy to develop safer, more efficient, and less expensive therapeutic agents for cartilage repair using iPSCs.

The potential of hiPSCs to regenerate cartilage has not been investigated in a preclinical or clinical study [216]. However, the use of hiPSCs in cartilage research may promote their applications in other fields, including tissue engineering, drug screening, and modeling of various diseases related to cartilage or even bone. With more defined protocols (e.g., uniform EB generation, defined production of outgrowth cells, and enhanced chondrogenic differentiation), it may be possible to generate spheroids of hiPSCs that readily undergo chondrogenic differentiation (Figure 6). Furthermore, the use of xeno-free components, GMP practices, and other quality control methods, such as the removal of tumorigenic cells, is required for clinical use. These process may allow the production of animal component-free cells

with low tumorigenecity from hiPSCs. The high cost of tissue engineering can also be reduced by using homozygous-HLA hiPSCs which requires minimal biological and chemical treatments. In summary, hiPSCs may open up a new era in cartilage regeneration.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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Research Article

Regeneration of Tracheal Tissue in Partial Defects Using Porcine Small Intestinal Submucosa

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Background. Surgical correction of tracheal defects is a complex procedure when the gold standard treatment with primary end-to-end anastomosis is not possible. An alternative treatment may be the use of porcine small intestinal submucosa (SIS). It has been used as graft material for bioengineering applications and to promote tissue regeneration. The aim of this study was to evaluate whether SIS grafts improved tracheal tissue regeneration in a rabbit model of experimental tracheostomy. *Methods*. Sixteen rabbits were randomized into two groups. Animals in the control group underwent only surgical tracheostomy, while animals in the SIS group underwent surgical tracheostomy with an SIS graft covering the defect. We examined tissues at the site of tracheostomy 60 days after surgery using histological analysis with hematoxylin and eosin (H&E) staining and analyzed the perimeter and area of the defect with Image-Pro® PLUS 4.5 (Media Cybernetics). *Results*. The average perimeter and area of the defects were smaller by 15.3% ($p = 0.034$) and 21.8% ($p = 0.151$), respectively, in the SIS group than in the control group. Histological analysis revealed immature cartilage, pseudostratified ciliated epithelium, and connective tissue in 54.5% ($p = 0.018$) of the SIS group, while no cartilaginous regeneration was observed in the control group. *Conclusions*. Although tracheal SIS engraftment could not prevent stenosis in a rabbit model of tracheal injury, it produced some remarkable changes, efficiently facilitating neovascularization, reepithelialization, and neoformation of immature cartilage.

1. Introduction

The trachea is composed of highly specialized tissues, which confer rigid support, longitudinal coating, and a functional epithelial covering [1, 2]. As a result of this complex structure, the treatment of tracheal defects and effective regeneration following injury are difficult [3, 4].

Tracheal defects are caused by various acquired and congenital abnormalities, including trauma, tuberculosis, cancer, and idiopathic causes [5]. Corrective surgery for

tracheal lesions remains a challenging procedure due to various potential complications, such as the formation of fistulae and tissue necrosis [6].

Treatment options for tracheal reconstruction depend on the defect size [7, 8]. It is known that the trachea, in cases of injury, can have its length reduced by half in adults and by about one third in young children [9]. The gold standard treatment is end-to-end primary anastomosis [10–12]. However, when defects exceed these limits, alternative treatments must be considered.

New technologies based on tissue engineering approaches have been proposed to assist regeneration of the trachea. Although diverse autologous and heterologous biomaterials have been used to repair tracheal lesions, most are neither effective nor reliable, especially for the long-term management of tracheal defects [13–17]. Porcine small intestinal submucosa (SIS) has been widely used in many areas of medicine as a graft material, including bladder and urethra reconstruction, heart valve replacement, diaphragmatic defect, and abdominal wall repair, amongst others, all of them with encouraging results [17–20]. Several studies have demonstrated SIS's ability to promote reepithelialization and complete infiltration of mesenchymal cells with new vascular growth [21–24].

Considering the difficulties encountered in the tracheal reconstruction process and the capacity of SIS to promote tissue regeneration, we carried out this study to evaluate whether SIS grafts could improve tissue regeneration of the trachea in a rabbit model of experimental tracheostomy.

2. Material and Methods

This is an experimental, interventional, and randomized study with 16 New Zealand white rabbits. Animals were randomly divided into two groups: the control group, which underwent only tracheostomy ($n = 8$), and the SIS group ($n = 8$), which underwent tracheostomy followed by SIS graft implantation at the tracheal defect site. The experiments were performed according to Law 6.638, May 8, 1979—Standards for the Practice, Teaching and Scientific Practice of Animal Vivisection. This project was presented to CEUA (Committee of Ethics in Research in Animal Use of the PUCPR) and approved under article number 640 (Annex 1).

2.1. Preparation of Porcine Small Intestinal Submucosa (SIS) Grafts. The SIS was obtained from a slaughterhouse. A segment of jejunum 20 cm from the duodenal-jejunal flexure was removed from recently sacrificed, healthy pigs. Subsequently, the mesentery was removed from the jejunal segment. The intestinal segment was inverted by exposing the mucosa, which was removed by scraping with a bistoury. After reinversion of the tissue, the seromuscular layer was removed by the same procedure, leaving only the jejunal submucosa (Figure 1). The SIS was washed in 0.9% isotonic saline solution (JP/Equiplex) and stored in 10% neomycin sulfate solution. Decontamination was performed with 8% chlorine dioxide saline solution (Veromax 80®, Veros Chemical) at a dilution of 0.04% using a shaker (109M Bureau, New Ethics) for 24 hours [20]. The ultimate preparation was that of an acellular, decontaminated, porcine small intestinal submucosa composed of structural proteins, like collagen and elastin; glycoproteins (fibronectin and laminin); glycosaminoglycans and proteoglycans (hyaluronan, heparan sulfate, heparin, and dermatan sulfate); and matricellular proteins (thrombospondins, osteopontin, and tenascins).

Figure 1: Mucosa of the inverted intestinal segment isolated.

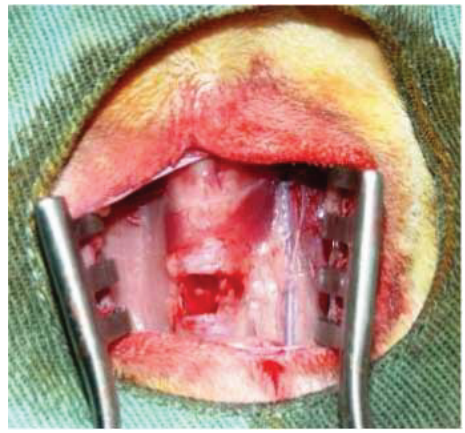

Figure 2: Trachea exposition with a 6×8 mm ($48\ mm^2$) defect made with a scalpel.

2.2. Tracheostomy and Tracheal Reconstruction. The study included sixteen New Zealand male rabbits, with an average weight of 6 kg (±0.5 kg) and was conducted in the laboratory of experimental surgical technique of PUCPR.

The animals were anesthetized with xylazine (10 mg/kg), ketamine (20 mg/kg), acepromazine maleate (0.05 mg/kg), and propofol (5 mg/kg), and they were treated with the prophylactic antibiotic (gentamicin sulfate 5 mg/kg) intravenously. The necks of the rabbits were shaved and disinfected with 10% povidone-iodine and 70% ethanol in preparation for surgical tracheostomy. Local anesthesia was conducted with 1 mL of lidocaine hydrochloride (2%) immediately before the skin incision. A vertical incision was made at the neck, and the strap muscles were divided along the midline. After fully exposing the trachea, a 6×8 mm ($48\ mm^2$) tracheal defect was excised with a scalpel (Figure 2). In the SIS group, the defect was covered with a rectangular SIS graft supported at the four vertices and continuously sutured with polypropylene 4.0 (Figures 3 and 4). The strap muscles were replaced and reinforced over the graft, and the skin was sutured. In the animals of the control group, the tracheal defect was kept open and left to heal by secondary intention. The strap muscles were replaced and reinforced over the defect, and the skin was sutured.

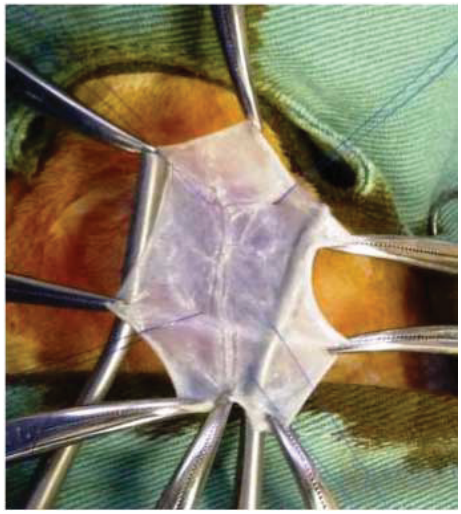

Figure 3: SIS group: implantation of the graft into the tracheal defect.

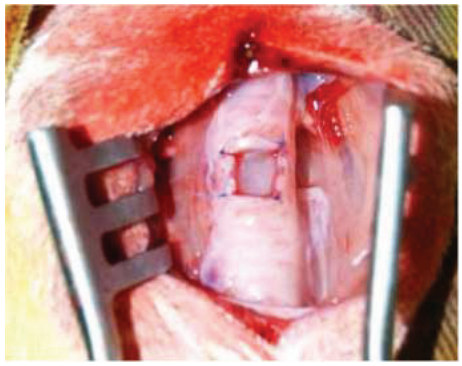

Figure 4: SIS group: defect covered by a rectangular graft supported by four points in the vertices with polypropylene 4.0.

Following surgery, rabbits were administered carprofen (2.2 mg/kg) for postoperative analgesia for three days.

Sixty days after undergoing surgical tracheostomy, rabbits were sedated and euthanized by intravenous administration of an overdose of pentobarbital (100 mg/kg).

The trachea of each animal was dissected from two centimeters above the main carina and removed for histopathological analysis (Figure 5). The tracheas were fixed with 10% formaldehyde for 72 hours. Dehydration of the samples was performed by successive baths of alcohol (concentrations of 70%, 80%, and 90%) and three baths of 100% ethanol for 1 hour. Following dehydration, the samples were embedded in liquid paraffin using two baths at 65°C in the same equipment. After cooling, histological sections were taken by means of a microtome (Leica model RM 2145, Solms, Germany).

2.3. Analysis of Perimeter and Area of the Tracheal Defects. Tracheal sections were macroscopically photographed to analyze the morphology of the tracheal lumen after surgery. The perimeters and areas of tracheal lumens in the defect

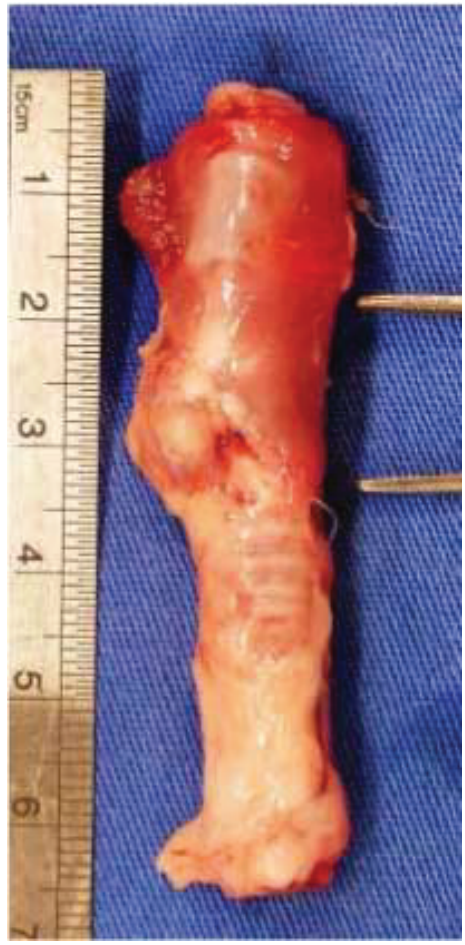

Figure 5: Trachea removed two centimeters above the main carina.

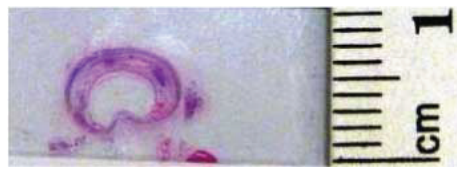

Figure 6: Photograph of the macroscopic section of the tracheal defect to analyze the morphology of the tracheal lumen.

regions of all sections were measured using Image-Pro PLUS 4.5 (Media Cybernetics) (Figure 6). In order to avoid differential operator bias, the same individual measured the variables three times, and the average values were taken for analysis. All photographs were taken with the same parameters, and a numerical scale was added to evaluate the defect dimensions in millimeters and square millimeters.

The results of the measurements of circumference and area of the defects were calculated as mean, median, minimum, maximum, and standard deviation values. Student's *t*-test for independent samples was used to compare between groups. Qualitative variables were compared using the Fisher exact test. p values < 0.05 were considered statistically significant. Data were analyzed with IBM SPSS Statistics V20 software.

Table 1: Tracheal defect perimeter: comparison between groups.

Group	n	Mean, mm	Median, mm	Minimum, mm	Maximum, mm	Standard deviation	p value
Control	8	1.703	1.697	1.342	2.005	0.229	
SIS	8	1.443	1.405	1.147	1.827	0.215	0.034

Student's t-test for independent samples, $p < 0.05$.

Table 2: Tracheal defect area: comparison between groups.

Group	n	Mean, mm^2	Median, mm^2	Minimum, mm^2	Maximum, mm^2	Standard deviation	p value
Control	8	0.151	0.151	0.090	0.197	0.039	
SIS	8	0.118	0.109	0.060	0.211	0.047	0.151

Student's t-test for independent samples, $p < 0.05$.

2.4. Histological Analysis. Histological sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy for identification of inflammatory reaction, fibrosis, neovascularization, and the presence of tissue regeneration characterized by evidence of reepithelialization and formation of new islands of cartilage.

Tissue regeneration was classified as present or absent. Other variables were also classified as present or absent. However, the present groups have also been divided into discreet, moderate, or severe presence for future analysis. The Fisher exact test was used to compare qualitative variables between the experimental groups. p values < 0.05 were considered statistically significant.

3. Results

3.1. Defect Perimeter and Area. Following a recovery period of 60 days, the average perimeter and area of the tracheal defects in control and SIS group animals were measured and compared (Tables 1 and 2; Figures 7 and 8). The average perimeter of the tracheal defect in the SIS group was 15.3% smaller than that of the control group, and the difference was statistically significant ($p = 0.035$). Similarly, the average area of the tracheal defect in the SIS group was 21.8% smaller than that in the control group, but this decrease was not statistically significant ($p = 0.151$).

3.2. Histological Analysis. The presence of inflammatory tissue, fibrosis, neovascularization, and tissue regeneration in the tracheal defect of control and SIS group animals was compared.

Although inflammatory cells were observed at the site of the graft in 54.5% of the SIS group animals as against 12.5% of the control group animals, the difference was not statistically significant ($p = 0.147$) (Figure 9). Necrosis was not observed.

There were also no statistically significant differences in fibrosis or neovascularization, although they were preferentially observed in the SIS group [9.09% against 0.0% ($p = 1.000$) and 27.27% against 12.5% ($p = 0.603$), resp.] (Figures 10 and 11). The SIS group presented evidence for the growth of new vessels in 3 of 8 animals.

A statistically significant difference in the presence of regenerated tissue was observed between the groups

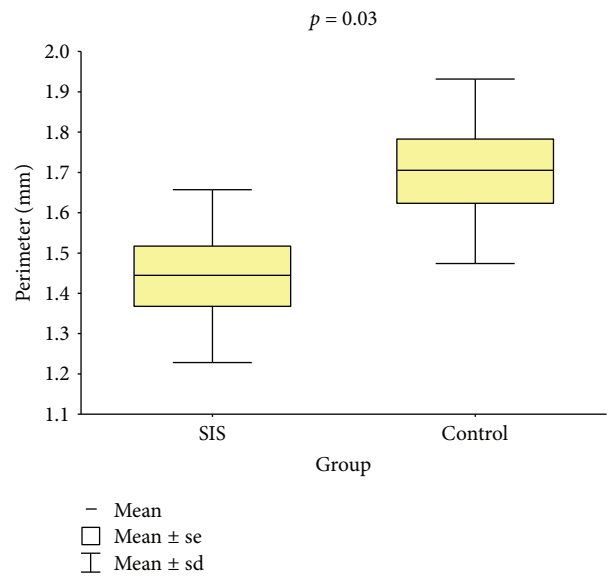

Figure 7: Tracheal defect perimeters of the groups.

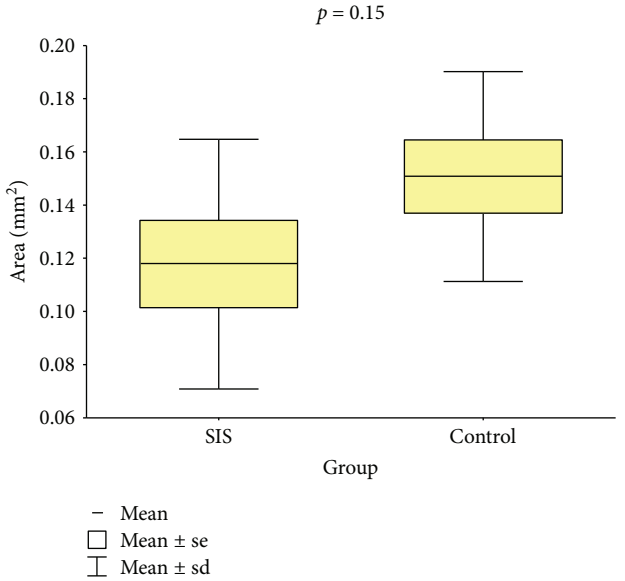

Figure 8: Tracheal defect areas of the groups.

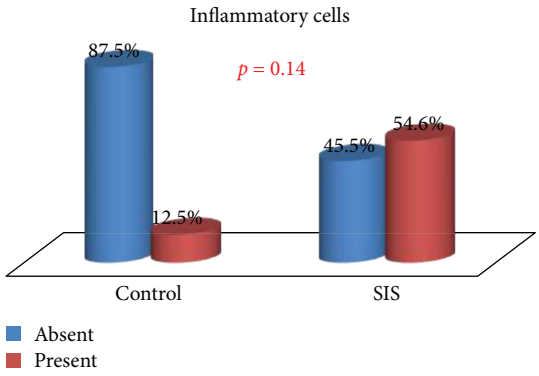

Figure 9: Inflammatory cells: group comparison.

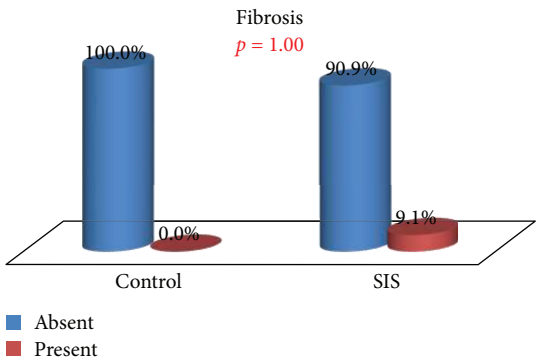

Figure 10: Fibrosis: group comparison.

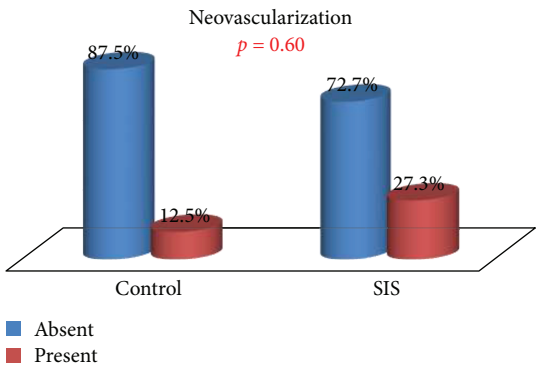

Figure 11: Neovascularization: group comparison.

($p = 0.018$) (Figure 12). In the control group, there was no detectable tissue regeneration in any of the samples, while in the SIS group, reepithelialization and newly formed cartilage were observed in 54.5% of the cases (Figures 13–17).

4. Discussion

Although most tracheal lesions can be treated with resection and primary anastomosis, defects that do not allow such treatment remain a challenge in medical practice [8, 9, 17, 20, 25]. During the last few decades, efforts in tracheal reconstruction have been aimed at treating a variety of malignant and benign diseases [7, 26] such as

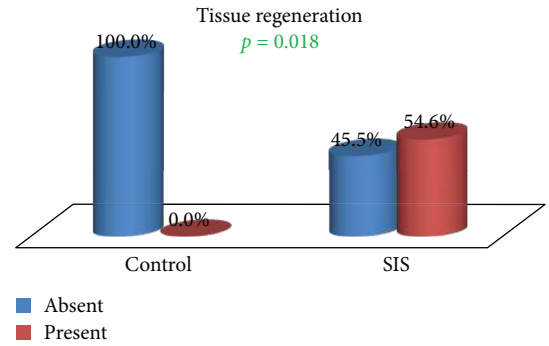

Figure 12: Tissue regeneration: group comparison.

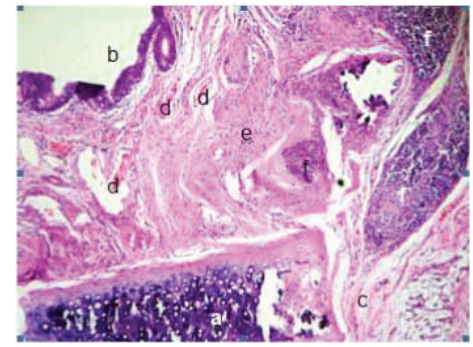

Figure 13: Control group without cartilaginous tissue regeneration (newly formed cartilage) at the site of the tracheal defect, H&E (100x): (a) mature cartilaginous tissue, (b) pseudostratified ciliated epithelium, (c) connective tissue, (d) blood vessels, (e) fibrosis, and (f) acute and chronic inflammatory tissue.

tumors, trauma, infections, birth defects, and the most common injuries caused by tracheostomy and tracheal intubation [10, 14, 17]. Biological membranes are considered the best option to perform these reconstructions as they provide a scaffold for reepithelialization of the defect and proliferation of cartilage tissue through regeneration factors, which promote the cellular matrix growth of the host tissue [1, 17, 20, 24, 27].

The ideal material for replacing the tracheal wall must be airtight, hard, longitudinally flexible, coated with epithelial tissue, and highly vascularized to prevent infection and to allow healing [28]. There are many materials available for tissue replacement, for example, Dacron®, polyurethane, costal and ear cartilage flaps, and allograft aorta [2, 5, 17, 20]. However, they present many complications, such as infection, extrusion, obstruction, stenosis, and chronic graft rejection [12, 17, 20, 29]. Alternative possibilities, discussed by Grillo [30], are the use of synthetic structures such as stents, which have two distinct disadvantages: Correction of the lesion is permanently prevented, and severe complications may develop from the stent. Removable silicone stents also hinder curative treatment and may induce granulation, especially in the subglottic region. However, granulation is sometimes reversible in contrast to problems caused by permanent stents, which include stenosis of the trachea.

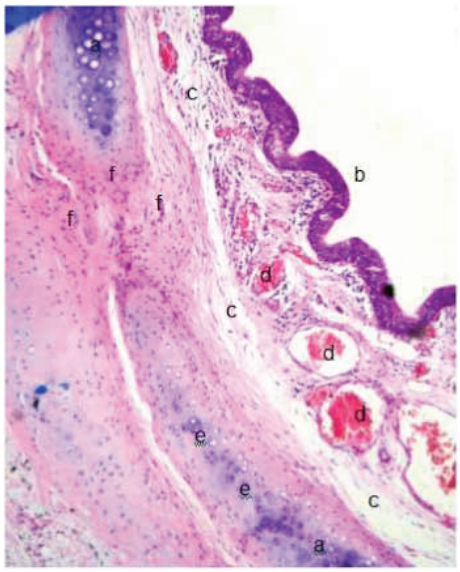

Figure 14: Tissue regeneration in the SIS group with reepithelialization, neovascularization, and newly formed cartilage, H&E (40x): (a) mature cartilaginous tissue, (b) pseudostratified ciliated epithelium, (c) connective tissue, (d) blood vessels, (e) immature cartilaginous tissue (newly formed cartilage), and (f) fibrosis.

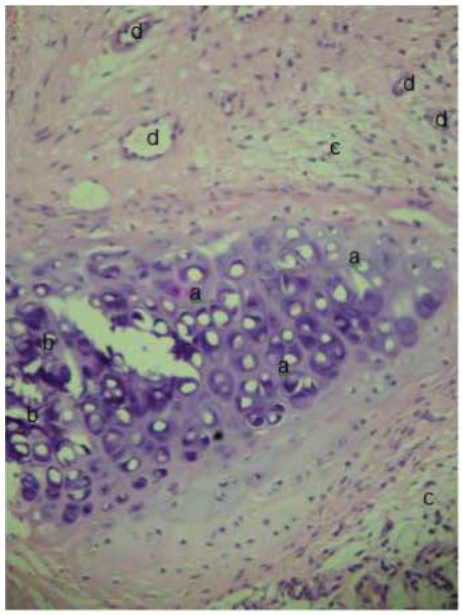

Figure 15: SIS group with immature cartilaginous tissue at the site of the tracheal defect, H&E (400x): (a) immature cartilaginous tissue (newly formed cartilage), (b) mature cartilaginous tissue, (c) connective tissue, and (d) blood vessels.

This study aimed at testing the use of SIS for repairing tracheal defects in rabbits by analyzing the dimensions of the tracheal defects after reconstruction and by evaluating the regeneration of the tracheal wall.

In order to perform this study, we created partial tracheal defects of 6×8 mm (48 mm^2) dimensions in our rabbit model. The size of this defect was chosen because a 6 mm resection of a circumferential segment from a rabbit trachea would cause a reduction of approximately 30% of the normal rabbit tracheal transverse section area and a decrease in tracheal lumen of up to 40% may occur without compromising the respiratory dynamics [31]. As the aim of this study was not to evaluate respiratory dynamics, animals were not submitted to respiratory distress. We considered it appropriate to use an approximately 30% tracheal lumen reduction to avoid a stenosis progression.

A surprising result of this study was the shorter average perimeter and smaller average area of defects in the SIS group. This was not expected as there was interposition of a tissue graft between the edges of the tracheal defect, avoiding decrease in tracheal lumen area during reparation. The average perimeter of the tracheal defect in the SIS group decreased by 15.3% compared to that of the control group ($p = 0.035$), and the average area decreased by 21.8% ($p = 0.151$). A decrease in tracheal lumen of up to 40% may be acceptable without compromising respiratory dynamics [31], as seen in our experimental animals, all of which survived the surgical procedure with no signs of obstruction or stenosis of the airway. Although the decrease in average perimeter was small and, in the case of the average area, not statistically significant, it points to the inability of this method in maintaining the tracheal structure. We believe that the decrease might have occurred due to contraction at the healing stage secondary to the inflammation and fibrosis induction caused by surgical injury. It might be possible that the use of a temporary endotracheal support (stent) could prevent this stenosis.

Histological analysis revealed an inflammatory tissue in both groups with no statistically significant difference ($p = 0.147$), but the presence of inflammatory cells at the site of the graft in SIS group without any signs of necrosis confirms biocompatibility, lack of antigenicity, and absence of rejection and is in agreement with the observations of other in vivo studies with SIS [22, 27].

There was also no significant difference when comparing the presence of fibrosis ($p = 1.000$) and neovascularization ($p = 0.603$) between the two groups. However, it was important to verify the occurrence of neovascularization in almost 38% of the animals in the SIS group, a result that is in accordance with the findings of Poulose et al. [19], who previously described the occurrence of neovascularization in the SIS graft matrix used in the vena cava of pigs.

Probably the most important finding in this study is that related to tissue regeneration. There was a statistically significant difference ($p = 0.018$) in favor of the SIS group relating to reepithelialization and formation of new cartilage in more than one half of the cases (54.5%), while there was no tissue regeneration in any of the samples of the control group. The occurrence of this level of tissue interaction supports the utilization of SIS as a graft material to support tissue regeneration [12, 27].

The tissue engineering industry is still seeking the ideal tracheal substitute that provides cell-matrix interaction with receptor cells for the promotion of migration, proliferation, and reepithelization of defects [1].

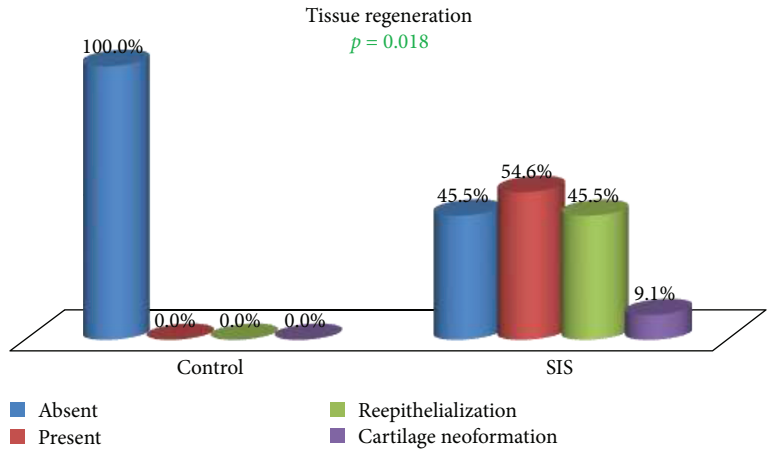

Figure 16: Tissue regeneration: group comparison.

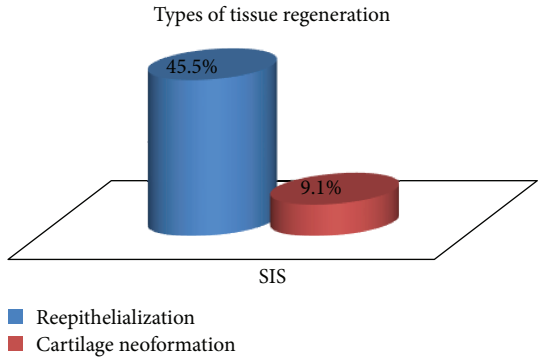

Figure 17: Tissue regeneration type within the SIS group.

Thus, porcine small intestinal submucosa, as a biodegradable tissue, is able to serve as a support for tissue remodeling [10, 15]. It has been widely used in many areas of medicine with satisfactory results in the regeneration of the aorta, vena cava, ligaments, skin, and other tissues [32]. The chemical and mechanical characteristics of SIS, combined with its low antigenicity, clearly make SIS a versatile and efficient option that appears to appropriately replace the tracheal tissue, although in our study it failed to prevent the occurrence of tracheal stenosis.

5. Conclusions

In summary, SIS showed some desirable properties when used as a graft material, partially replacing the tracheal wall in a rabbit model of tracheal injury, such as the capacity to promote tissue regeneration and to lower the risk of some serious postoperative complications like infection and graft extrusion or obstruction. However, it did not prevent the occurrence of tracheal stenosis but was successful in regenerating the tracheal wall by promoting efficient neovascularization, reepithelialization, and formation of new cartilage.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Annex 1: Committee of Ethics in Research in Animal decision. *(Supplementary Materials)*

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Review Article

Translational Application of Microfluidics and Bioprinting for Stem Cell-Based Cartilage Repair

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Cartilage defects can impair the most elementary daily activities and, if not properly treated, can lead to the complete loss of articular function. The limitations of standard treatments for cartilage repair have triggered the development of stem cell-based therapies. In this scenario, the development of efficient cell differentiation protocols and the design of proper biomaterial-based supports to deliver cells to the injury site need to be addressed through basic and applied research to fully exploit the potential of stem cells. Here, we discuss the use of microfluidics and bioprinting approaches for the translation of stem cell-based therapy for cartilage repair in clinics. In particular, we will focus on the optimization of hydrogel-based materials to mimic the articular cartilage triggered by their use as bioinks in 3D bioprinting applications, on the screening of biochemical and biophysical factors through microfluidic devices to enhance stem cell chondrogenesis, and on the use of microfluidic technology to generate implantable constructs with a complex geometry. Finally, we will describe some new bioprinting applications that pave the way to the clinical use of stem cell-based therapies, such as scaffold-free bioprinting and the development of a 3D handheld device for the in situ repair of cartilage defects.

1. Introduction

Cartilage defects, due to trauma or progressive joint degeneration, can impair the most elementary daily activities, such as walking or running. Due to the limited self-repair ability of cartilage, these lesions can easily evolve into osteoarthritis (OA), leading to the complete loss of articular function and to the subsequent need for joint replacement [1]. In the last decades, the limitations of standard surgical treatments for cartilage repair have triggered the development of cell-based therapies. Autologous chondrocyte implantation (ACI) has been the first cell-based approach to treat cartilage defects [2, 3], and more lately, stem cells have been proposed as an alternative cell source for cell-based cartilage repair [4, 5]. Among the various types of adult stem cells, mesenchymal stem cells derived from bone marrow (BMSCs) have been widely used for cartilage applications due to their well-demonstrated chondrogenic potential [6, 7]. Besides BMSCs, more lately, adipose-derived mesenchymal stem cells (ADMSCs) obtained from different adipose depots, including knee infrapatellar fat pad, have gained growing interest as an alternative cell source for cartilage repair [8–10].

In the development of stem cell-based therapies for tissue regeneration, bioprocessing optimization is required to exploit the remarkable potential of stem cells. In particular, efficient cell differentiation protocols and the design of proper biomaterial-based supports to deliver cells to the injury site need to be addressed and overcome through basic and applied research [11]. In this scenario, microfluidic systems have attracted significant interest implementing platforms, in which the control of local environmental conditions, including biochemical and biophysical parameters, is exploited to study and direct stem cell fate [12, 13]. Indeed, microfluidic technology enables the precise control over fluids at the microscale, thus allowing mimicking of the natural cell microenvironment by continuous perfusion culture or by creating chemical gradients [14]. Because of these features, microfluidic devices can be efficiently used to investigate the plethora of factors that guide stem cell differentiation towards a specific cell lineage, testing several conditions with minimal requirements in terms of cell number and amount of reagents to perform large experiments [15]. So far, a suite of microfluidic devices has been developed to investigate the influence of both biochemical and biophysical factors on stem cell differentiation in order to outline new protocols for stem cell chondrogenesis [16–18]. Recently, microfluidic technology has also been used to fabricate advanced systems for 3D bioprinting to produce microchanneled scaffolds for the enhancement of nutrient supply [19] or to encapsulate cells within microspheres or fibers [20–22]. 3D bioprinting is a novel research field that is showing excellent potential for the development of engineered tissues, allowing the fabrication of heterogeneous constructs with biochemical composition, mechanical properties, morphology, and structure comparable to those of native tissues [23, 24]. As reported in several recent reviews [23, 25–28], this technology has the potential to overcome major problems related to the clinical translation of tissue engineering products for cartilage repair, which has been so far limited due to the poor results obtained in terms of construct functionality. Indeed, cartilage properties are determined by its complex architecture characterized by anisotropic orientation of collagen fibers and density gradients of chondrocytes, which even express slightly different phenotypes [29, 30]. 3D bioprinting, due to its ability to control material and cell positioning, appears as a promising approach to replicate the complexity of zonal variability in terms of cell densities and extracellular matrix (ECM) properties [31, 32]. Moreover, this technique offers other advantages, such as the possibility to reproduce subject-specific geometry and topography starting from medical images to create cell-laden constructs fitting to the defect of the specific patient [33].

In this review, we will describe how microfluidics and bioprinting can provide different insights in the field of mesenchymal stem cell-based cartilage repair and contribute to the development of novel therapeutic strategies. Specifically, since microfluidic and bioprinting technologies share the use of hydrogel-based materials, in the first section, we will focus on the optimization of these materials to mimic the composition and the mechanical properties of the articular cartilage. We will then describe the use of microfluidic devices for the identification of biochemical and biophysical factors driving stem cell chondrogenesis that could be implemented during the *in vitro* maturation of bioprinted constructs. In addition, we will describe studies whereby microfluidic and 3D bioprinting technologies have been applied to generate implantable constructs with a complex geometry. Finally, we will describe some new bioprinting applications that pave the way to the clinical use of stem cell-based therapies, such as scaffold-free bioprinting to generate clinically relevant constructs using 3D cell spheroids as building blocks and the development of a 3D biofabrication handheld device for the in situ repair of cartilage defects.

2. Microfluidics and Bioprinting to Trigger the Translation of Cell-Based Therapy

2.1. Advancements in Hydrogel-Based Materials. In several clinical applications, stem cells are directly injected into the target tissue without any biomaterial carrier. This process leads to limited stem cell engraftment at the treatment site, mainly due to leakage of cell suspension during injection [34]. Since the regeneration potential of stem cells is strongly correlated with the number of cells retained at the lesion site, improving stem cell engraftment is of utmost importance [34]. The use of hydrogel carriers has been introduced to overcome this limitation, by promoting cell retention at the desired site and providing the implanted cells with a microenvironment supporting cell viability and functions [34, 35]. In the context of cartilage regeneration, hydrogels have been widely applied, because of their numerous advantages. These highly hydrated polymeric networks that can be either natural or synthetic can be used for cell embedding as well as for incorporating growth factors and ECM components. Furthermore, hydrogels can be easily tailored in different geometries and, if properly designed, can provide the cells with an environment similar to that of native cartilage [36, 37].

Because of their intrinsic features, hydrogels have been applied as a 3D matrix for cell culture in microfluidic devices [38] as well as bioinks for 3D bioprinting. In particular, their increasing use in bioprinting has triggered the efforts in the optimization of hydrogel-based materials, in terms of composition, growth factor enrichment, and mechanical properties. Indeed, despite the promising advantages of bioprinting, one of the major challenges is the absence of a material that can be considered as the ideal bioink that satisfies all the specific requirements, as described in a number of recent reviews [39–42]. Regarding extrusion-based bioprinting process, the bioink should present shear thinning behavior to allow extrusion through the printer nozzle. At the same time, the bioink should be characterized by quick shear recovery to maintain the printed shape, showing adequate mechanical properties to guarantee a proper environment for embedded cells and long-term shape fidelity, manipulation, and ease of handling. Finally, the bioink should be biocompatible allowing long-term culture of bioprinted cells. The bottleneck of this technology is represented by the complexity to combine rheological/mechanical properties and biological properties, which are often mutually exclusive. To overcome this issue,

there are two different approaches: either to improve the biocompatibility of materials characterized by adequate printing properties or to improve the printability of biocompatible materials [43]. For instance, Armstrong et al. [44] have formulated a new Pluronic-alginate multicomponent bioink with BMSCs to generate bone and cartilage structures. In this study, Pluronic was used as a sacrificial template in order to provide structural stability during printing, before chemical crosslinking of alginate took place, as well as to generate micropores and/or anisotropic microchannels in the construct to increase nutrient diffusion after its removal. Similarly, Kesti et al. [45] have improved the printability of a photocrosslinkable methacrylated hyaluronan (HAMA) by adding poly(N-isopropylacrylamide)-grafted hyaluronan (HA-pNIPPAM), a thermoresponsive polymer with good cytocompatibility used as a sacrificial template. In this way, the authors created a bioink in liquid state at room temperature that reticulates at body temperature. After thermal gelation, the biopolymer was stabilized through free radical polymerization of HAMA to achieve a long-term mechanical stability. Finally, the HA-pNIPPAM was eluted through medium washing at 4°C resulting in a glycosaminoglycan-based scaffold. This procedure allowed printing scaffolds with a diameter of 10 mm and a height of 2.8 mm, characterized by high biocompatibility as shown by articular chondrocyte viability.

The problem of the printability of soft biocompatible materials was also addressed by Müller et al. [46] developing a novel alginate sulfate-nanocellulose bioink for cartilage applications. Notwithstanding the innate biocompatibility of hydrogels derived from natural biopolymers such as hyaluronic acid, chitosan, or alginate, the rheological behavior of their solutions is often not suitable for 3D bioprinting. To overcome this problem, Müller and colleagues increased alginate viscosity through the addition of nanocellulose, changing also the behavior from Newtonian-like to shear thinning. The results of this study showed that articular chondrocytes embedded in alginate sulfate-nanocellulose were viable and synthesized type II collagen, proving the suitability of the newly developed biomaterial for cell-based cartilage repair. Composite bioinks combining nanofibrillated cellulose (NFC) with alginate (NFC/A) and hyaluronic acid (NFC/HA) were developed by Nguyen et al. [47]. In particular, in the case of cartilage, the NFC mimics the bulk collagen matrix, alginate simulates proteoglycans, and hyaluronic acid substitutes for the hyaluronic acid found in the native cartilage matrix. Noticeably, both alginate and nanofibrillated cellulose are xeno-free and FDA-compliant materials and hence can be easily translated into clinical use. The authors showed that both composite bioinks are printable; however, low proliferation and phenotypic changes of printed human-derived-induced pluripotent stem cells (iPSCs) were observed in the case of NFC/HA. On the other hand, iPSCs printed in NFC/A produced a relevant amount of hyaline-like cartilaginous tissue-rich and hyaline-like cartilaginous tissue-expressed chondrogenic markers, such as aggrecan. Differently from the aforementioned study [47] where the different constituents of the bioink mix resembled the different components of the articular cartilage, the approach developed by Levato et al. [48] to recapitulate the cartilage composition and architecture is based on the intrinsic ability of primary cells to produce specific ECM. In fact, these authors developed a zonal-like model using two different cell sources, chondrocyte progenitor cells (CPCs) and BMSCs encapsulated in gelatin methacrylamide (GelMa) hydrogels. By combining CPC- and MSC-laden bioinks, a bioprinted model of the articular cartilage was generated, consisting of defined superficial and deep regions, each with distinct cellular and ECM composition. Noticeably, the authors showed that their bioprinting method, which uses Pluronic F-127 as sacrificial ink to support GelMa during the biofabrication process, allows fabricating clinically relevant anatomical structures. However, to match the mechanical properties of the articular cartilage, the bioprinted material should undergo an extensive *in vitro* maturation before implantation or reinforcement of a supporting material. Indeed, hydrogels show low compressive stiffness resulting to becoming unsuitable for application in the fabrication of load-bearing tissues. For this reason, several strategies have been exploited to reinforce hydrogels using stiffer materials [27]. For instance, Daly et al. [49] engineered mechanically reinforced hydrogels by codepositing soft bioinks, such as agarose, alginate, and GelMa, with polycaprolactone (PCL) filaments. In this way, the authors were able to obtain BMSC-laden constructs with bulk compressive modulus similar to the native articular cartilage. Noticeably, this approach allowed at the same time enhancing the printability of soft hydrogels and matching the mechanical properties needed to withstand high mechanical loading within a joint environment, a result that is hardly achievable when using standard hydrogels [23]. Another interesting work that focuses on the reinforcement of bioinks was performed by Kang et al. [50] who developed an integrated tissue-organ printer (ITOP). This device includes a multiple cartridge system that allows the deposition of cell-laden composite hydrogels in combination with PCL polymer and an external sacrificial Pluronic F-127 hydrogel, which reinforces the material properties and supports the structure, respectively, during printing. Through a 3-axial motorized stage system and an air pressure-based controller, the ITOP is able to precisely regulate the dispensing volume of each material enabling the production of constructs with structural integrity and complex geometry. The promising results obtained using this system to generate a human-scale ear-shaped bioprinted construct indicate that this approach would be also suitable to print clinically relevant constructs recapitulating the structures and features of the native articular cartilage.

Despite the aforementioned approaches, the formulation of the optimal bioink for cartilage tissue regeneration is still to be achieved. Furthermore, the intrinsic complexity of the native ECM is often neglected in 3D bioprinting experiments. It is well known that the ECM microenvironment plays a key role in directing the differentiation of stem cell through receptor ligand interactions and mechanotransduction [51]. Hence, to successfully generate an instructive cell niche, the tissue-specific cell-ECM interactions have to be recapitulated. To this purpose, Pati and colleagues [52] have recently proposed the use of a decellularized extracellular

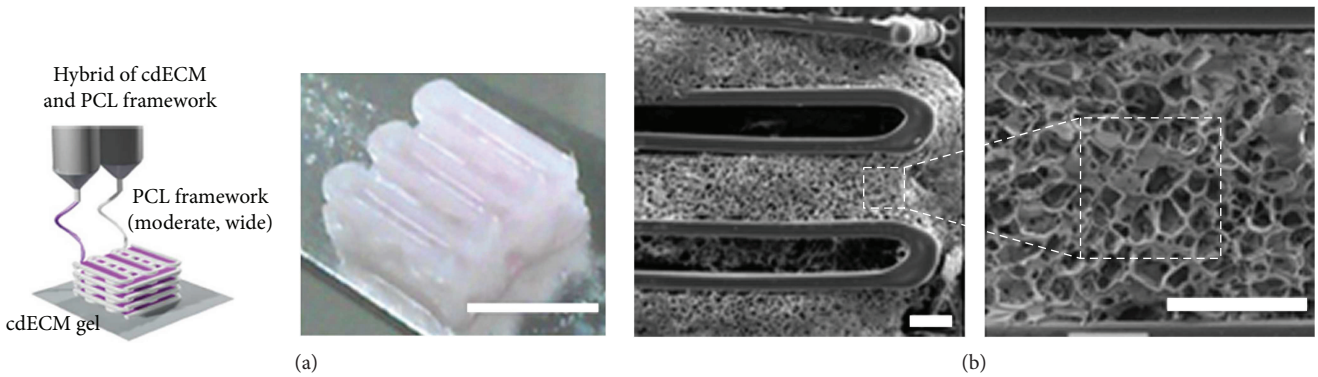

Figure 1: Bioprinting of 3D constructs using a bioink based on a tissue-specific decellularized matrix. (a) Bioprinting process for the obtainment of hybrid structures made of decellularized cartilage ECM (cdECM) and PCL for cartilage repair. Scale bar = 5 mm. (b) SEM images of the bioprinted construct. Scale bars = 400 μm (adapted from [52]).

matrix (dECM) as bioink to maintain the complexity of the native tissue (Figure 1). In particular, the articular cartilage was decellularized, solubilized, combined with inferior turbinate tissue-derived mesenchymal stromal cells (TMSCs), and printed with a layer-by-layer technique using a PCL polymeric framework to support the structure during printing and gelation. The obtained results showed that dECM scaffolds provided a biocompatible microenvironment for cell proliferation and outperformed the control materials in directing tissue-specific lineage commitment, as revealed by the increased expression of chondrogenic markers. Remarkably, this study demonstrated that bioprinting with dECM bioink is an attractive option that paves new ways for both *in vitro* and *in vivo* tissue reconstruction. Additionally, the developed material could be also particularly beneficial in the field of *in vitro* cartilage models that currently use standard and nonspecific hydrogels, such as type I collagen and fibrin, as a 3D matrix for cell culture. For instance, the dECM hydrogels, gelling at 37°C, could be easily used in microfluidic models to better mimic the chondral environment and to provide the cells with an instructive cell niche, representing a significant step forward in the development of biomimetic chondral models. This study perfectly shows how the efforts in a research field strongly related to the *in vivo* application, such as 3D bioprinting, can lead to important advancements in other apparently unrelated fields, such as *in vitro* biomimetic models, representing a perfect example of the convergence between different scientific areas.

2.2. Evaluation of Biochemical and Biophysical Factors. Stem cell differentiation protocols exploit developmental signals to instruct the cells and drive them towards a specific lineage. The generation of models for the screening of multiple growth factors is a crucial step to define differentiating signals able to recapitulate *in vitro* the developmental processes leading to chondrogenesis *in vivo*. Indeed, the identification of biochemical and biophysical parameters able to trigger stem cell chondrogenesis can significantly impact the design of proper culture conditions for *in vitro*-generated constructs in terms of medium composition as well as the development of dynamic culture systems for the application of biophysical stimuli. In this scenario, the results obtained using *in vitro* models could be implemented during the *in vitro* maturation of biofabricated constructs before their implantation, in order to achieve the chondrogenic priming of the engineered tissue. The definition of the most suitable combination of differentiating signals often requires the screening of several growth factor combinations, as well as concentration ranges and timing, which easily results in a complex experimental setup based on many levels of interactions among multiple parameters. In this context, microfluidic technology offers several advantages related to the minimal number of cells required to test a high number of experimental conditions and the use of very low amounts of costly growth factors. Even more importantly, microfluidic models feature an unprecedented spatial and temporal control over the cell microenvironment. Indeed, the controlled perfusion of the culture medium within microchannels allows maintaining more uniform culture conditions than standard static approaches, providing the stable supply of nutrients and growth factors as well as the removal of waste products [13, 53]. Remarkably, controlled fluid flow can also be exploited to automatically obtain gradients of growth factors in the same microfluidic platform using serial dilution generators (SDGs). This strategy was recently applied by Occhetta et al. [16] who developed a microfluidic platform implementing two different SDGs to generate either a wide range of concentrations of soluble factors (logarithmic scale) or a narrower concentration window (linear scale) (Figure 2(a)). This device was specifically designed to induce the condensation of BMSCs within fluidically connected microchambers, enabling the formation of 3D micropellets with uniform size and shape. Using this platform, the authors were able at the same time to uniformly generate 3D cell micropellets into defined spatial configurations and culture them under a continuous laminar flow with defined concentrations of transforming growth factor- (TGF-) β3 spanning over four orders of magnitude. This screening led to identify the lowest TGF-β3 concentration (0.1 ng/mL) as capable of inducing chondrogenesis and maintaining the proliferative ability of BMSCs, while the highest TGF-β3 concentration (100 ng/mL) induced the disaggregation of the micropellets. Noticeably, the results of this study demonstrated that the developed model allows replicating the 3D expansion step

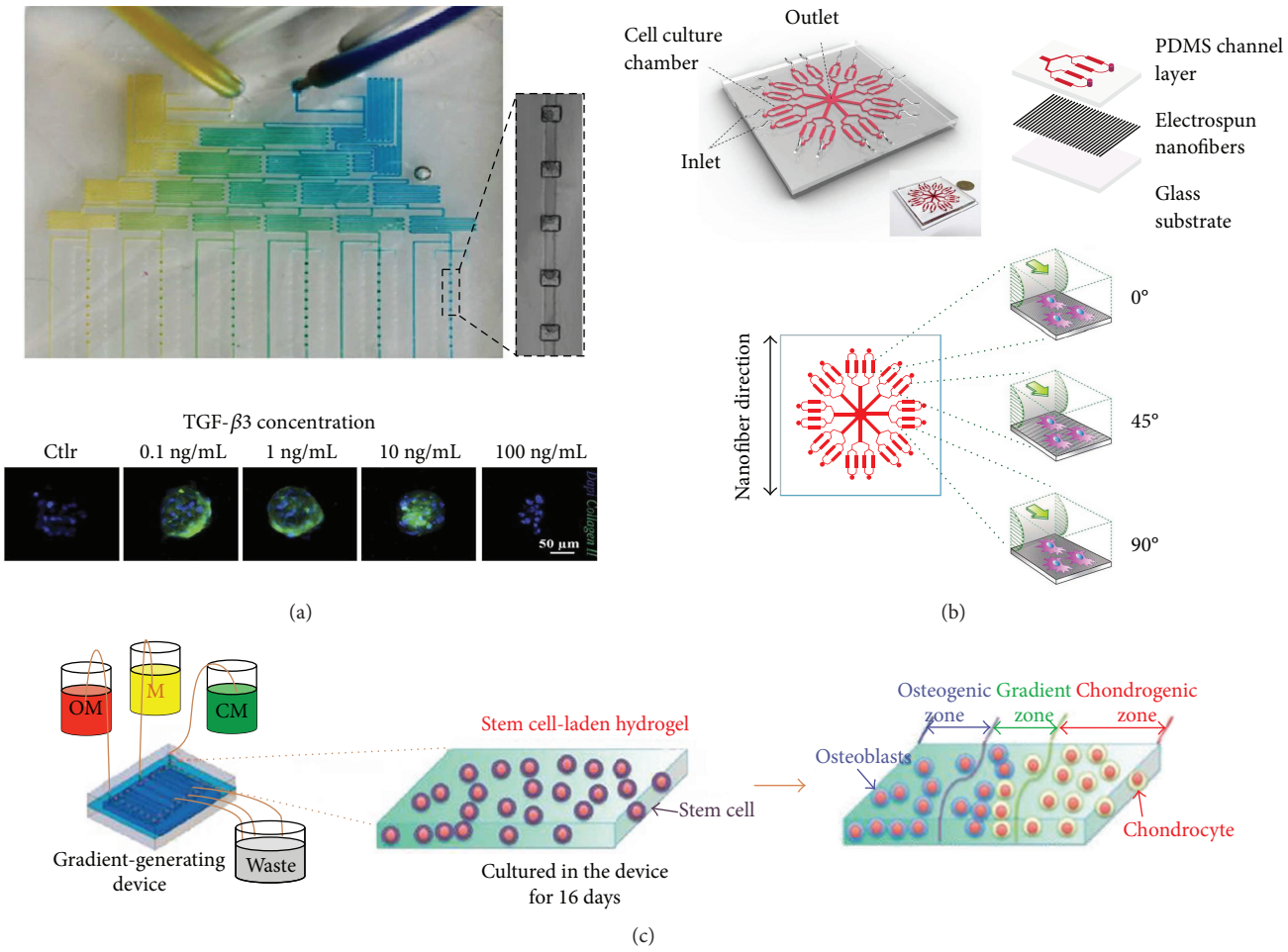

Figure 2: Microfluidic models for the screening of biochemical and biophysical factors. (a) Photograph of a microfluidic device including a serial dilution generator to develop a gradient of growth factors and a 3D culture area for the generation and perfusion of 3D cell spheroids. This device was exploited to generate a gradient of TGF-β3 and to identify the concentration able to induce type II collagen expression in micropellets (adapted from [16]). (b) Schematic illustration of a microfluidic device with integrated electrospun nanofibers to study the influence of 3 different flow directions with respect to fiber orientation on stem cell chondrogenesis (adapted from [58]). (c) Schematic representation of a microfluidic device for the development of the osteochondral interface. A system formed by two serpentines and a central channel, respectively, filled with osteogenic medium (OM), chondrogenic medium (CM), and cell culture medium (M), allows the generation of osteogenic and chondrogenic growth factor gradient to obtain spatially controlled differentiation of MSCs (adapted from [59]).

occurring during the early stages of limb development, overcoming one of the main limitations of other 3D models, such as macropellets, which experience a reduction in cell number over time due to the formation of a necrotic core [54]. The use of SDGs has been also implemented in microfluidic platforms designed and exploited to select the most suitable concentration and/or combination of growth factors to favor articular chondrocyte proliferation, either in monolayer culture [55] or hydrogel-based culture [56]. The low number of cells that can be obtained from patients' biopsies is one of the main limiting factors in ACI procedures, and hence, the optimization of the protocol for chondrocyte expansion represents a crucial step to improve the outcome of this clinical approach. Another strategy to overcome this limitation is represented by combining MSCs and articular chondrocytes as proposed by Higuera et al. [57] who developed an implantable screening device that allows the analysis of multiple coculture conditions both *in vitro* and *in vivo*. The device consists in a 3D-printed platform formed by arrays of micro- to millimeter-scale wells that can be subcutaneously implanted in nude mice to evaluate the influence of the cell source on the accumulation of ECM. Because of this system, the authors identified the optimal ratio of MSCs and articular chondrocytes to achieve cartilaginous ECM deposition, demonstrating the existence of optimal conditions for the crosstalk between these two cell types.

Besides biochemical factors, the role of biophysical factors as determinants in stem cell differentiation is also gaining more and more attention. The development of a biomimetic microenvironment allows a more precise study of cell behavior in physiological-like conditions. In this scenario, microfluidics has major advantages compared to conventional 2D culture due to the possibility of replicating some aspects of the *in vivo* 3D environment in terms of both biochemical and physical stimuli. In this context, Rivera and Baskaran [17] have developed a microfluidic device

to investigate simultaneously the influence of shear stress and biomolecular gradients on BMSC alignment and chondrogenesis. The 3-week exposure to TGF-β1 gradients via fluid flow enhanced the formation of chondrogenic aggregates with an increased cell elongation in flow direction when compared to a constant TGF-β1 concentration. The cellular alignment was more evident in confluent regions with respect to nonconfluent regions demonstrating that both shear stress and cell-cell contact influenced BMSC behavior. Hence, exposing BMSCs to gradients of chondrogenic factors and shear stress appears as a promising strategy to induce chondrogenesis that can be implemented to generate engineered tissues with superior features compared to standard culture. An alternative microfluidic approach was exploited to investigate the effect of sequential mechanical and biochemical stimuli on stem cell differentiation [18]. To this purpose, a shear stimulation system controlled by a syringe pump was used to apply a physiological shear stress of 1.5 Pa to a suspension of BMSCs flowing through the tube. Shear-stimulated BMSCs were then exposed to biochemical factors to determine if preconditioning BMSCs with a mechanical stimulus could enhance their response to chondrogenic factors. Remarkably, this study highlighted that BMSCs retain the memory of a single shear stimulation experience and that 3 weeks later, the commitment towards the chondrogenic lineage was still superior in shear-stimulated compared to nonstimulated cells, demonstrating the potential of this approach to influence stem cell fate and responsiveness to differentiating factors in a short timeframe.

Recent advances in microfluidic technology have also allowed the incorporation of nanostructures in 3D microfluidic models to create a more biomimetic microenvironment and understand the mechanotransduction dynamics modulating stem cell fate. In particular, Zhong et al. [58] integrated aligned nanofibers obtained by electrospinning into a microfluidic platform to investigate the simultaneous role of topographical cues and mechanical cues provided by fluid flow (Figure 2(b)). The authors designed a microfluidic device containing independent microchambers with multiple orientations with respect to electrospun nanofibers to allow fluid flow to form different angles with the nanofibrous substrate. The results showed that BMSCs preferentially elongated along the nanofiber direction and that chondrogenesis was improved by the presence of perpendicular flow. In this condition, the expression of type II collagen was higher, whereas a significant increase of type I collagen was observed in cells under parallel flow, demonstrating how specific mechanotransduction signaling pathways regulate BMSC differentiation by translating mechanical stimuli into biochemical signals.

From the perspective of stem cell-based restoration of the articular cartilage, the simultaneous triggering of efficient chondrogenesis and osteogenesis of stem cells in the spatially defined region of a 3D scaffold appears to be a promising strategy to develop *in vitro* models for the study of bone-cartilage crosstalk and, hence, to achieve new strategies to improve the integration of the chondral graft. In this context, the microfluidic technology provides powerful tools to engineer interfacial tissues by exploiting the intrinsic multidifferentiation ability of stem cells. These constructs can be either used as a disease model to investigate the role of pathogenic signals that may affect both cartilage and the subchondral bone or to generate implantable constructs, representing a *trait d'union* between *in vitro* and *in vivo* applications. Recently, Shi and colleagues [59] have developed a microfluidic system able to generate gradients of chondrogenic and osteogenic growth factors to steer the spatially controlled chondrogenesis and osteogenesis of ADMSCs embedded in the same hydrogel (Figure 2(c)). This gradient-generating system includes a bottom layer consisting of a PDMS pool filled with a stem cell-laden agarose hydrogel covered with a microporous membrane and with a top layer containing two lateral serpentine channels and a central linear channel. To produce a biomimetic transitional phase between osteogenic and chondrogenic zones, a culture medium without any differentiation factors was introduced into the central channel while chondrogenic and osteogenic media were introduced into the two lateral serpentines. After 25 days of culture, the spatially controlled differentiation of stem cells into chondrocytes and osteoblasts was achieved and a region mimicking the bone-cartilage interface was observed in the central region of the hydrogel. The implementation of this type of gradient-generating system in a more complex and clinically relevant setup may pave the way to the *in vitro* engineering of interfacial tissues starting from a single cell source seeded in a single biomaterial. This platform could also be used to study the pathogenesis of diseases involving both the articular cartilage and the subchondral bone, such as OA. Indeed, the generated construct may represent a reliable *in vitro* model of the osteochondral interface and the two lateral serpentines could be used to generate a gradient of pathogenic signals (e.g., proinflammatory cytokines) starting from either the chondral or the bony side. A similar approach has been used by Lin and colleagues [60] who developed a microphysiological model of the osteochondral unit integrating a microfluidic system into a multichamber bioreactor. This system was exploited to achieve spatially defined chondrogenic or osteogenic differentiation of BMSCs loaded in a methacrylated gelatin-based scaffold and to induce an OA-like response through the targeted treatment of either the chondral or the bony compartment with the proinflammatory cytokine interleukin- (IL-) 1β. Using this approach, the authors demonstrated that the exposure of the bony layer to IL-1β resulted in a stronger catabolic response in the chondral layer than the direct application of IL-1β to the chondral component, indicating the active communication between the two tissues. 3D bioprinting techniques have been also exploited to generate anisotropic microscale multiphase 3D tissue models with potential impact in *in vitro* drug testing, discovery, and development as reported by Gurkan et al. [61]. In this study, the generation of an interfacial tissue was achieved by printing BMSCs in nanoliter hydrogel droplets encapsulating either bone morphogenetic protein- (BMP-) 2 or TGF-β1 to drive their differentiation towards different lineages. The authors showed that phenotypic pathway and network analysis can be performed using the genomic expression data obtained from the model, demonstrating

the potential of bioprinted anisotropic tissues as functional *in vitro* 3D tissue models.

2.3. Improvement of Construct Architecture. In stem cell-based cartilage applications, the use of hydrogel biomaterials has been introduced to improve cell retention at the injury site and to provide the implanted cells with a favorable microenvironment. However, the use of bulk hydrogels has some disadvantages including a high risk of ectopic chondrogenesis and an inefficient supply of oxygen and nutrients due to the limited diffusion within hydrogels, which is often restricted to 200 μm and results in a necrotic core [62].

With advances in engineering technologies, such as soft lithography and 3D bioprinting, microfluidic channels and complex geometries have been engineered into hydrogels to improve perfusion for the delivery of oxygen and nutrients and removal of metabolic waste products for the embedded cells [19, 63–67]. To obtain printable microfluidic channels, Zhang and coworkers [19] have combined 3D bioprinting and microfluidics for the generation of cell-embedding hollow filaments. In this study, coaxial nozzles were fabricated using three fluid-dispensing tips and assembling a feed tube, an outer tube, and an inner tube. The feed tube was used to deliver the alginate solution into the cavity formed between the outer and inner tubes, while the $CaCl_2$ crosslinking solution was fed through the inner tube to create the hollow filament. By modulating the flow rate of alginate and $CaCl_2$ solutions, the authors were able to tune the ratio between the core diameter and the fiber diameter demonstrating the great flexibility of this technique. Chondrocyte progenitor cells (CPCs) encapsulated within the hollow alginate fibers showed a high cell viability, proving the cytocompatibility of this process. Noticeably, the expression of chondrogenic markers was enhanced in encapsulated CPCs compared to monolayer culture, indicating that alginate hollow filaments provide an ideal environment for CPCs to differentiate and carry out their cartilage-producing function. This strategy allows fabricating 3D constructs loaded with progenitor cells, yielding the viability and functionality of the cells seeded in the central region, which usually have limited access to nutrients and oxygen. Furthermore, as envisioned by the authors, this approach can be implemented by printing CPC spheroids between filaments and pumping a culture medium through the hollow channels to promote the formation of a cartilage-specific matrix in tissue constructs with a clinically relevant size. As aforementioned, the insufficient supply of nutrients and oxygen and the inefficient waste removal are major disadvantages when engineering in vitro 3D thick tissues. Embedding microfluidic networks within 3D hydrogel scaffolds represents a promising approach to improve perfusion through thick tissues. Choi and coworkers [65] have presented a strategy to control the distributions of soluble chemicals within the scaffold with convective mass transfer via microfluidic networks embedded within the cell-seeded biomaterial. The authors exploited a lithographic technique to generate functional microfluidic serpentines in a calcium alginate hydrogel seeded with articular chondrocytes and characterized convective and diffusive solute transfer, demonstrating that microfluidic channels enable efficient exchange of solutes with the bulk of the scaffold and quantitative control of the soluble signals experienced by the cells. This approach was also suitable to generate two independent microfluidic networks in the same scaffold, which could be particularly relevant in view of the administration of different growth factors to induce the spatially controlled differentiation of stem cells seeded within the same hydrogel to engineer interfacial tissues. A similar strategy was adopted by Goldman and Barabino [66] to design agarose constructs embedding a microfluidic serpentine in order to enhance the viability of encapsulated articular chondrocytes and the production of type II collagen and glycosaminoglycans (Figure 3(a)). To this purpose, a PDMS mold was used to generate a cell-laden agarose layer integrating a microfluidic serpentine (425×425 μm square cross-section) that was then sealed against a planar slab of a cell-laden agarose solution to complete the construct. This study showed that the incorporation of a microfluidic network in cell-laden agarose gels allows improving proliferation and ECM biosynthesis in tissue-engineered constructs of relevant thickness (2.5 mm and 5 mm thick) compared to bulk hydrogels.

Considering the exploitation of microfluidics as a biofabrication technology, interesting studies have been recently published whereby microfluidics was used to produce 3D scaffolds with uniform pore sizes [68, 69]. Specifically, Chung et al. [68] used a microfluidic device including two concentric tapered channels to generate bubbles enclosed within liquid alginate droplets by pumping nitrogen gas and aqueous alginate solution through the inner and the outer channels, respectively. These bubbles spontaneously self-assembled into a liquid foam that was then exposed to a $CaCl_2$ solution to induce alginate crosslinking and generate a solid foam. This approach generated scaffolds with highly ordered and interconnected pores with controlled size. In a following study [69], the same group showed that this honeycomb porous scaffold, which is characterized by a more ordered structure than traditional alginate sponges, well supported chondrocyte growth and phenotype maintenance demonstrating that this highly organized scaffold prepared with an economical microfluidic device holds potential for future applications in the field of cartilage tissue engineering.

The recent combination of 3D bioprinting with advanced microfluidic printheads has recently found application in many areas, leading to unprecedented advances in the biofabrication of complex tissue constructs with high spatial resolution [70]. In the context of cartilage repair, a system based on two coaxial needles has been used to fabricate 3D scaffolds via bioprinting composed of ECM biomimetic hydrogels loaded with BMSCs (Figure 3(b)) [71]. In details, the authors have developed a bioprinting system formed by an external nozzle and an inner nozzle, dispensing $CaCl_2$ and different alginate-based hydrogel solutions, respectively. In this way, as the two solutions came into contact, hydrogel fibers formed immediately at the tip of the inner nozzle through a gelation process that allowed producing 3D hydrogels with high resolution. After 3D bioprinting, the constructs underwent a secondary UV crosslinking to guarantee an efficient bonding among fibers belonging to

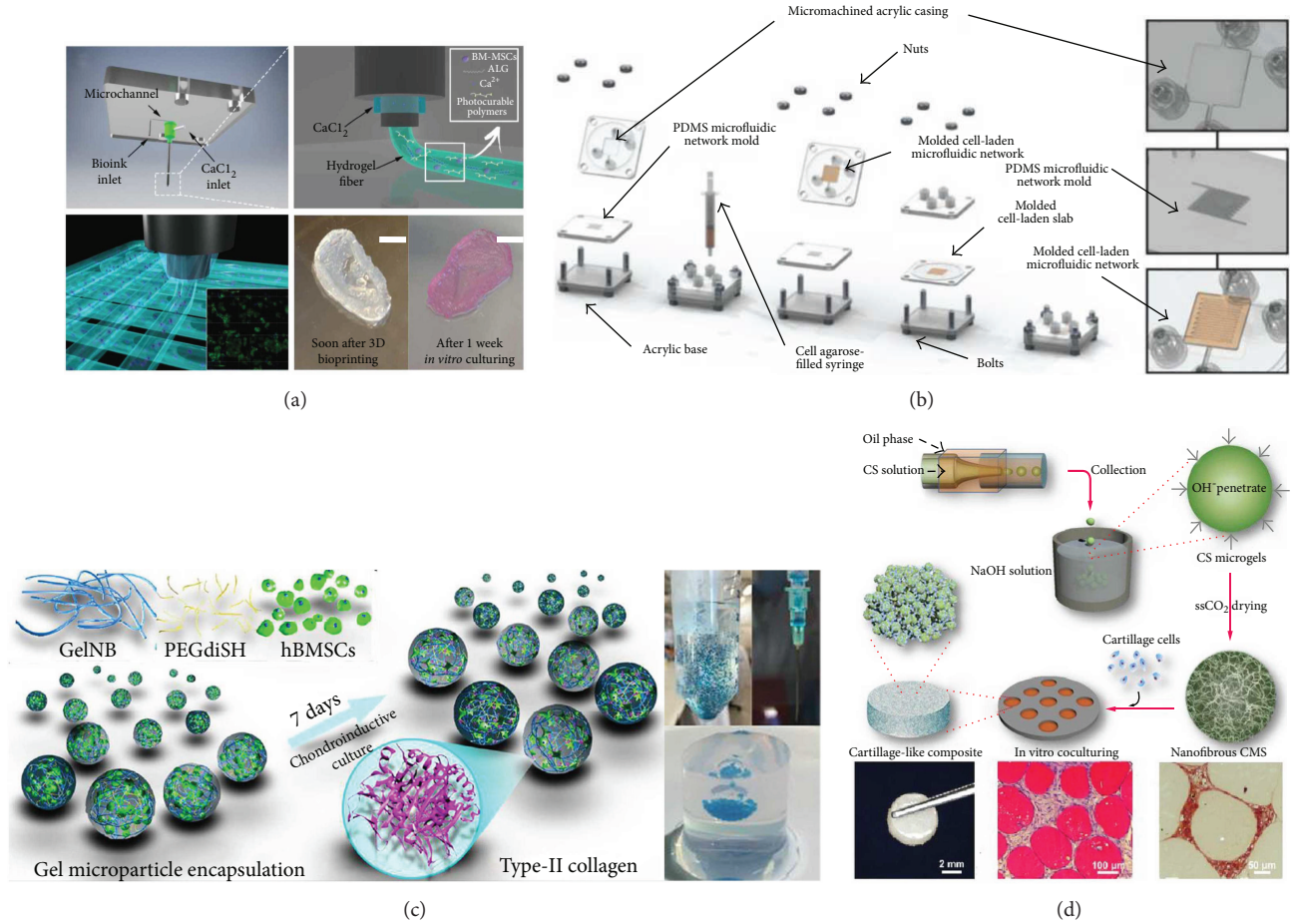

Figure 3: Application of microfluidic and bioprinting technologies for the development of 3D cartilaginous constructs. (a) Schematic representation of the custom-made dispensing coaxial system: calcium chloride flows in the external nozzle while the bioink is supplied through the inner one. Hydrogel fibers form immediately at the tip of the inner nozzle when the two solutions come into contact. Real-size neonatal ear can be obtained with a high printing resolution (≈100 μm). Scale bar = 10 mm (3D bioprinting method reported in [71]). (b) Fabrication process of a cell-laden agarose construct with an incorporated microfluidic serpentine to enhance oxygen and nutrient transport (adapted from [66]). (c) Schematic diagram of chitosan microsphere production through a coaxial glass microcapillary device. Each nanofibrous microsphere is seeded with articular chondrocytes, and the deposition of newly generated ECM tightly bridges the microspheres into a clinically relevant 3D construct (adapted from [21]). (d) Schematic representation of hydrogel microspheres with encapsulated BMSCs produced by a simple syringe-based system. The obtained microspheres can be injected into the lesion site, as demonstrated by the injection into an agarose gel model, which mimics an articular cartilage defect (adapted from [20]).

adjacent layers determining the overall mechanical properties of the scaffolds. In particular, structures with a 5 mm height were printed depositing 50 layers with 100 μm thickness. 3D bioprinting experiments performed with BMSCs showed that ionic crosslinking of alginate and UV crosslinking were not detrimental to cell survival, proving the biocompatibility of this approach.

Microfluidic technology has been also exploited to achieve cell microencapsulation generating cell-laden microgels in a high-throughput manner, as reported in several recent reviews [72–74]. Remarkably, the use of these microgels as building blocks that can be combined to obtain relevant constructs offers a major advantage with respect to bulk hydrogels, since the large surface-to-volume ratio promotes a more efficient mass transport and enhanced cell-matrix interactions. In the context of cartilage repair, Li and colleagues have developed a simple and cheap microfluidic device to encapsulate BMSCs in hydrogel-based microspheres that can be crosslinked using visible light [20] (Figure 3(c)). Specifically, the device was composed of an ordinary pipette tip and two tubes connected to two syringes: one containing a precursor hydrogel solution (aqueous phase) and BMSCs and the other one loaded with oil (oily phase). First, the pipette chamber was filled with oil, and then, the dispersed hydrogel phase was pumped into the tube at a constant rate to generate the microspheres through the silicone tube. In this way, the authors were able to generate microspheres with different diameters (ranging from 300 to 600 μm) by adjusting the flow rate ratio between the aqueous and the oily phase. The authors showed that BMSCs encapsulated into the microspheres achieved a superior chondrogenesis compared to the bulk hydrogel and that microspheres could be injected into a cavity simulating a focal cartilage lesion with a 20-gauge hypodermic needle,

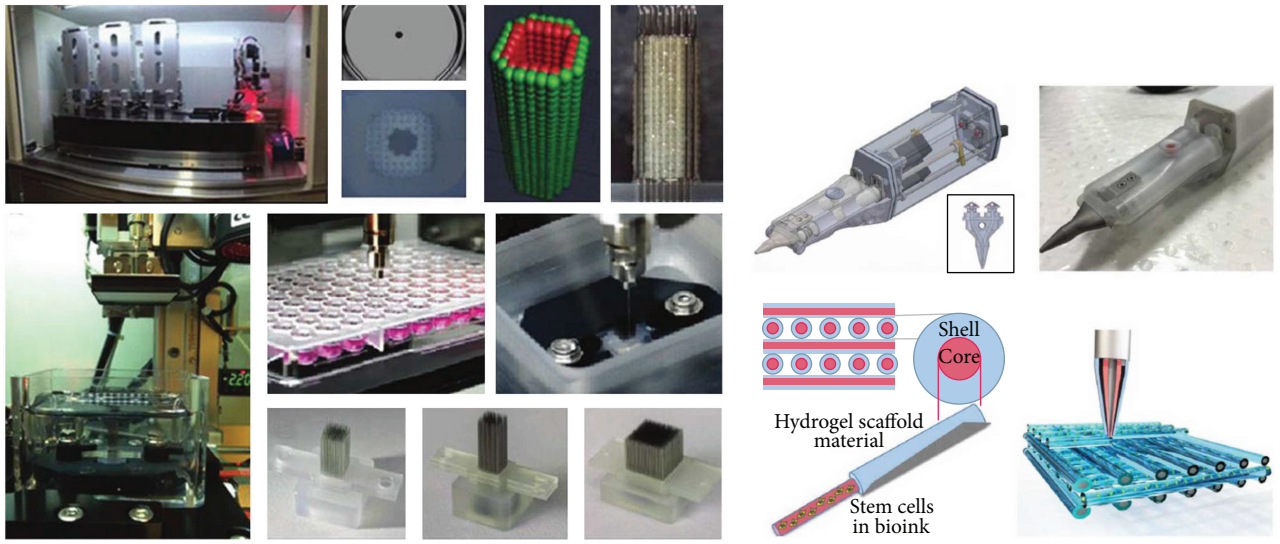

Figure 4: 3D bioprinting approaches towards the clinical translation of cell-based therapies. (a) Kenzan Method, based on an automated system for cell spheroid bioprinting, allows the deposition of 3D spheroids on a microneedle array as a support for the production of scaffold-free 3D constructs [75]. (b) Schematic illustration of Biopen, a handheld device constituted of two bioink chambers, a collinear nozzle, and a UV crosslinking source for the in situ deposition of cell-laden methacrylated hydrogels (adapted from [79]).

thus proving the feasibility of intra-articular microsphere injection and the clinical relevance of this approach.

Microfluidic technology has also been exploited as a manufacturing technique for the preparation of microparticles to generate clinically relevant 3D constructs, as reported by Zhou and colleagues [21] (Figure 3(d)). Specifically, a coaxial glass microcapillary device was assembled by round and square glass capillaries, and chitosan and cyclohexane solutions were flown to generate an inner aqueous and an outer oil phase, respectively. The generated emulsion was collected in an alkaline solution to induce the gelation of chitosan microspheres (ϕ 165–425 μm), which were then seeded with articular chondrocytes and cultured for 7 days. The authors showed that this period was sufficient for articular chondrocytes to tightly bridge chitosan microspheres through ECM into bigger aggregates that were then transferred into molds (ϕ 5 mm, h 2 mm) and cultured in static conditions for 14 additional days. At the end of culture, histological analysis showed that the spaces among the microspheres were filled with cartilage-specific ECM rich in glycosaminoglycans. Furthermore, the generated constructs were able to withstand several compression cycles and displayed a certain degree of elasticity, indicating that the microspheres were tightly bonded together by the chondrocytes and the secreted ECM and demonstrating the validity of this bottom-up approach for cartilage tissue engineering applications.

2.4. New Approaches towards Clinical Practice. A particular 3D bioprinting approach to obtain highly organized constructs for tissue regeneration has been invented by Professor Nakayama, using cell spheroids as building blocks (Figure 4(a)) [75]. This innovative method belongs to the biomaterial scaffold-free approach, for which exogenous materials are not required. Differently from previous studies whereby spheroids were manually assembled into 3D clinically relevant constructs using cylindrical molds [76, 77], in the "Kenzan Method," a 3D bioprinter is used to robotically place cell spheroids in microneedles, which are used as a temporary support during the fusion of spheroids. Each array is composed by 160 μm thick microneedles, which are 500 μm distant from each other, and therefore, spheroids should have a diameter of a hundred micrometers to get in contact and form ECM in order to achieve a compact construct. After the spheroid fusion, the constructs are removed from the needle support and cultivated for the postprinting maturation phase in which the holes formed by the needle are resorbed due to their cell "healing" capacity. Remarkably, although this technique differs from the standard approach of 3D bioprinting for the presence of preformed cell spheroids and for the absence of hydrogel-based materials, it represents a valid method to produce constructs with a clinically relevant size, avoiding potentially detrimental processes, which can occur during standard 3D bioprinting procedures.

Another interesting approach that shares some fundamental principles with 3D bioprinting, such as the use of living cells and biomaterials as building blocks, is represented by a 3D bioprinting pen, called "Biopen", which was developed by O'Connell et al. [78]. This new approach, which represents one of the most relevant applications in view of the clinical translation of bioprinting, was designed to overcome the issues related to the traditional procedure for tailoring implants to the anatomy of the defect. This process involves the use of medical imaging data to create implant design before the chondral repair procedure. However, such a method does not take into account the initial steps of surgery in which the surgeon removes the excess

of fibrous tissue around the defect, thus varying its size and shape. Differently from the standard bioprinting approach, Biopen does not use a CT/MRI image for the development of a digital model of the defect to direct the deposition of cells and biomaterials, but the bioprinting process is manually operated by the user in a direct writing fashion during the surgical procedure. This feature that represents the major difference between the Biopen approach and standard 3D bioprinting is also the main advantage of this device allowing the fabrication of constructs that perfectly fit the shape and size of the chondral defect. This handheld fabrication tool is composed of three main components: an inner 3D-printed core that contains two collinear ink chambers, a custom titanium extruder nozzle, and a UV source. The extrusion process is controlled by the user through a foot pedal-based pneumatic system that allows depositing each ink individually and/or simultaneously. In a preliminary phase, Biopen has been tested in 2D deposition processes to verify the printing stability and the ability to create compositional gradients controlling the relative extrusion rates of the two chambers. The obtained results demonstrated the printing capacities and consistency of fabricated objects using a GelMa/HAMA hydrogel. In the latter phase, biological experiments have been performed to evaluate the effects of the printing process on ADMSCs. In a subsequent work, the same research group performed a pilot study to evaluate the surgical applicability of Biopen to repair critical full thickness chondral defects (ϕ 8 mm) in an ovine model [79]. Differently from the above-described device, the new version of Biopen is characterized by a coaxial extrusion system that allows depositing a biphasic hydrogel constituted by an inner "core" of GelMa-HAMA bioink laden with ADMSCs from infrapatellar fat pad and an outer "shell" of GelMa-HAMA bioink mixed with the photoinitiator (Figure 4(b)). The presence of this outer shell warrants photocuring of the construct during printing. The obtained results showed that Biopen was able to deliver 3D-printed scaffolds perfectly fitting the shape and depth of the defect, without causing any sign of inflammation or infection. Moreover, constructs printed through Biopen showed a higher amount of newly generated cartilage if compared with both negative untreated controls and defects treated by microfracture technique, evidencing chondrocyte columnar alignment and maintenance of subchondral bone integrity after 8 weeks from implantation. Promising results were obtained also evaluating the mechanical properties of Biopen-extruded scaffolds, which yielded values of instantaneous Young's modulus, equilibrium modulus, and maximum stress similar to those of the native articular cartilage. The promising outcomes reported in these studies [78, 79] and the recent results regarding the optimization of the bioprinting conditions to achieve high cell viability and relevant structural stiffness [80] may pave the way to the use of Biopen to build up mm- to cm-scale 3D structures. More importantly, because of its ability to directly control the deposition of biomaterials during the surgical process, this device can represent an exciting advance in the translation of bioprinting into clinical practice, not only for cartilage regeneration but also in other applications where tissue regeneration is critical.

3. Outlook

Although stem cell-based therapies have emerged as a novel treatment in cartilage-based repair, their success is often limited due to multiple factors, such as inefficient differentiation of stem cells towards the chondrogenic lineage and/or poor stem cell engraftment and survival after transplantation. In addition, an important aspect that is often neglected is that stem cells are usually delivered to an inflamed environment and, hence, have to face a plethora of catabolic signals that may negatively affect the outcome of cell-based approaches.

In this scenario, microfluidics can provide important advances in the selection of biochemical and biophysical factors able to direct the fate of stem cells that can be implemented in new protocols for stem cell differentiation and in the design of dynamic culture systems. It is also possible to envision an exploitation of microfluidic models personalized with patient-derived stem cells for the screening of the most suitable differentiation protocol for each patient-specific stem cell population. This would allow the optimization of personalized differentiation protocols. Additionally, since the use of growth factors during *in vitro* culture may pose obstacles in the clinical translation of stem cell-based therapy, the possibility to direct stem cell fate uniquely using biophysical factors, such as shear stress, appears to be fascinating. Microfluidic models hence represent an invaluable tool to define the biophysical stimuli that should be used to direct stem cells towards the chondrogenic lineage without using growth factors. Furthermore, microfluidic devices can be used to develop organotypic models of the whole articular joint capable to recapitulate either a physiological or a pathological environment [81], perfectly matching the concept of organs-on-chips for the study of tissue development, organ physiology, and disease etiology [82, 83]. The models that recapitulate the osteochondral unit on a single chip are a striking evidence of the potential of microfluidics in this context. Indeed, the comprehension of the inflammatory events involving the articular cartilage and subchondral bone may help in defining complementary anti-inflammatory treatments to promote the survival and the engraftment of implanted stem cells. Recently, a new strategy to mold and culture composite 3D cellular constructs featuring different cell types and/or biomaterials, with high spatial control in microfluidic channels, has been developed [84]. This technique allows obtaining a continuous gel-gel interface, with no need for pillars to delimit the hydrogels, and paves the way to the development of microfluidic models including chondral and osseous compartments with highly specific features in terms of cell and ECM composition. Using this approach, it would be possible to combine a miniaturized model of the articular cartilage with a miniaturized model of the subchondral bone, including a calcified ECM, osteoblasts, osteoclasts, and endothelial cells [85]. Bioprinting could be also used to generate microfluidic models of the articular joint, since it enables the printing of multiple materials and different types of living cells

in a programmable manner with high spatial resolution, and has proven to hold a great potential for fabricating organs-on-a-chip recapitulating the intrinsic complexity of the native tissue/organ [86, 87].

On the other hand, microfluidics-based 3D bioprinting approaches could be used to overcome the problem of poor cell engraftment at the lesion site, by transplanting stem cells embedded in an ECM-mimicking environment. 3D bioprinting is rapidly becoming a first-choice approach for several advanced applications in tissue engineering. Despite the appealing advantages offered by this technology, such as rapid production of cellularized constructs with high accuracy and repeatability independently of scaffold geometry, a major ongoing challenge that still needs to be addressed is related with bioink formulation. In fact, an ideal bioink should provide, on one side, a proper matrix for cell maturation, differentiation, and neomatrix synthesis while still keeping, on the other side, a high printing resolution. So far, this issue has been addressed only to a minor extent and a continuous research is carried out to find new solutions. In the case of cartilage regeneration, 3D bioprinting represents a suitable technology to recapitulate the tissue structure in terms of chondrocytes and ECM organization. In particular, the complex zonal organization might be reproduced in the future through the development of more accurate systems for multimaterial deposition. Furthermore, the formulation of bioinks should be refined to better promote stem cell differentiation towards the chondrogenic lineage with the synthesis of new polymers or with the formulation of blends or composite bioinks that would eventually result in enhanced quality of the neodeposited matrix. Another major issue that must be overcome in order to boost the translation of engineered constructs for cartilage regeneration into the clinic is related to their poor mechanical properties. In fact, native cartilage has a Young's modulus of around 700–800 kPa, which is between one and two orders of magnitude higher compared to the constructs obtained via 3D bioprinting. This is a key issue that would require many efforts to be overcome. A possible solution may be found by employing more sophisticated culture systems that may lead to more functional cartilage tissue by providing controlled mechanical and biochemical stimuli. However, then, we need to be sure that biofabricated mature tissue will properly integrate with surrounding natural cartilage. So far, 3D bioprinting has already demonstrated its capacity to build complex artificial structures. However, the future work must be focused on enhancing the functionality of such constructs to prompt applications in real clinical scenarios. Finally, in situ 3D bioprinting can enable the achievement of thick tissues directly into the lesion site in a one-step approach, by translating bioprinters in the surgery room. Despite challenges, this computer-aided technology holds a great potential since it would allow overcoming the need for preshaping or reshaping of the scaffold based on the defect geometry and achieving high precision in the deposition of cells and biomaterials. Because of these features, we envision that in situ 3D bioprinting will produce significant advances in the regeneration of the entire articular units or in the treatment of complex articulations, such as the carpometacarpal joint.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Carlotta Mondadori and Valerio Luca Mainardi are equally contributing authors.

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Review Article

Stem Cells for Cartilage Repair: Preclinical Studies and Insights in Translational Animal Models and Outcome Measures

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Due to the restricted intrinsic capacity of resident chondrocytes to regenerate the lost cartilage postinjury, stem cell-based therapies have been proposed as a novel therapeutic approach for cartilage repair. Moreover, stem cell-based therapies using mesenchymal stem cells (MSCs) or induced pluripotent stem cells (iPSCs) have been used successfully in preclinical and clinical settings. Despite these promising reports, the exact mechanisms underlying stem cell-mediated cartilage repair remain uncertain. Stem cells can contribute to cartilage repair via chondrogenic differentiation, via immunomodulation, or by the production of paracrine factors and extracellular vesicles. But before novel cell-based therapies for cartilage repair can be introduced into the clinic, rigorous testing in preclinical animal models is required. Preclinical models used in regenerative cartilage studies include murine, lapine, caprine, ovine, porcine, canine, and equine models, each associated with its specific advantages and limitations. This review presents a summary of recent *in vitro* data and from *in vivo* preclinical studies justifying the use of MSCs and iPSCs in cartilage tissue engineering. Moreover, the advantages and disadvantages of utilizing small and large animals will be discussed, while also describing suitable outcome measures for evaluating cartilage repair.

1. Introduction

Articular cartilage covers the ends of the bone; due to its slightly compressible and elastic nature and lubricated surface, it provides the joint with shock absorption and lubrication [1, 2]. Hyaline cartilage is comprised of 95% extracellular matrix (ECM) (dry weight) and only 5% of sparsely distributed chondrocytes [3]. This matrix primarily consists of type II collagen and proteoglycans (PGs). Negatively charged glycoproteins are able to attract water, allowing the cartilage to resist compressive forces [4]. Despite the fact that chondrocytes only make up about 5% of hyaline cartilage tissue, they are integral for cartilage function and homeostasis [4]. These cells are of mesenchymal origin and are responsible for synthesizing cartilage ECM [3]. Hyaline cartilage is an avascular tissue which, in part, explains the limited regeneration capacity following injury. The lack of vasculature makes it difficult for progenitor cells to be recruited to the site of injury and hinders the supply of nutrients necessary for tissue regeneration [1, 5].

Cartilage loss can occur as a consequence of traumatic injury, leading to focal defects or through chronic degeneration. Both partial thickness and full thickness cartilage defects occur [6]. Since full thickness lesions extend into the subchondral bone, they have access to bone marrow cells and therefore have a higher probability of spontaneous regeneration than partial thickness lesions, which only

involve the avascular cartilage tissue [6]. Eventually, cartilage defects will lead to activity-related pain, swelling, and decreased mobility and will frequently progress to osteoarthritis [1, 7]. In the United States alone, over 27 million adults suffer from osteoarthritis, leading to a substantive clinical and financial burden [8, 9].

There are currently no drugs available to effectively heal cartilage defects. When cartilage defects develop into osteoarthritis, the condition can only be managed by a multidisciplinary approach including pharmacotherapy, physiotherapy, or joint replacement surgery [10]. However, several surgical interventions can be performed in order to prevent progression towards osteoarthritis [1]. Current techniques include arthroscopic lavage and debridement, microfracture induction, and autologous chondrocyte implantation [11]. Although these techniques have been proposed to restore normal joint function and minimize further degeneration, they often do not offer a long-term clinical solution. There is a clinical need to develop regenerative medicine approaches to permanently restore articular cartilage [11].

Both adult mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) are promising stem cell sources to achieve cartilage regeneration [5, 7, 12–14]. However, the use of adult MSCs still faces considerable challenges such as cell senescence and donor variability [7, 15]. iPSCs may provide a suitable alternative in order to overcome the limitations of adult MSCs [7]. iPSCs possess unlimited self-renewal and pluripotency, similar to embryonic stem cells (ESCs), but lack the ethical concerns associated with the use of ESCs [1]. However, it remains to be determined whether differentiated iPSCs are able to form a bona fide cartilage [1]. Furthermore, more research is required to alleviate any concerns for tumorigenic effects before this technology can progress to preclinical and clinical usage [16, 17]. Before any of these possible treatment options can be introduced into the clinic, they first have to be tested in suitable and translational animal models [9]. A wide variety of animal models is available to investigate cartilage regeneration ranging from small animal models, such as mice and rats, to larger animals such as canine, porcine, caprine, ovine, and equine models. Smaller animal models are cost-effective and easy to house and offer a variety of genetically modified or immunocompromised strains. However, due to their small joint size and thin cartilage, their translational value is limited [9]. Larger animal models on the other hand more accurately approximate the human situation but are associated with greater logistical, financial, and ethical considerations [9].

In this review, recent *in vitro* data and preclinical studies justifying the use of MSCs and iPSCs in cartilage tissue engineering are summarized. Since preclinical studies require translational animal models, the advantages and disadvantages of small and large animal models will be discussed, while also focusing on suitable outcome measures for evaluating cartilage repair.

2. *In Vitro* Evidence of Chondrogenic Differentiation of Stem Cells

For stem cell-based cartilage regeneration, MSCs are of particular interest because, in comparison to chondrocytes, they have high availability and both easy isolation and expansion [18]. In addition, their *in vitro* chondrogenic differentiation has been proven [19]. More recently, *in vitro* studies on iPSCs indicated promising results for their use in cartilage repair [20, 21]. However, a number of challenges have to be overcome and further optimization is still needed before both stem cell types can be used as a safe and effective therapeutic option for promoting cartilage repair [1, 14, 22–24].

2.1. Mesenchymal Stem Cells. Adult MSCs were first identified in bone marrow [25], but afterwards, other MSC niches have been discovered in both adult and fetal tissues, including adipose tissue [26], placenta [27], umbilical cord [28], dental pulp [29, 30], and peripheral blood [31], and in the synovial membrane [32]. As defined by the International Society for Cellular Therapy (ISCT), MSCs must be able to differentiate into chondrocytes under specific *in vitro* conditions [33]. In addition, MSCs possess additional properties making them a suitable cell source for cartilage regeneration. High cell numbers can be produced, and the immunomodulatory characteristics of MSCs allow for their allogeneic use [34].

Pellet and monolayer cultures are the two main culture systems that have been developed to study *in vitro* chondrogenic differentiation. The 3D pellet system is the most representative *in vitro* model for the condensation of mesenchymal cells that is observed during the initiation phase of chondrogenesis in the process of endochondral ossification [35, 36]. Moreover, cocultures with chondrocytes in both 2D and 3D culture systems could push MSCs towards the chondrogenic lineage [37–39] and growth factors, such as insulin-like growth factor (IGF) [40] and members of the fibroblast growth factor (FGF) [41] and transforming growth factor-beta (TGF-β) [42–44] families, can be added to the differentiation medium to enhance chondrogenic differentiation. Additionally, the chondrogenic differentiation potential of MSCs and the production of ECM proteins can also be stimulated by combining MSCs and biomaterials in 3D scaffolds [45–52] or by manipulating the oxygen tension [53].

In vitro studies mainly focus on bone marrow-derived MSCs (BM-MSCs), followed by MSCs derived from adipose tissue and synovial membrane because of their easy isolation and close proximity to cartilage and joints, respectively [16]. A correlation between the chondrogenic potential of MSCs and their tissue source has been suggested. BM-MSCs showed a superior chondrogenic differentiation capacity compared to MSCs from other origins [54–56]. These differences might be explained by variations in gene expression and pathway activation [57]. Therefore, an adapted differentiation protocol could compensate for lower chondrogenic differentiation capacities [57, 58].

Despite their promising chondrogenic potential *in vitro*, several challenges are linked to the use of MSCs in cartilage regeneration. The most common issue is terminal differentiation towards hypertrophic cells [36]. Moreover, mineralization and vascularization have also been reported after transplantation [35, 59]. In addition, cartilage tissue derived from *in vitro* differentiated MSCs resembles fibrocartilage with inferior mechanical properties and healing capacity [22]. Another limitation is the inter- and intradonor

heterogeneity of MSCs which could influence chondrogenic differentiation potential of cells [60], depending on comorbidities, tissue source, and culture methods [24]. Furthermore, serial passaging, needed to obtain sufficient cell numbers for *in vivo* studies, has been reported to affect chondrogenic differentiation of BM-MSCs [61]. Finally, supplementation of the culture media with high and repeated doses of growth factors does increase the costs of stem cell-based therapy and might be associated with several side effects including synovial fibrosis, osteophyte induction, and other osteoarthritic-like symptoms [62, 63].

2.2. Induced Pluripotent Stem Cells. Part of the issues associated with MSCs can be circumvented by using iPSCs. iPSCs are an ideal patient-specific unlimited cell source for autologous tissue regeneration. Promising *in vitro* results have already been demonstrated in the cartilage engineering field for iPSCs generated from various cell types [20, 21, 23, 64, 65]. Nevertheless, Guzzo et al. stressed the influence of cell type origin on their chondrogenic capacity, where superior properties could be assigned to iPSCs from chondrogenic origin [66], which may be due to the preservation of the epigenetic memory [67].

Analogous to MSCs, indirect cocultures of iPSCs with primary chondrocytes could directly induce the formation of chondrocytes [20]. Furthermore, iPSCs could be committed to the chondrogenic lineage in high-density pellet culture systems, enhanced by the addition of growth factors from the TGF-β superfamily. Nevertheless, the resulting cartilage is a heterogeneous combination of hypertrophic, articular, and fibrocartilage [68]. This heterogeneity could be reduced by first differentiating iPSCs towards an intermediate cell population, such as MSCs [68, 69] or embryonic cell types [23, 65, 70]. An alternative approach to further enhance the chondrogenic potential is seeding iPSCs into scaffolds [71].

Although iPSCs express higher proliferation rates [72] and similar or superior chondrogenic differentiation potential [14, 64] compared to MSCs, other limitations remain associated with these stem cells. Patient-specific autologous iPSC generation and transplantation are very expensive. Allogeneic therapy would be more attractive, but immune rejection cannot be excluded [73]. Analogous to MSCs, it remains uncertain whether the regenerated cartilage induced by iPSCs preserves the mechanical and functional properties of native articular cartilage. Furthermore, also for iPSCs, the presence of hypertrophic signals under *in vitro* conditions, even though to a lesser extent than for MSCs, might indicate the formation of low-quality cartilage tissue by iPSCs [14, 23]. Safety issue is the most important concern that hampers their general use [74]. The potential reactivation of pluripotency in iPSCs or iPSC-derived chondrocytes should be addressed [75]. Moreover, when using retrovirally transduced iPSCs, where the retroviral gene is integrated in the host, a higher risk for teratoma formation in cell transplants is reported [76]. Therefore, adequate phenotyping of (fully) chondrogenic committed iPSCs is needed before transplantation of cells in (pre)clinical use. Several approaches have been proposed to develop iPSCs with a lower risk for tumorigenicity [69, 75, 77–79]. Nakagawa et al. generated iPSCs without Myc from mouse and human fibroblasts and reduced the tumorigenicity of cells [77]. Fusaki and colleagues induced transgene-free human pluripotent stem cells by means of a vector based on the Sendai virus, which does not integrate into the host [78]. Nejadnik et al. used the integration- and viral-free minicircle reprogramming technique to reduce the reactivation of pluripotency in the used human iPSC-derived chondrocytes [69]. Alternatively, transgene-free iPSCs can be used as generated by Wu and colleagues [80]. Additionally, iPSC-derived chondrocytes could be engineered to express a suicide gene in order to eliminate the cells, which was reported to be efficient in ESCs and BM-MSCs [81, 82].

3. Mechanisms of Action of Stem Cell-Based Therapies for Cartilage Regeneration

Stem cell-based therapies were initially developed as a cell replacement therapy due to the chondrogenic differentiation potential of stem cells [14, 23, 52, 83, 84]. Moreover, differentiated MSCs, ESCs, and iPSCs secrete PGs and collagen II [23, 85–88] which are essential components of cartilage tissue. However, it has been shown that upon intra-articular transplantation, MSCs induce cartilage replacement, but the principal source of repair tissue is derived from endogenous cells [89]. Therefore, it is postulated that the paracrine effect of the transplanted cells on the damaged host environment is mainly responsible for stimulating cartilage regeneration (Figure 1). MSCs that were exposed to tumor necrosis factor alpha (TNF-α) and IL-1β were shown to upregulate the expression of several growth factors, anti-inflammatory mediators (*vide infra*), and anticatabolic factors ultimately leading to (stem) cell-mediated cartilage regeneration (reviewed in [90, 91]). The main growth factors associated with cartilage regeneration that are secreted by MSCs belong to the TGF-β superfamily [92]. Moreover, adipose tissue-derived mesenchymal stem cells (AT-MSCs) were demonstrated to diminish MMP-13 expression upon transplantation, potentially counteracting collagen degeneration in pathological cartilage [93]. In addition to the paracrine effect of soluble factors, extracellular vesicles (EVs), released by MSCs, have been shown to influence cartilage regeneration (Figure 1). Reports on stem cell EV-mediated cartilage repair are scarce. Studies showed that MSC-EVs promoted the formation of new cartilage and the deposition of collagen II and glycosaminoglycans (GAGs) [94]. Additionally, EVs from MSCs that overexpressed miR-140-5p stimulated chondrocyte migration and proliferation [95]. Moreover, it was recently reported that BM-MSCs secrete hyaluronan-(HA-) coated EVs [96], which may allow MSC homing to cartilage defects in a receptor-mediated way via CD44. Although stem cell EVs have shown beneficial effects in cartilage repair, it should be noted that EVs may also have damaging effects in arthritis [13].

Furthermore, it has been demonstrated that MSCs possess immunomodulatory properties (Figure 1) [97]. Given the immune component underlying cartilage degeneration, modulating the immune response might contribute to reducing cartilage loss in diseases where an uncontrolled immune response is detrimental [98, 99]. BM-MSCs, for example,

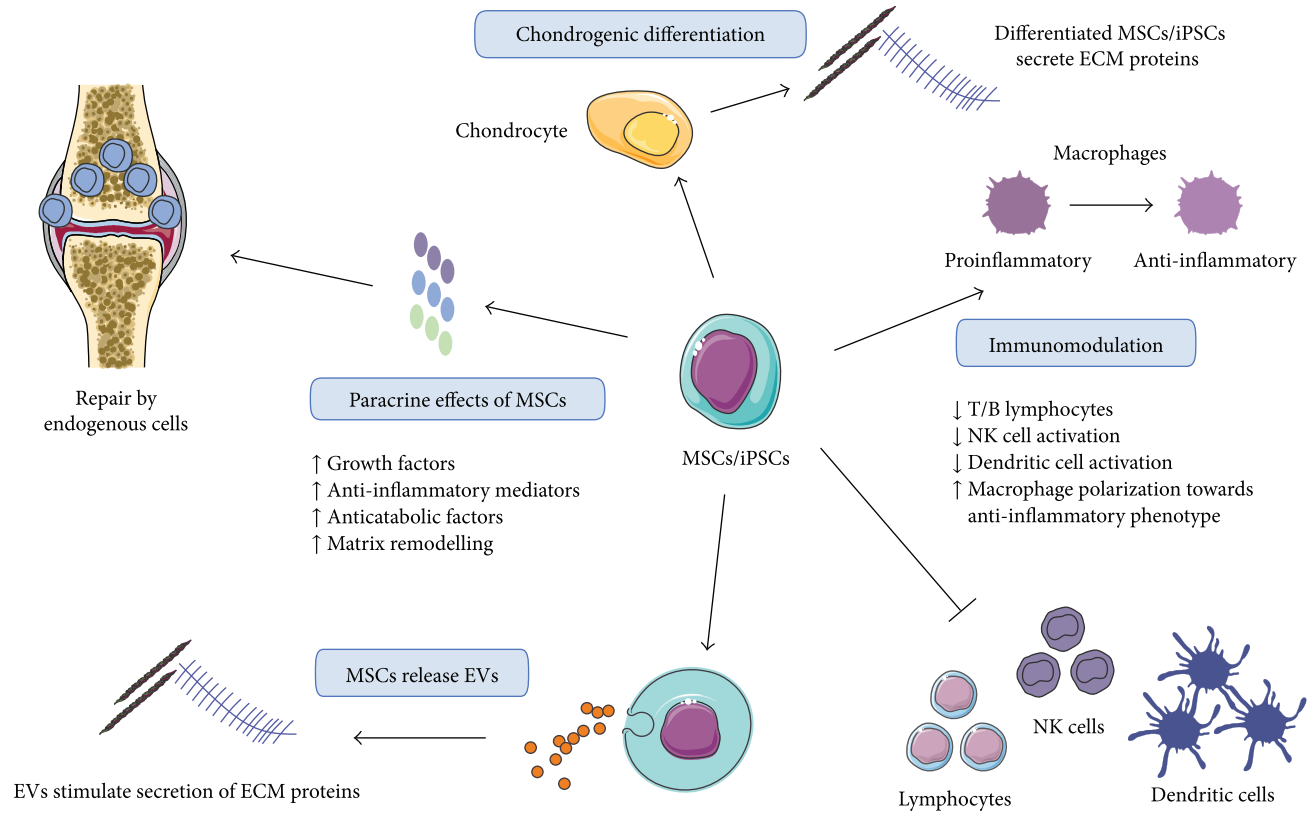

Figure 1: Mechanisms of action of stem cell-based therapies in cartilage regeneration. First, stem cells could be applied as cell replacement therapy because of their chondrogenic differentiation potential. Differentiated mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) secrete proteoglycans and collagen II. Secondly, it is suggested that the tissue is regenerated by endogenous cells under the influence of paracrine factors secreted by stem cells. Extracellular vesicles (EVs) contribute to stem cell-mediated cartilage regeneration by promoting the formation of new cartilage and the deposition of collagen II and GAGs. Finally, immunomodulatory effects are also observed. *This image was created using Servier Medical Art.*

have been shown to suppress T-cell proliferation [100, 101] and to induce T-cell apoptosis [102]. The resulting debris stimulated phagocytes to produce TGF-β which increased the number of regulatory T cells [102]. Moreover, T-cell proliferation was inhibited by BM-MSCs that produced prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) [103, 104]. These factors were also shown to inhibit NK cell activation [105]. Also, MSCs derived from the dental pulp possess immunomodulatory properties [106, 107]. The proliferation, activation, maturation, and antigen presentation of dendritic cells were also inhibited by MSC subtypes [108–112], and macrophage/microglia polarization was shifted towards an anti-inflammatory phenotype after exposure to MSCs, their secretome, or EVs [110–115]. Additionally, MSCs were able to modulate the B cell response by paracrine actions [116, 117]. Next to MSCs, iPSC- or ESC-derived MSCs could also inhibit lymphocyte proliferation and function [118–121] and NK cell function [120].

4. The Importance of a Translational Animal Model and Appropriate Outcome Measures

While *in vitro* studies and models offer a substantial amount of information about the potential of stem cells for cartilage repair [122, 123], more in-depth knowledge about their behavior *in vivo* should be derived from immunocompetent animal models [124]. In orthopedic research, to move new technologies from bench to bedside, strict preclinical studies using translational animal models are required [125]. Preclinical studies evaluating the healing of cartilage defects have been performed using both small and large animal models including murine, lapine, porcine, caprine, ovine, canine, and equine models [16, 124]. The following section will focus on the advantages and disadvantages of utilizing small and large animals for cartilage repair studies as well as some key factors in study design and the usage of validated outcome measures.

4.1. Choice of Animal Model: Small versus Large Animal Models. Articular cartilage defects have been created in small animals, such as mice [84], rats [126–129], and rabbits [130–132]. Smaller animal models are cost-effective and easy to house, and rodents are available in a variety of genetically modified strains with minimal biological variability [9, 124]. However, the small joint size, the thin cartilage [133, 134], altered biomechanics [135, 136], and increased spontaneous intrinsic healing [137] hamper the study of the regenerative capacity of stem cells and these mechanisms of healing cannot be fully extrapolated to human cartilage repair [9, 124]. Rodents have mainly been

used to assess chondrogenesis of cell-based therapies by subcutaneous [138], intramuscular [139], and intra-articular [140] implantations of cells [9]. Of all small animals, the rabbit model is the most utilized model in cartilage regeneration studies because of the slightly larger knee joint size in comparison to rodents [16]. Despite their limited translational capacity, small animals can be very useful as a proof-of-principle study and to assess therapy safety before moving on to preclinical studies using larger animals [9, 125].

Large animal models play a more substantial role in translational research because of a larger joint size and thicker cartilage; however, their preclinical use is often hindered by high costs and difficulties in animal handling. A variety of large animal models have been used to investigate cartilage repair strategies, including horses [141–143], dogs [144], sheep [145–149], goats [150, 151] and (mini)pigs [152–155], each with their own strengths and limitations.

The knee anatomy [156–158], cartilage thickness [133, 159], biomechanical loading environment [124] and the subchondral bone properties [136] of the above-mentioned species differ variously from the human condition [124, 160]. An advantage of using the porcine model is the cartilage thickness of 1.5 mm–2 mm, compared to human cartilage thickness of 2.4 mm–2.6 mm [152, 159]. Dogs, in contrast, have thinner cartilage (0.95 mm–1.3 mm) compared to human cartilage [124, 159]. For the goat, cartilage thickness has been reported between 0.8 mm and 2 mm, whereas cartilage thickness in sheep ranges from 0.4 mm to 1.7 mm [124, 159]. Of all animal models used in cartilage regeneration studies, the horse's cartilage thickness (1.75 mm–2 mm) provides the closest approximation to the human situation [133, 136, 159, 161].

In a comparative anatomical analysis, the goat stifle displayed strong anatomic similarities to the human knee except for a long trochlear groove with medial and lateral ridges and the intercondylar notch width [124, 156]. According to Osterhoff et al., the ovine stifle is very similar to the human knee except for the femoral intercondylar notch width, the patellofemoral joint's biomechanics, and the proximal tibia's cortical bone stock [158]. More recently, Vandeweerd and colleagues described several anatomical features in the ovine stifle [157]. Although the goat and ovine stifles are very similar to the human knee, these few anatomical differences remain and should be taken under consideration when selecting them as a suitable animal model [156–158], which, for instance, can have an impact on the volume of the synovial cavity. In addition to similar knee anatomy, the caprine model has been reported to have similar stifle biomechanics compared to human knees [124, 162]. While the horse model offers defect sizes comparable to human defect dimensions, the increased weight and the fact that the horse spends much of its time in standing position place defects under significant loading and this continuous loading cannot be diminished [159]. Nevertheless, this constant loading environment in the horse stifle joint could be argued to be beneficial for translational cartilage repair studies since the human knee provides a less challenging load environment [163].

Moreover, since numerous repair strategies rely on the subchondral repair mechanisms, subchondral bone properties must be considered when selecting the appropriate repair model [136]. According to Chevrier et al., the subchondral properties of the rabbit trochlea are similar to the human medial femoral condyle (MFC) [136]. The goat offers advantages in subchondral bone consistency, thickness, and trabecular structure, which are more similar to the human structure in comparison to either small animals, ovine models, or canine models [9, 124]. A major disadvantage of the ovine and equine models is the dense and hard subchondral bone, while the caprine model has a softer subchondral bone [9, 159]. In addition, subchondral bone cysts in sheep [145, 164] and goat [165] have been reported when the subchondral bone is involved in cartilage repair mechanisms [166].

Ultimately, when selecting the best repair model, comparable anatomy and joint function are not the only important aspects, but other factors need to be taken into consideration when performing translational preclinical studies (Table 1). A factor requiring major consideration is the choice of defect location [124]. Clinically, most defects are made on the femoral condyles or the trochlear groove [160]. However, defect position influences cartilage repair response as demonstrated in caprine and ovine models leading to contradictory results [147, 162]. These differences in repair potential are due to differences in cartilage thickness, loading mechanics, and subchondral bone properties within the knee and between species [136, 147, 162]. In addition, defects may occur where higher loads are expected [167]. Ideally, these areas should be used when defects are induced. Therefore, it is important to identify the prevalence of naturally occurring defects in animal models and to assess where the lesion should be created based on the biomechanics of the joint of the animal [124, 167]. The ovine model is a well-documented model, where the most frequent naturally occurring cartilage defects in the ovine knee occur on the axial aspect of medial tibial condyle (MTC) and on the MFC [167]. Critical size chondral and osteochondral defects have been reported in rats, rabbits, dogs, (mini)pigs, sheeps, goats, and horses (as shown in [125, 159, 168]). Skeletal maturity and animal age also affect repair mechanisms of cartilage defects, especially when the subchondral bone is fractured for induction of repair [136, 137, 166, 169, 170]. Experimental models in animals that have reached skeletal and articular cartilage maturity are needed before the effect of any novel regenerative strategies on adult cartilage repair can be clinically evaluated. According to the International Cartilage Repair Society (ICRS) recommendations, selection of the age of an experimental animal should be based on cartilage maturity rather than on skeletal maturity (closure of the growth plate) [166]. Cartilage maturity can be defined as the time point where a cartilage defect is not spontaneously repaired and at the presence of a well-defined zonal architecture, an intact continuous layer of calcified cartilage, and minimal vascular penetration in the subchondral bone plate [166]. This would confirm that the articular cartilage has the adequate cellular, biomechanical, and biochemical properties. Therefore, in preclinical cartilage repair studies, animals at the age of

Table 1: Key factors for the selection of a translational animal model for cartilage repair.

Aspect	Remark/recommendation
Anatomy and biomechanics	(i) Large difference in anatomy and biomechanics remains between animal models and humans
Cartilage thickness	(i) Large animals provide closer proximity to the human condition (ii) Depends on topographic location in joint
Subchondral bone properties	(i) Effect on repair mechanisms (ii) Depends on topographic location in joint
Defect dimensions and location	(i) Critical size chondral or osteochondral (ii) Location of defect influences cartilage repair (iii) Femoral condyles or trochlea (iv) Defect should be made based on the biomechanics of the joint of the animal
Age and gender	(i) Age and gender may have effect on repair mechanism (ii) Inclusion of skeletally mature animals with mature cartilage (human—near puberty) (a) Rabbit—8 months (b) Dog—24 months (c) Pig—18 months (d) Sheep—24 months (e) Goat—24 months (f) Horse—24 months (iii) Gender effects must be taken into consideration (iv) Use animals with short range of ages and with similar sex
Study duration	(i) Depends on type of study (ii) Proof-of-principle (<6 months) versus late-stage study (6 months–12 months)
Surgical and practical considerations	(i) Unilateral versus bilateral repair models (a) Unilateral models: evaluation of locomotion, range of motion and gait, better immobilization, and no influence of contralateral technique (b) Bilateral models: minimize interanimal variability (ii) Postoperative management should be tolerated (iii) Ethical permission for small animals and ruminants is easier to obtain (iv) Surgical feasibility must be taken into account (v) Financial costs to house and handle differ variously between animals (vi) Availability of facilities, competent personnel, and equipment
Validated outcome measures	(i) At baseline, *in vivo* and post mortem (ii) Clinical response and kinematics (iii) Biological fluid collection (iv) Noninvasive compositional imaging MRI (v) Ex vivo high resolution magnetic resonance imaging (MRI) or microcomputed tomography (μCT) (vi) Tracking and monitoring (vii) Macroscopic/arthroscopic scoring (viii) Histological and histomorphometric scoring (ix) Mechanical testing (x) Biomolecular and biochemical testing

cartilage maturity, defined based on the aforementioned conditions, should be used (Table 1) [166].

While the choice of animal age, critical defect dimensions, and location in preclinical studies is often justified, gender selection is frequently overlooked. Regenerative strategies to address cartilage lesions and osteoarthritis have not sufficiently considered possible gender differences [171]. Therefore, potential gender effects must be taken more into consideration during analysis. Epidemiological studies demonstrated the presence of sex differences in osteoarthritis prevalence and incidence with females being at a higher risk to develop more severe knee osteoarthritis after reaching menopausal age [171]. Several researchers examined the role of sex hormones in osteoarthritis, including in ovine and murine models [167, 172–174]. Ma and colleagues showed that sex hormones, both testosterone and oestrogen, have a crucial influence on the advancement of osteoarthritis in mice. Testosterone aggravated the disease in male mice evidenced by the fact that orchiectomized mice showed a less severe osteoarthritis than intact males. Healthy female mice showed less severe osteoarthritis than ovariectomized females, demonstrating the protective role of female hormones [174]. In a biomechanical study in sheep, ovariectomy in females induced a detrimental effect on the intrinsic properties of the articular cartilage in the knee [172]. In human subjects, differences in knee joint volume and articular surface areas between men and women have been described [175]. Moreover, gender differences in cartilage composition and gait mechanics in young healthy, middle-aged healthy, and osteoarthritis cohorts are reported [176]. These

differences might influence functional outcome after repair [177]. Thus, effective and well-designed regenerative preclinical studies are required and should lead to a better understanding of gender-specific differences in the mechanisms involved in cartilage re- and degeneration. Since osteoarthritis and cartilage biology are reported to be sex-dependent, the inclusion of female animals is essential for preclinical cartilage repair studies. If both sexes are included, an equal number of males and females per study group with short ranges of ages should be used. Moreover, results should be reported for both genders and per study group [171]. In addition, for large animals, it is more difficult to manage male animals, since sexual behaviour and mounting may increase loads on high limbs.

Obviously, the recommended study duration for evaluating cartilage repair in preclinical animal models is different for proof-of-concept or pilot studies (<6 months) versus late-stage preclinical studies in large animal models (>6 months) [124, 125, 166]. However, for late-stage preclinical studies, caution must be exercised when the study ends within a year or when no interval follow-up investigations are implemented since the repaired tissue can vary at earlier phases of healing and the sustainability of the repaired tissue is time-dependent [148, 153, 166]. Follow-up methods of noninvasive imaging are necessary [178, 179]. Ovine models allow for imaging techniques such as magnetic resonance imaging (MRI) [166, 180], while the equine model is much more difficult, or impossible, due to the size of animal versus size and costs of high-field MRI. Furthermore, the nature of the regenerative strategy, such as the use of autologous or allogeneic cell therapy, also needs to be considered. Other key issues in cartilage repair models are the choice of bilateral versus unilateral surgery and acute versus chronic defects [148, 166]. Bilateral repair models are suitable to minimize interanimal variability and to increase the number of treated limbs but are only useful if the treatments are not reciprocally influencing the opposite limbs [181]. Unilateral models, in contrast, ensure that the treatment is not influenced by the contralateral technique. In addition, these models allow easier joint immobilization and are exposed to less initial weight bearing on the operated limb. More importantly, unilateral models permit better evaluation of locomotion, range of motion, and gait [166].

The choice of animal model is also influenced by practical aspects such as ethical considerations, costs, and availability of housing accommodations, materials, and competent personnel [160]. Nowadays, it is increasingly difficult to obtain ethical permission for the usage of dogs and horses, while working with reformed sheep or goats is much easier to justify. Surgical limitations, such as the ability of the animal to tolerate anaesthesia and postsurgical recovery protocols or the possibility of second-look access, could influence the choice of a specific animal model [141, 142, 166, 182]. The ovine model, for instance, is particularly easy to handle, cost-effective, and easy to anaesthetize.

4.2. Follow-Up and Outcome Measures. Preclinical animal studies analyzing the capacity of new technologies in cartilage regeneration frequently suffer from a lack of noninvasive follow-up and outcome measures and are therefore often forced to use endpoint outcome measures such as histology and destructive mechanical testing (Table 1). Additionally, there is an increasing need for standardized technologies with a diagnostic significance over the whole defect and adjacent tissues, while incorporating reflections of costs, care, and ethics and mimicking the clinical investigations in human clinical trials [166, 178].

For longitudinal *in vivo* studies, it is advised to assess the animal at baseline and at different time points. Depending on the animal, healthy joint status at the start of the study should be evaluated via diagnostic imaging modalities since variability in cartilage thickness, bone structure, and the prevalence of naturally occurring cartilage defects and other lesions associated with osteoarthritis can occur among species [167, 183–185]. More specifically, spontaneously occurring cartilage lesions have been described in canine, equine, and aging ovine models [9, 166, 167]. Canine and equine models should be screened for naturally occurring osteoarthritis, since they can have lesions associated with osteoarthritis or osteochondritis dissecans [9, 166]. Noninvasive imaging of articular cartilage defects can be performed by magnetic resonance imaging (MRI) [186–188] or computed tomography arthrography (CTA) [185, 189, 190]. CTA has been shown to be more accurate than MRI to detect cartilage defects in humans [185, 191]. Recently, Hontoir et al. described CTA to be an accurate imaging method for detecting articular cartilage defects in the ovine stifle [185]. Additionally, the same authors compared the sensitivity and specificity of 3-Tesla (3-T) MRI and CTA to identify structural cartilage defects in the equine metacarpo/metatarsophalangeal. Hontoir and colleagues showed that CTA is superior to MRI due to its shorter acquisition time, enhanced correlation to macroscopic assessment, and its specificity and sensitivity in identifying articular cartilage defects; nonetheless, MRI has the advantage to assess soft tissues and subchondral bone [189].

For the visualization of cartilage, diagnostic imaging techniques such as ultrasound, computed tomography (CT), and MRI can be used [125, 178]. More recently, novel quantitative MRI and CT techniques are being adopted as outcome measures after cartilage repair [178, 188, 190]. Compositional imaging MRI is being progressively applied to assess the biochemical composition of cartilage for the longitudinal follow-up of cartilage repair studies [179]. More specifically, T2 mapping combined with delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) seems to be a good compositional imaging modality to monitor cartilage repair and to discriminate between a collagen network with zonal organization and healthy cartilage [179, 192]. Combining multiple imaging techniques may yield a better understanding of both the collagen and PG content of the repaired defect [193]. T2 mapping provides information about the interaction of water molecules and the collagen network, while dGEMRIC evaluates GAG concentration within the cartilage [194]. In human patients, Kurkijarvi et al. demonstrated that combining datasets from dGEMRIC and T2 relaxation time mapping provides additional information on cartilage repair [192]. Recently, T2 mapping and dGEMRIC were used for assessing cartilage repair after allograft chondrocyte

implantation in a rabbit model, where dGEMRIC data showed a high correlation with histological and biochemical data [194]. In goat models, T2 mapping and dGEMRIC have also been used as outcome measures in a study evaluating cartilage repair after microfracture in an osteochondral defect of both the medial and lateral femoral condyles [195]. Alternatively, T1ρ has been used as a complementary imaging tool to T2 mapping which allows for the examination of PGs and the collagen organization [179]. However, one of the major issues of using T1ρ is reaching an adequate resolution with an acceptable acquisition time [179]. More recently, van Tiel and colleagues showed that dGEMRIC is more robust in accurately measuring cartilage GAGs *in vivo* in patients compared to T1ρ mapping [196].

Although substantial progress has been made in real-time *in vivo* cartilage imaging, spatiotemporal tracking of stem cells *in vivo* using MRI, bioluminescence imaging (BLI), fluorescence imaging (FLI), or nuclear imaging methods should be the focus when developing novel imaging techniques [178]. Superparamagnetic iron oxide (SPIO) particles are used for cartilage tissue engineering to monitor transplanted cells [197, 198]. However, SPIO particles are associated with several drawbacks such as the inability to distinguish viable cells from dead cells and from cells engulfed by phagocytes [199]. One of the possibilities to minimize particle transfer to other cells is the use of reporter genes. BLI compatible reporter genes such as red/green luciferases have already been used for cartilage tissue engineering to track transplanted cells [200]. In addition, by labeling cells with an additional chondrogenic reporter gene, cell differentiation can be monitored by means of dual bioluminescence labeling [201]. While this optical imaging method offers a sensitive technique to track stem cells, its use in larger animal models is limited because of a loss of signal intensity from deeper tissues due to scattering [202].

At baseline and at longitudinal intervals, clinically relevant examinations of cartilage repair and functional improvement should be carried out. These should be performed by a veterinary surgeon familiar with observing clinical signs and locomotion by assessment of changes in joint palpation, quantitative monitoring of pain, and changes in joint function or locomotion by gait analysis [125, 166, 203–206]. In rats, several scoring systems have been published to measure lameness, stride length and limb rotation, dynamic force application, and hind limb motion [206]. Moreover, for large animal models, kinematic marker analysis, ground reaction force measurements, and observational gait assessment have been progressively used in osteoarthritis-related gait alterations in canine, ovine, and equine models [206]. Several scaling systems have been documented in the literature, such as the American Association of Equine Practitioners (AAEP) lameness scale in the horse ranging from zero to five [207]. In ovine models, a numeric ranking scale can be used to determine comfort, movement, and flock behaviour [204]. A more detailed lameness scoring system has been published by Kaler et al. ranging from "normal" (0) to "unable to stand or move" (6) [203]. Overall, clinical assessment and gait monitoring are indispensable in order to increase the translational value of preclinical animal studies to human clinical trials and to the clinic.

Biomarkers represent an additional tool to evaluate normal and pathological processes or to evaluate the interventional repair strategies [208, 209]. These biomarkers may be identified and quantified via enzyme-linked immunosorbent assays (ELISA) or other protein assays in synovial fluid or other biological fluids such as in the blood and urine [208, 209]. Synovial and other biological fluid collections should be performed at baseline and multiple time points [166], since synovial fluid biomarkers have the capacity to reflect the articular environment before treatment and could possibly inform on postoperative outcomes [208]. In small animal models, however, it can be difficult to obtain sufficient amounts of biological fluid at multiple time points necessary for biomarker analysis [210]. To solve this, the use of paper or alginate to obtain small amounts of synovial fluid has been described to be successful and effective [211]. Because of the relatively larger joint size in large animal models, a collection of synovial fluid and serum biomarkers can be more easily performed [161]. Nevertheless, a major difficulty to perform repeated collections is the increased inflammation in the joint due to iatrogenic damage. Biomarkers of particular interest are markers for cartilage or synovium metabolism or markers involved in pathological pathways, such as inflammation [209]. Recently, biological (synovial) fluid markers in osteoarthritis were thoroughly reviewed by Nguyen and colleagues [209]. Besides analyte quantifications to assess changes in inflammation and cartilage turnover, volume and physical characteristics of the synovial fluid, such as viscosity, could also be used as an outcome measure in preclinical studies [166].

At the end of *in vivo* studies, cadaver tissue can undergo ex vivo high-resolution MRI [212, 213] and μCT [214] to evaluate structural improvements. Hereafter, macroscopic/arthroscopic scoring, histological and histomorphometric scoring methods, quantification of collagen and GAG expression by immunohistochemistry, collagen organization by polarized light microscopy and subchondral bone, and adjacent tissue integration are all outcome methods that should ideally be performed [214–218].

Nowadays, many histological scoring systems are available, contributing to the confusion on the use of an appropriate scoring method for a specific research question and study settings [219]. Moreover, it is unclear which scoring systems are validated and how study results can be compared between studies using different scoring methods [219]. The variety of histological scoring systems for the analysis of normal or osteoarthritic *in vivo* repaired or *in vitro* tissue-engineered cartilage was thoroughly reviewed by Rutgers et al. [219]. Normal cartilage can be distinguished from osteoarthritic cartilage via the Histological-Histochemical Grading System (HHGS) or HHGS-related systems and the Osteoarthritis Research Society International (OARSI) scoring method [219]. Of the various scoring systems available for the analysis of *in vivo* repaired cartilage, the ICRS II score seems most suitable in humans. In preclinical cartilage repair studies, the validated Pineda score or O'Driscoll score is advisable [219]. Other histological scoring systems

for preclinical cartilage repair are widely used. In addition to the Pineda score, the Wakitani score is an elementary scoring system, reflecting not more than five parameters [220]. The Pineda score assesses four histological parameters: cell morphology, matrix staining, lesion filling, and osteochondral junction [220]. The O'Driscoll score is a more complex histological scoring method which also assesses surface regularity, structural integrity, cellularity, chondrocyte clustering, adjacent bonding, and adjacent cartilage degeneration. In addition to the O'Driscoll score, also the Fortier and Sellers scores are more comprehensive scoring systems [220]. Orth et al. showed that both elementary and comprehensive histological scoring systems are appropriate to quantify articular cartilage repair [220]. However, complex scoring systems provide more descriptive data about the character of the repair tissue [220]. The use of validated scores, such as the Pineda score or the O'Driscoll score, may significantly increase comparability of information and should thus stimulate consistency between studies. Importantly, histological and biochemical evaluations are complementary tools to assess experimental articular cartilage repair *in vivo* [219]. A key goal of regenerating mature cartilage tissue is to regenerate a tissue with biochemical/biomolecular and mechanical properties resembling those of native cartilage tissue. Small biopsies for biochemistry (water content, GAGs/PG content, and collagen content) and/or biomechanical testing should ideally be gathered before fixation of the repaired tissue for histology [217]. In addition to typical end-point destructive measures to assess mechanical properties, indentation testing provides a nondestructive compressive technique for in situ mechanical evaluation [178, 221]. Large animal models allow the harvest of a large amount of repaired tissue in order to have parallel histological, biochemical, and biomechanical analyses of the repaired area postmortem [166, 222].

Finally, the combined utilization of *in vivo* clinical tests and assessment of locomotion, *in vivo* noninvasive imaging methods, and postmortem evaluation of tissue structure with validated scoring systems, biochemical composition, and mechanical properties will deliver a robust outcome analysis in order to improve the translational value of animal models in cartilage repair.

5. *In Vivo* Evidence of Stem Cells in Cartilage Regeneration

Within the field of cartilage regeneration, numerous preclinical studies have been published demonstrating the favorable effects of cell-based approaches on the repair of cartilage defects. Although the cartilage contains an inherent progenitor cell population [223–225], to our knowledge, robust scientific reports describing their *in vivo* regenerative potential in particular defects are currently lacking. Given their aforementioned *in vitro* properties, certain pluripotent and multipotent stem cell populations are considered to be credible candidates for stem cell-based repair and regeneration of cartilage tissue. iPSCs, for example, have been shown to successfully repair cartilage defects in a variety of rat models, following predifferentiation towards a chondrogenic lineage [14, 69, 226]. However, due to their pluripotent nature, the use of these stem cells still bears the risk of tumorigenesis [1]. Saito and coworkers, for instance, reported the formation of an immature teratoma in one animal, following a prolonged transplantation period of predifferentiated iPSCs in the knee joints of immunocompromised mice [74].

With regard to multipotent stem cell populations, one of the most frequently applied stem cell sources in the repair and regeneration of articular cartilage defects are MSCs. BM-MSCs in particular have been used in a wide variety of small and large animal models [16, 227]. Zhang et al., for example, recently demonstrated the regeneration of meniscal tissue after transplantation of BM-MSC-seeded poly(ε-caprolactone) (PCL) scaffolds in rabbits [228]. Formation of hyaline-like cartilage tissue was also observed after the treatment of a canine osteochondral defect with autologous BM-MSCs [144]. Sridharan and coworkers reported the successful repair of a rat trochlear knee defect after transplantation of high density BM-MSC/fibrin aggregates [229]. Similar results were found by Itokazu et al., indicating osteochondral repair in nude rats after transplantation of human BM-MSC cell sheets [230].

Although BM-MSCs are reported to have a predominantly positive effect on cartilage repair and regeneration, their invasive collection as well as the limited yield of stem cells during this procedure encourages the search for alternative tissue sources of MSCs [231, 232]. Substantial amounts of AT-MSCs, for example, can be relatively easy to be obtained through liposuction, and their intrinsic behavior does not seem to be affected by donor-related characteristics, such as age [231, 233]. Recent work of Mehrabani and coworkers demonstrated the successful formation of hyaline cartilage tissue after intra-articular injection of AT-MSCs in the knee joints of rabbits [234]. Implantation of scaffold-free spheroids of AT-MSCs into an osteochondral defect in two adult (mini)pigs led to the regeneration of the original cartilage tissue [235]. While hypoxic preconditioning of AT-MSCs had no effect on their *in vivo* chondrogenic potential [236], pretreatment of these stem cells with activated platelet-rich plasma substantially improved articular healing after transplantation in immunocompromised mice [237]. However, in comparison to BM-MSCs, AT-MSCs exhibit a significantly lower osteogenic and chondrogenic differentiation potential both *in vitro* and *in vivo* [55, 238–240].

Synovium-derived MSCs, on the other hand, not only display a higher proliferation potential in comparison to other sources of MSCs but also show a more pronounced production of cartilage-specific ECM when transplanted into an osteochondral defect in rabbits [238, 241, 242]. Similar findings were reported by Nakamura and coworkers, indicating the successful formation of cartilage tissue after intra-articular injection of allogeneic synovium-derived MSCs in the knee joints of pigs [243].

With regard to the mechanisms underlying the favorable effects of MSCs in cartilage repair, it remains unclear whether chondrogenic differentiation is a necessary prerequisite for cartilage tissue engineering as an increasing amount of evidence suggests that both the secretion of paracrine factors and the subsequent attraction of resident cells can also mediate tissue regeneration [7, 244]. In order to promote

these complex interactions, MSCs may be combined with chondrocytes in coculture systems, supported by exogenous growth factors and/or biomaterials to recreate the most optimal microenvironment for cartilage repair and regeneration [245]. Sabatino et al., for example, reported the successful production of cartilage grafts in a proof-of-principle mouse model. After subcutaneous transplantation of (precultured) collagen sponges containing BM-MSCs and articular chondrocytes, an increased GAG and collagen type II content was observed [246]. Similar results were found by Cai et al., indicating the formation of cartilage-specific ECM after subcutaneous transplantation of AT-MSCs and auricular chondrocytes supported by Pluronic F-127 [247]. In addition to subcutaneous transplantation, cocultures have also been directly applied in cartilage defects. Successful regeneration of meniscus tissue was demonstrated after transplantation of a polyvinyl alcohol/chitosan scaffold containing an AT-MSC/chondrocyte coculture in New Zealand rabbits suffering from a unilateral, medial meniscectomy. However, no significant differences were observed between the coculture scaffolds and the scaffolds merely containing articular chondrocytes [248].

In terms of delivery methods, numerous researchers used a scaffold-free intra-articular injection of stem cells. Nam and colleagues conducted a pilot study to test the effects of an intra-articular injection of autologous mesenchymal stromal cells on the repair outcomes of bone marrow stimulation (BMS) surgery in a caprine model. Results showed that the intra-articular injection of BM-MSCs following BMS intervention induced better cartilage repair outcomes [150]. In another study, MSCs were injected with hyaluronic acid (HA) and this resulted in good defect coverage at 12 weeks postinjection in a pig model [249]. The major advantage of an intra-articular injection of stem cells is the simplicity of the administration, but it would only be useful in early stages of cartilage injury. Additionally, intra-articular injection can lead to cell dispersion and an insufficient amount of cells reaching the defect required for repair [227]. One way to solve this is by using a local adherent technique for transplanting MSCs to the cartilage defect. Koga et al. showed in a pig model that placing an MSC suspension on the cartilage lesion for 10 minutes resulted in adherence of more than 60% of cells to the defect and induced cartilage regeneration [250]. Similarly, Nakamura and colleagues recently showed the same adherent technique with synovial MSCs in a pig model [243]. Unfortunately, by using these techniques, the transplanted cells lack an ECM, which makes it challenging to exploit the function of cells since the 3D environment is reported to be crucial in the processes of cell proliferation and differentiation [251]. To address this, a novel scaffold-free 3D tissue-engineered construct (TEC) has recently been developed, composed of native ECM, synthesized by MSCs [251]. In addition, MSCs seeded in acellular cartilage matrices/sheets also showed successful cartilage repair [252, 253].

Scaffolds are preferably biocompatible or biodegradable and can be implemented via a minimally invasive surgical procedure. Furthermore, they should provide rigid mechanical properties and offer some additional advantages such as adequate nutrient transport and adhesion to the defect [254]. Stem cells have been combined with a wide variety of natural and synthetic biomaterials to support and promote cartilage repair and regeneration [16, 231, 255–257]. This combinatorial approach has led to several successful *in vivo* applications of cell-seeded biomaterials for cartilage repair [14, 51, 52]. Of the various scaffold materials, the most commonly explored are hydrogels, which are cross-linked water-swollen systems [254]. Hydrogels gained a lot of interest because of their ability to homogeneously contain cells in a 3D environment and the minimal invasive injection procedure [254, 258, 259]. Natural hydrogels based on polysaccharides, such as chitosan, HA, alginate, and agarose, have been reported to support cartilage regeneration [254]. Recently, it was shown that MSCs isolated from the dental pulp cultured in an alginate scaffold successfully regenerated articular cartilage [260]. HA-based hydrogels are one of the most extensively used hydrogels in cartilage repair [254] and have been reported to improve cartilage specific-matrix deposition of MSCs [254, 259, 261]. In a direct comparative study in rats between several hydrogels such as alginate, pluronic, HA, and chitosan with human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs), the combination of hUCB-MSCs-HA resulted in superior cartilage repair on a macroscopic and histological level [262]. Similarly, combining hUCB-MSCs with a HA hydrogel promoted cartilage regeneration in an osteochondral defect minipig model [155]. With regard to natural biomaterials and hydrogels based on proteins such as collagen, gelatin, fibroin, and fibrin, Wilke et al. described an early chondrogenic response after intra-articular injection of a fibrin gel containing BM-MSCs in horses [142]. Fibrin is a commonly used natural protein with chondrogenic-inducing properties [254, 259]. However, one of the major disadvantages of using fibrin gels is the fast degradation [263], resulting in less beneficial results *in vivo* [264]. More recently, platelet-rich fibrin (PRF) has gained more interest to provide a 3D environment for stem cells, consisting a strong fibrin network and supportive platelets. PRF has the ability to support the proliferation of MSCs and favors cytokine enmeshment and cellular migration [144]. Kazemi and colleagues showed that the use of BM-MSCs seeded on PRF could be a novel method for articular cartilage regeneration, where the PRF creates a suitable environment for stem cell proliferation and differentiation by secreting growth factors [144]. Alternatively, collagen is abundant in native articular cartilage and is therefore widely used in preclinical animal studies as a stem cell carrier [254]. The application of collagen combined with BM-MSCs led to fully repaired cartilage tissue in a porcine model [265]. However, the softness of collagen gels is one of the major concerns for *in vivo* cartilage repair [254]. Recently, a natural type II collagen hydrogel, fibrin sealant, and adipose-derived stem cells have been recommended as a positive combination for articular cartilage repair in rabbits [266]. In addition, transplantation of synovium-derived MSCs in a combination of collagen type I/HA/fibrinogen composite gel induced the formation of hyaline cartilage tissue in a lapine osteochondral defect model [242]. One of the major limitations of natural hydrogels is the low mechanical strength [259], which needs further modifications or combinations with

other natural or synthetic polymers (composite scaffold). A number of advantages were also reported for synthetic polymers, such as a controlled degradation and good mechanophysical properties [267]. Poly-(lactic-(co-glycolic)) acid (PL(G)A), for example, is one of the most widely applied synthetic scaffold materials in stem cell-based cartilage tissue engineering [16]. Recent work of Yin et al. showed the regeneration of articular cartilage after transplantation of a TGF-β1-immobilized PLGA scaffold seeded with AT-MSCs into a full-thickness cartilage defect in New Zealand white rabbits [268]. Similar findings of successful cartilage engineering were reported earlier for BM-MSCs, indicating the successful formation of hyaline cartilage tissue by connective tissue growth factor- (CTGF-) modified stem cells contained within a sodium hydroxide-treated PLGA scaffold [269]. Caminal et al., however, only demonstrated a transient improvement caused by BM-MSC-seeded PLGA scaffolds in sheep with a critical size chondral defect [270]. Recently, hiPSCs-MSCs were seeded onto a PLGA scaffold and transplanted into a cartilage defect in a rabbit model. Results showed that the hiPSCs-MSCs-PLGA scaffold experimental group had the potential to repair cartilage defects *in vivo* [132]. One of the major disadvantages of using such synthetic polymers is the possibility to elicit an immune response [267]. Moreover, synthetic biomaterials lack biocompatibility and biological activity [259]. As previously mentioned, it is better to combine a synthetic polymer with a natural polymer to improve biological activity [259]. Many other scaffold materials have been tested in an attempt to improve cartilage repair by using MSCs. A wide overview of most used scaffolds can be found in the review article published by Goldberg and colleagues [16].

In order to make human stem cells applicable to human clinical translation, more in-depth knowledge about their *in vivo* behaviour should be derived from larger immunocompetent animals. Several researchers used human MSC transplantation in smaller nonimmunosuppressed animals and reported no graft rejection [271, 272]. Transplantation studies in larger animal models such as immunocompetent dog and swine reported similar results and even point out immunosuppressive capacities which are related to the MSC transplantation [273, 274]. Dayan and colleagues demonstrated that transplantation of human MSCs in ovine immunocompetent animal models showed clinical safety and efficacy suggesting that immunocompetent sheep can serve as a suitable preclinical large animal model for testing human stem cells [275]. In the unfortunate case of detecting an immunogenic response following human stem cell transplantation into the animal model, generally known immunosuppressive drugs can be administered at the time of transplantation. Alternatively, autologous transplantation of stem cells can be considered. However, one of the disadvantages of using autologous stem cells isolated from larger animals is the lack of well-characterized species-specific stem cells and protocols for their culturing and differentiation [276]. For autologous iPSCs in preclinical research in animal models, it appears that iPSCs in farm animals have not yet received the deserved attention [277]. Ogorevc et al. described only 32 studies addressing the development of iPSCs in pig, cattle, horse, sheep, goat, and rabbit [277]. In addition, large animal commercial products, such as antibodies, reagents, and microarrays, are not widely available [276]. Nevertheless, beyond any doubt, for translational research in cell therapy, testing human stem cells in preclinical animal models which are immunocompetent should gain more attention.

6. Conclusion

Despite the multiple promising mechanisms of action of stem cell-based therapies for cartilage repair, supported by advances in bioengineering and biomaterials to exploit the full potential of stem cells, it is not yet possible to achieve engineered cartilage possessing identical properties as native cartilage [17, 278]. Several concerns have to be addressed when considering these therapies for large-scale human translation.

Before moving to the clinic with a universally applicable therapy, issues involving the heterogeneity of MSC sources as well as the heterogeneity within MSC populations, isolation methods, and differentiation protocols should be addressed [24]. Other factors such as aging [279], serial passaging [61], and the presence of comorbidities in the donor [60] can restrict the chondrogenic differentiation potential of MSCs. Furthermore, MSCs mainly produce collagen type I, while the main collagen subtype in the cartilage is collagen II. This needs to be taken into account to avoid the production of fibrocartilage or ossified hypertrophic cartilage [280]. Biomaterials can be used in combination with stem cells in order to support and promote cartilage repair and regeneration [16, 278]. In the future, biomaterials can offer enhanced control of cell fate, enable sustained and localized release of paracrine factors, and facilitate remodeling of newly formed tissue [7].

Despite conflicting preclinical results, the use of allogeneic MSCs is gaining support as it avoids donor site morbidity and allows for single-stage procedures, thereby reducing the financial burden and increasing the simplicity of cell-based therapies [16]. Since different stem cell sources show inherent differences in their differentiation potential, secretome, and ECM profile, various MSC sources should be compared to select the most promising one for allogeneic therapies [7]. However, applying therapies involving allogeneic MSCs on a large scale requires cell banking possibilities and long-term safety and efficacy studies in order to assess possible immune rejection.

iPSCs have been explored as a possible alternative to MSCs due to their superior self-renewal capacity [1], proliferation rate [72], and chondrogenic differentiation potential [14, 64]. However, the most important obstacle in the use of iPSCs is the risk for teratoma formation after transplantation [1]. Therefore, before this technology can progress to clinical translation, research into the control of cell phenotype and cell fate is required in order to alleviate the concerns for tumorigenesis [16].

Under ideal circumstances, novel therapies would reach the market after *in vitro* data were used to inform preclinical studies, which in turn led to human clinical trials [16].

Researchers should be aware that every animal model is associated with its advantages and disadvantages and the choice of the model should match the research hypothesis and is important to ensure the translation to the clinic [9, 124]. Furthermore, the current lack of standardized protocols (i.e., cell delivery route and number of transplanted cells) as well as the wide variety of different outcome measures used to evaluate preclinical studies makes it difficult to draw definite conclusions regarding the potential use of stem cell-based approaches in cartilage tissue engineering through direct comparison of studies. Furthermore, gender differences in most animal studies have not been adequately investigated and should gain more attention. Moreover, the same applies for *in vitro* studies, where researchers, using primary cells or cell lines, often do not compare results between sexes. For cell lines, the gender of the cell line provided is frequently not mentioned, leading to conclusions which cannot be drawn for the whole population [171].

Despite these hurdles, at least 19 clinical trials have been registered using stem cell-based therapies for cartilage repair procedures [278]. Unfortunately, the quality of the existing clinical data is rather limited, but more recently registered clinical trials are showing improvement in the study design and methodology. This might in part be explained by the methodical recommendations developed by the ICRS [281]. This consensus statement includes guidelines for the statistical study design, patient recruitment, and considerations for appropriate control groups in order to help clinicians achieve high-quality data [281].

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Jean-Michel Vandeweerd and Ivo Lambrichts contributed equally to this work.

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Research Article

Different Chondrogenic Potential among Human Induced Pluripotent Stem Cells from Diverse Origin Primary Cells

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Scientists have tried to reprogram various origins of primary cells into human induced pluripotent stem cells (hiPSCs). Every somatic cell can theoretically become a hiPSC and give rise to targeted cells of the human body. However, there have been debates on the controversy about the differentiation propensity according to the origin of primary cells. We reprogrammed hiPSCs from four different types of primary cells such as dermal fibroblasts (DF, $n = 3$), peripheral blood mononuclear cells (PBMC, $n = 3$), cord blood mononuclear cells (CBMC, $n = 3$), and osteoarthritis fibroblast-like synoviocytes (OAFLS, $n = 3$). Established hiPSCs were differentiated into chondrogenic pellets. All told, cartilage-specific markers tended to express more by the order of CBMC > DF > PBMC > FLS. Origin of primary cells may influence the reprogramming and differentiation thereafter. In the context of chondrogenic propensity, CBMC-derived hiPSCs can be a fairly good candidate cell source for cartilage regeneration. The differentiation of hiPSCs into chondrocytes may help develop "cartilage in a dish" in the future. Also, the ideal cell source of hiPSC for chondrogenesis may contribute to future application as well.

1. Introduction

Reprogramming mature somatic cells into human induced pluripotent stem cells (hiPSCs) opened a new strategy in tissue engineering and regenerative medicine. The delivery of transcription factors (i.e., OCT4, SOX2, KLF4, and c-Myc) transits somatic cells into a state similar to embryonic stem cells. The pluripotency and unlimited proliferation makes hiPSCs an ideal cell source for the production of transplantable regenerative cell sources.

In the early years of hiPSC generation, skin fibroblasts were usually used for reprogramming. The first hiPSCs generated by Yamanaka himself was generated from human skin dermal fibroblasts (DF). DFs may be convenient to cultivate in vitro; however, it can be obtained only through an invasive punch biopsy procedure. Somatic cells that are easy to obtain from an individual were required as a substitute. Today, hiPSCs are generated from various cell sources such as blood cells, keratinocytes, urine cells, and more [1–3].

Previous reports suggest that the original primary cell source of hiPSCs can subsequently affect the in vitro differentiation ability. Some report that the differentiation ability is biasing the cells towards the tissue of origin [4–8]. Epigenetic memory such as DNA methylation is thought to be responsible for the different differentiation capacity [9, 10].

Through our previous studies, we successfully generated hiPSCs from peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) [11, 12]. We also generated hiPSCs using fibroblast-like synoviocytes (FLSs) isolated from the synovium in the knee joint of osteoarthritis (OA) patients [13, 14]. CBMC-derived hiPSCs were generated with CBMCs with homozygous human leukocyte antigen (HLA) types for future use in regenerative

medicine. Using the CBMC-derived hiPSCs, we confirmed the potential in chondrogenic differentiation by generating chondrogenic pellets that are about 1-2 mm in size [15, 16]. After 21 days of differentiation using CBMC-hiPSCs in chondrogenic differentiation medium containing human BMP2 and TGFβ3, the chondrogenic pellets showed increased expression of chondrogenic markers (i.e., ACAN, COL2A1, COMP, and SOX9). Extracellular matrix (ECM) proteins were positively detected in the CBMC-hiPSC-derived chondrogenic pellets and the quality was comparable with that generated using actual mesenchymal stem cells (MSCs), which is a generally used cell source for in vitro chondrogenesis.

It is well known that the adult articular cartilage lacks natural healing ability [17–20]. Chondrogenesis using hiPSCs was attempted for several years with hiPSCs generated from various origin cells (i.e., adipose-derived stem cells, fibroblasts, and articular chondrocytes) [15, 21–25]. All cell sources successfully went through chondrogenesis. However, to our knowledge, the capacity of chondrogenic differentiation between hiPSCs generated from different origins was not confirmed. This study aims to compare the chondrogenic efficacy of hiPSCs generated from PBMCs, DFs, FLSs, and CBMCs.

2. Materials and Methods

2.1. Dermal Fibroblast and Fibroblast-Like Synoviocyte Isolation and Maintenance. Skin samples were extracted by skin punch biopsy procedures. Synovium samples were received by arthroscopic synovectomy or total knee replacement surgery. The delivered skin and synovium was chopped and homogenized. Chopped tissues were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) containing with 0.01% collagenase. Tissues were digested with collagenase for 4 hours at 37% with vigorous shaking. Cells were washed and resuspended in DMEM supplemented with 20% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin solution (Gibco). Cells were cultured until more than 80% confluence was achieved.

2.2. Peripheral Blood and Cord Blood Mononuclear Cell Isolation and Maintenance. Blood was delivered in heparin tubes. Fresh blood was diluted with phosphate buffered saline (PBS) and placed onto Ficoll-Paque reagent. The samples were centrifuged for 30 minutes at 850 ×g. Isolated PBMCs were transferred to a new tube and washed with PBS. Cell pellet was resuspended in StemSpan media (STEMCELL Technologies, Vancouver, Canada) containing CC110 cytokine (STEMCELL Technologies) for expansion. Cells were incubated for 5 days before reprogramming. Fresh media was added if needed.

2.3. hiPSC Generation Using Dermal Fibroblasts and Synoviocytes. Cells were washed and treated with trypsin/EDTA. Cells were counted and 3×10^5 cells per well was obtained. Cells were then resuspended in 20% DMEM and seeded into a 6-well plate. Yamanaka factors were delivered by sendai virus (Invitrogen, Carlsbad, CA, USA). The next day, prealiquoted sendai virus was treated to the cells and incubated in 5% CO_2, 37°C for 48 hours. After 48 hours, virus-containing media were removed and changed every other day for 6 days. On day 7, media were changed to hiPSC media. Cells were cultured until colonies appeared. Media were changed daily after they were transited to E8 media.

2.4. iPSC Induction Using Peripheral Mononuclear Blood Cells and Cord Blood Mononuclear Cells. Reprogramming and characterization was performed as previously described [12]. Cells transfected with sendai virus containing Yamanaka factors were maintained in a vitronectin-coated dish (Thermo Fisher Scientific, Waltham, MA, USA), and media were changed daily with fresh E8 medium (STEMCELL Technologies) before use. Cells were maintained in vitronectin-coated dishes. Media were changed daily.

2.5. Chondrogenic Differentiation Using Pellet Culture. The protocol was followed by the procedure shown in our previous studies [15, 16]. A 1 : 1 mixture of TeSR-E8 and AggreWell media (STEMCELL Technologies) was added to 2×10^6 hiPS cells for EB generation. EBs were maintained in E8 media for 3 days and then in E7 media for additional 3 days. EBs were harvested and resuspended in outgrowth (OG) cell induction media consisted of DMEM, 20% FBS, and 10% penicillin/streptomycin. EBs were counted and 50–70 EB per cm^2 was seeded onto a gelatin-coated dish. OG cells were induced from the attached EBs for 3 days at 5% CO_2, 37°C. Next, cells were detached and the remaining EB clumps were removed using a 40 μm cell strainer (BD Technologies, Franklin Lakes, NJ, USA). Single OG cells were harvested and plated onto a new gelatin-coated dish (1–5×10^4 cell per cm^2). Cells were used up to passage 5. OG cells were counted and 3×10^5 cells per pellet were prepared. Cells were harvested in a 15 mL conical tube and media were changed into chondrogenic differentiation media (CDM; DMEM supplemented with 20% knockout serum replacement, 1x nonessential amino acids, 1 mM L-glutamine, 1% sodium pyruvate, 1% ITS+ Premix, 10^{-7} M dexamethasone, 50 mM ascorbic acid, and 40 μg/mL L-proline) supplemented with 10 ng/mL recombinant human bone morphogenetic protein 2 (BMP2), and transforming growth factor beta 3 (TGFβ3). Cells resuspended in CDM were centrifuged at 750 ×g for 5 minutes. Generated pellets were maintained for 21 days and media were changed every 3 days.

2.6. Polymerase Chain Reaction. Chondrogenic pellets or cells were harvested and stored at −80°C. Samples were snap frozen with liquid nitrogen and ground using a pestle. Ground samples were incubated with TRIzol (Thermo Fisher Scientific), and mRNA was extracted according to the manufacturer's instructions. Using RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific), 2 μg of the extracted total RNA was used to synthesize cDNAs. Real-time PCR reactions included 2 μL of diluted cDNA (1 : 10 dilution). Real-time PCR was carried out using LightCycler® 480 Instrument II (Roche, Basel, Switzerland). The geometric mean of GAPDH was used as an internal control to normalize the results.

Table 1: Primers used for real-time PCR against pluripotent markers and chondrogenic markers.

Description	Target name	REFSEQ_ID	Direction	Primer sequence	Size
Pluripotency marker	OCT4	NM_203289.5	Forward	GGGAAATGGGAGGGGTGCAAAAGAGG	151
			Reverse	TTGCGTGAGTGTGGATGGGATTGGTG	
	KLF4	NM_004235.4	Forward	TTCCCATCTCAAGGCACAC	158
			Reverse	GGTCGCATTTTTGGCACT	
	SOX2	NM_003106.3	Forward	GGGAAATGGGAGGGGTGCAAAAGAGG	151
			Reverse	TTGCGTGAGTGTGGATGGGATTGGTG	
	NANOG	NM_024865.2	Forward	AAAGGCAAACAACCCACT	270
			Reverse	GCTATTCTTCGGCCAGTT	
Viral vector detection marker	SeV		Forward	GGATCA CTA GGTGATATCGAGC	181
			Reverse	ACCAGACAAGAGTTTAAGAGATATGTATC	
	KOS		Forward	ATGCACCGCTACGACGTGAGCGC	528
			Reverse	ACCTTGACAATCCTGATGTGG	
	Klf4		Forward	TTCCTGCATGCCAGAGGAGCCC	410
			Reverse	AATGTATCGAAGGTGCTCAA	
	c-Myc		Forward	TAACTGACTAGCAGGCTTGTCG	532
			Reverse	TCCACATACAGTCCTGGATGATGATG	
Chondrogenic marker	SOX9	NM_000346	Forward	GACTTCCGCGACGTGGAC	99
			Reverse	GTTGGGCGGCAGGTACTG	
	SOX5	NM_001261415.2	Forward	CAGCCAGAGTTAGCACAATAGG	104
			Reverse	CTGTTGTTCCCGTCGGAGTT	
	SOX6	NM_033326.3	Forward	GGATGCAATGACCCAGGATTT	141
			Reverse	TGAATGGTACTGACAAGTGTTGG	
	ACAN	NM_001135.3	Forward	TCGAGGACAGCGAGGCC	85
			Reverse	TCGAGGGTGTAGCGTGTAGAGA	
	COL2A1	NM_001844	Forward	GGCAATAGCAGGTTCACGTACA	79
			Reverse	CGATAACAGTCTTGCCCCACTTA	
	CHAD	NM_001267	Forward	GATCCCCAAGGTGTCAGAGAAG	66
			Reverse	GCCAGCACCGGGAAGTT	
	PRG4	NM_005807.4	Forward	AAAGTCAGCACATCTCCCAAG	199
			Reverse	GTGTCTCTTTAGCGGAAGTAGTC	
	COL1A1	NM_000088.3	Forward	TCTGCGACAACGGCAAGGTG	146
			Reverse	GACGCCGGTGGTTTCTTGGT	
	COL10A1	NM_000493.3	Forward	CAGGCATAAAAGGCCCAC	108
			Reverse	GTGGACCAGGAGTACCTTGC	
	RUNX2	NM_001024630	Forward	CCAGATGGGACTGTGGTTACTG	65
			Reverse	TTCCGGAGCTCAGCAGAATAA	
Housekeeping gene	GAPDH	NM_002046.5	Forward	ACCCACTCCTCCACCTTTGA	101
			Reverse	CTGTTGCTGTAGCCAAATTCGT	

The detection of viral vectors was followed by the manufacturer's instruction produced in the reprogramming sendai virus kit (Invitrogen). Reverse transcription reaction was performed with the synthesized cDNAs. Integration of viral vectors was confirmed using four primers; SeV, KOS, KLF4, and c-Myc. The primer sequences are provided in Table 1.

2.7. Histological Analysis of Pellets. Chondrogenic pellets were washed with PBS. Pellets were fixed in 4% paraformaldehyde for 2 hours at room temperature (RT). Dehydration was performed with increasing sequential ethanol solutions. Additional clearing was done with sequential ethanol-xylene mixtures and pellets were infiltrated with paraffin overnight. Paraffin blocks were fixed and 7 μm sections were obtained using a microtome. Before staining the sections, slides were placed in a 60°C oven for at least 10 minutes. Slides were immediately deparaffinized using xylene. Slides were rehydrated with decreasing sequential ethanol series and were rinsed with running tap water for 1 minute each.

Table 2: Information of generated hiPSCs.

hiPSC type	No	Sex	Cell type	Description
DF-hiPSC	1	F	Dermal fibroblast	Donor number 1
	2	M		Donor number 2
	3	M		Donor number 3
PBMC-hiPSC	1	F	Peripheral blood Mononuclear cell	Donor number 1
	2	M		Donor number 2
	3	F		Donor number 4
FLS-hiPSC	1	F	Synovial fibroblast	OA patient number 1
	2	F		OA patient number 2
	3	F		OA patient number 3
CBMC-hiPSC	1	F	Cord blood mononuclear cell	Donor number 5
	2	M		Donor number 6
	3	M		Donor number 7

Toluidine blue staining was done by incubating the hydrated slides in 0.04% toluidine blue (Sigma Aldrich, St. Louis, Missouri, USA) solution for 10 minutes. Slides were washed in running tap water and dried for 10 minutes until complete dryness. After the staining process, slides were dehydrated with an increasing sequential ethanol series. Ethanol was cleared with 2 cycles of 100% xylene and slides were mounted with VectaMount™ Permanent Mounting Medium (Vector Laboratories, Burlingame, CA, USA).

2.8. Immunohistochemistry. Slides were placed in a 60°C oven for 10 minutes and deparaffinized with 2 cycles of xylene. Slides were rehydrated and were incubated in boiling citrate buffer (Sigma Aldrich) for antigen unmasking. After cooling the unmasked slides, endogenous peroxidase activity was blocked by treating the slides with 3% hydrogen peroxide (Sigma Aldrich). Slides were washed and blocked with tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA). Primary antibodies were diluted in blocking solution (collagen type II, 1/100; collagen type I, 1/200; collagen type X, 1: 250, Abcam). Slides were incubated with diluted primary antibody at 37°C for 1 hour. Slides were washed with TBS containing 0.1% tween-20. Secondary antibodies (1/200; Vector Laboratories) diluted in blocking buffer were treated for 40 minutes at RT. After washing out the secondary antibody, slides were treated with ABC reagent drops (Vector Laboratories) for 30 minutes. DAB solution (Vector Laboratories) was followed and incubated for 5 minutes. Slides were washed and counterstained with Mayer's hematoxylin (Sigma Aldrich) for 1 minute. Slides were dehydrated and cleared. Slides were mounted and staining was confirmed under a bright-field microscope.

2.9. Statistical Analysis. Comparison of each of cell type-derived chondrogenic pellets and hiPSCs was analyzed by Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test. Analysis was done using the software GraphPad Prism 5 ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ for statistically significant differences).

3. Results

3.1. Generation of hiPSCs from a Different Origin. Human iPSCs were generated from DFs, PBMCs, FLSs and CBMCs. The information of each cell line is shown in Table 2. Somatic cells were obtained from three individual donors per cell type. Some of the PBMCs and DFs were obtained from the identical donor. FLSs were isolated from the joint of three osteoarthritis patient's synovium. Three hiPSC clones were generated from each hiPSC and three chondrogenic pellets were analyzed individually from each cell line.

Primary cells were isolated from skin tissue, blood, synovium, and cord blood (Figure 1(a)). Primary cells were transduced with sendai viral vectors containing Yamanaka reprogramming factors. After several passaging and purification, reprogrammed cells exhibited a colony with compact morphology (Figure 1(b)). Pluripotency of the generated hiPSCs was confirmed with several markers. Colonies of each cell line showed positive expression of OCT4 and TRA-1-60 (Figure 1(c)). The colonies were also positively stained against alkaline phosphatase (AP) (Figure 1(d)). By real-time PCR, the relative expression of OCT4, SOX2, NANOG, and KLF4 was assessed in hiPSCs generated from different somatic cells (Figures 1(e)–1(h)). Interestingly, the expression of pluripotent markers was higher in PBMC and CBMC primary cells. Also, KLF4 expression was comparably high in FLSs as well. All of the pluripotent markers were expressed highest in DF-hiPSCs. The tendency of each pluripotent marker showed an identical pattern of expression. The silencing of integration of reprogramming viral vectors was confirmed in the hiPS cell lines (Figure S1). We have confirmed that the generated hiPSCs were viral vector free. Taken all together, we successfully reprogrammed nonintegrated hiPSCs from DFs, PBMCs, FLSs, and CBMCs with confirmed pluripotency.

3.2. Chondrogenic Pellet Generation Using hiPSCs from a Different Origin. In our previous studies, we confirmed the chondrogenic differentiation ability of CBMC-hiPSCs [15, 16]. The protocol we used is simply described in

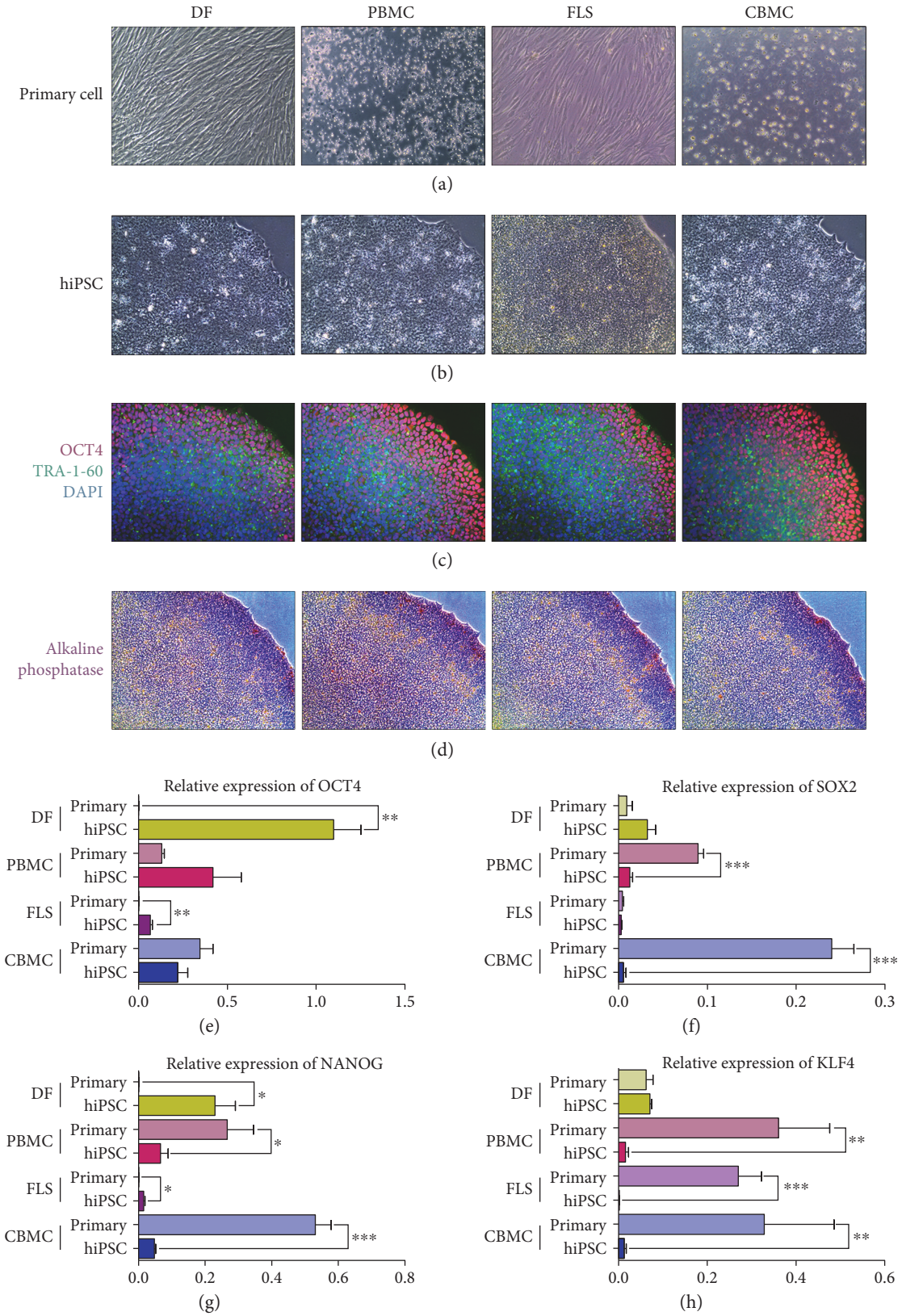

Figure 1: Characterization of DF-, PBMC-, FLS-, and CBMC-hiPSCs. (a) The morphology of each primary cell type: dermal fibroblast (DF), peripheral blood mononuclear cell (PBMC), fibroblast-like synoviocyte (FLS), and cord blood mononuclear cell (CBMC). (b) The morphology of the generated hiPSCs. (c) Immunohistology image of the hiPSC colonies stained against OCT4 and TRA-1-60. (d) Image of colonies stained against alkaline phosphatase (AS). (e) Relative expression of pluripotent markers in each cell line and primary cells ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$).

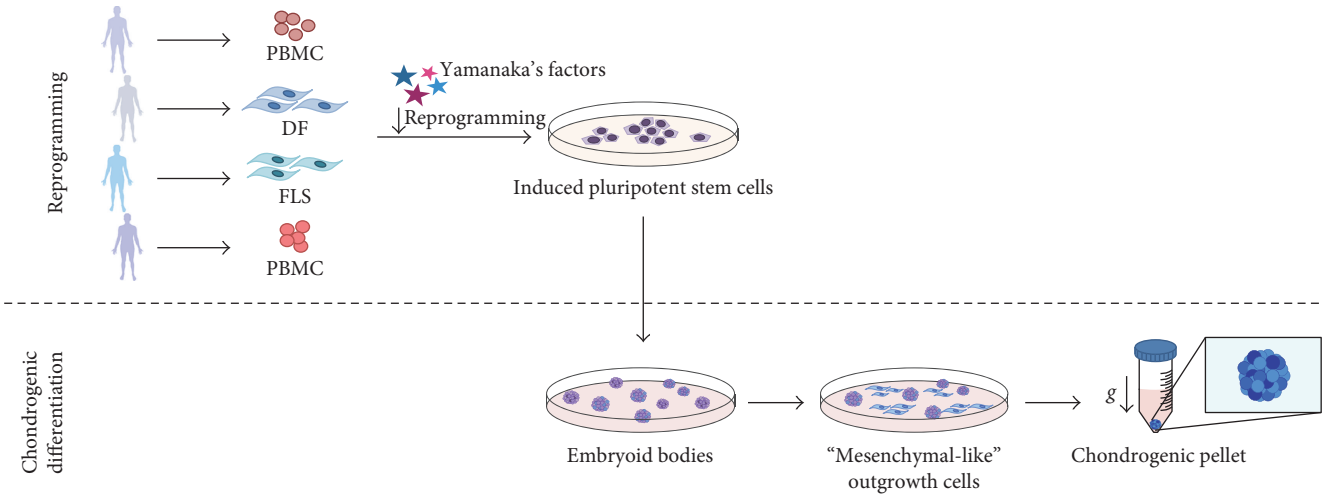

Figure 2: Scheme of experiment. Human iPSCs generated from different originated somatic cells were condensed into embryoid bodies (EB). EBs were attached to gelatin-coated dishes and outgrowth (OG) cells were induced. Using the OG cells, chondrogenic pellets were generated with centrifugal force and human growth factors.

Figure 2. Roughly, embryoid bodies (EBs) were generated using hiPSCs. EBs were cultivated for about 1 week and attached onto a gelatin-coated plate for outgrowth (OG) cell induction, which are cells that sprout out from the bottom of EBs. OG cells are known to have the similar characteristics of MSCs [26]. Koyama et al. referred them as "mesenchymal progenitor cells." Sprouted OG cells were then cultivated and used for chondrogenic differentiation. After 21 days of differentiation towards the chondrogenic lineage, pellets showed similar characteristics to that of cartilage (i.e., lacuna).

On day 3 of differentiation, OG cells derived from each hiPSC were condensed into a pellet (Figure 3(a)). OG cells derived from DF-hiPSCs and CBMC-hiPSCs condensed faster than the other two, and PBMC-hiPSCs took the longest time to form a pellet. After 21 days of differentiation, chondrogenic pellets were harvested (Figure 3(b)). CBMC- and DF-derived chondrogenic pellets had the largest size, indicating larger amounts of accumulated ECM proteins (Figure S2). On the other hand, FLS-derived pellets had the smallest size; however, there was no significant difference when the size were measured. ECM proteins were detected by toluidine blue staining (Figure 3(c)). Pellets generated from CBMC-hiPSCs showed high ECM accumulation through staining. PBMC- and DF-hiPSC-derived pellets also showed highly accumulated ECM proteins. However, FLS-hiPSC-derived pellets showed less accumulation. Cell death in the inner area was also detected, despite the small pellet size. The results of staining against collagen type II showed a similar tendency (Figure 3(d)). The staining in FLS-hiPSC-derived chondrogenic pellets showed hallow areas in the inner area. The other three cell types showed similar expression of collagen type II. The expression of collagen type I (Figure 3(e)) and type X (Figure 3(f)) was confirmed as well. The expression of both collagen types was highly detected in the inner area of the FLS-derived chondrogenic pellet. The other cell type-derived pellets had similar levels of collagen types I and X.

3.3. Chondrogenic Gene Expression in Chondrogenic Pellets Originating from Different Cell Source-Derived iPSCs. The gene expression of chondrogenic pellets generated from CBMC-derived hiPSCs ($n = 27$) was examined and compared with pellets derived from DF- ($n = 27$), PBMC- ($n = 27$), and FLS-hiPSC- ($n = 18$) derived pellets. SOX9 is generally known as the transcriptional activator that is crucial for chondrogenesis. The expression of SOX9 was highest in CBMC-hiPSCs (Figure 4(a)). The expression was significantly higher than that of PBMC-hiPSCs or FLS-hiPSCs. SOX5 and SOX6 are closely related to the DNA-binding proteins that enhance the function of SOX9 [27]. The expression of SOX5 and 6 was also confirmed in the chondrogenic pellets. The expression of SOX5 in CBMC-hiPSC-derived pellets was significantly higher than DF- and FLS-hiPSC-derived chondrogenic pellets (Figure 4(b)). PBMC-hiPSC-derived pellets had similarly high expression of SOX5. This was also shown in SOX6 as well (Figure 4(c)). Aggrecan (ACAN) and collagen type II (COL2A1) are the major proteoglycan proteins in the articular cartilage that provides the hydrated gel structure. The expression of ACAN correlated to that of SOX9 (Figure 4(d)). ACAN expression was significantly higher in CBMC-hiPSC-derived chondrogenic pellets. Similar to SOX9, the expression pattern was not significant between DF-hiPSC-derived pellets and CBMC-hiPSC-derived pellets. Also, CBMC-hiPSC-derived pellets were the only type that showed significantly increased marker expression compared to its primary cell source. ACAN expression in PBMC- and FLS-hiPSC-derived pellets was relatively lower than the other two. In the case of COL2A1 expression, however, DF-hiPSC-derived pellets had the lowest expression (Figure 4(e)). Identical to ACAN expression, CBMC-hiPSC-derived pellets were the only type that showed significantly increased COL2A1 expression compared to its primary cell source. Lubricin (PRG4) is a proteoglycan that acts as lubricant in the joint. Interestingly, the expression of lubricin was significantly high in FLS-hiPSC-derived pellets (Figure 4(f)). On the other hand,

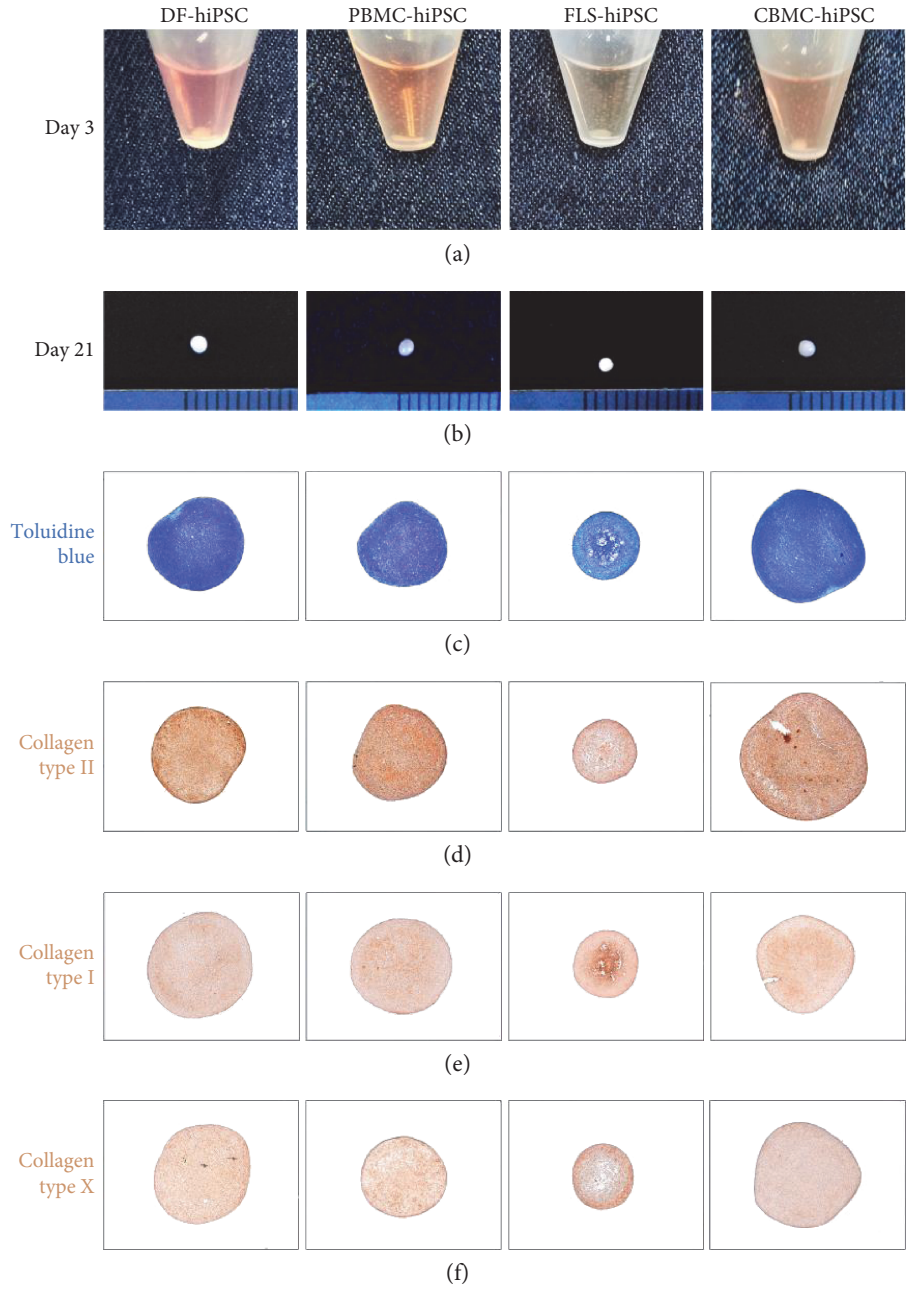

Figure 3: Chondrogenic pellets generated from hiPSC-derived from different originated somatic cells. (a) Morphology of outgrowth cells 3 days after differentiation. (b) Chondrogenic pellets after 21 days of chondrogenic differentiation. (c) Toluidine staining and (d) collagen type II immunohistology images of the chondrogenic pellets generated from each cell source-hiPSCs. (e) Collagen type I and (f) collagen type X staining images of chondrogenic pellets.

hiPSCs derived from PBMCs, FLSs, and CBMCs all had a relatively high expression of PRG4. Collagen type I (COL1A1) is a general marker for fibrocartilage, which is a less lubricated form of cartilage. As it is also a fibrotic marker, the expression was higher in FLS-hiPSC-derived pellets (Figure 4(g)). COL1A1 expression was high in both DF- and FLS-hiPSC-derived pellets. PBMC-hiPSC-derived pellets had the lowest expression of COL1A1 and FLS-hiPSC-derived pellets showed the highest expression. Yet, despite these differences, the results lacked significance. Collagen type X (COL10A1) is a marker for hypertrophy. The expression of COL10A1 was mostly all lower than other markers (Figure 4(h)). Hypertrophy can lead to osteogenecity, and RUNX2 is another marker indicating early calcification or osteogenesis of cartilage. RUNX2 expression was the highest in DF-hiPSC-derived chondrogenic pellets (Figure 4(i)). The expression was significantly lower in PBMC-hiPSC-derived pellets compared to CBMC-hiPSC-derived pellets. However, the expression of RUNX2 was relatively lower than all the other markers in every cell

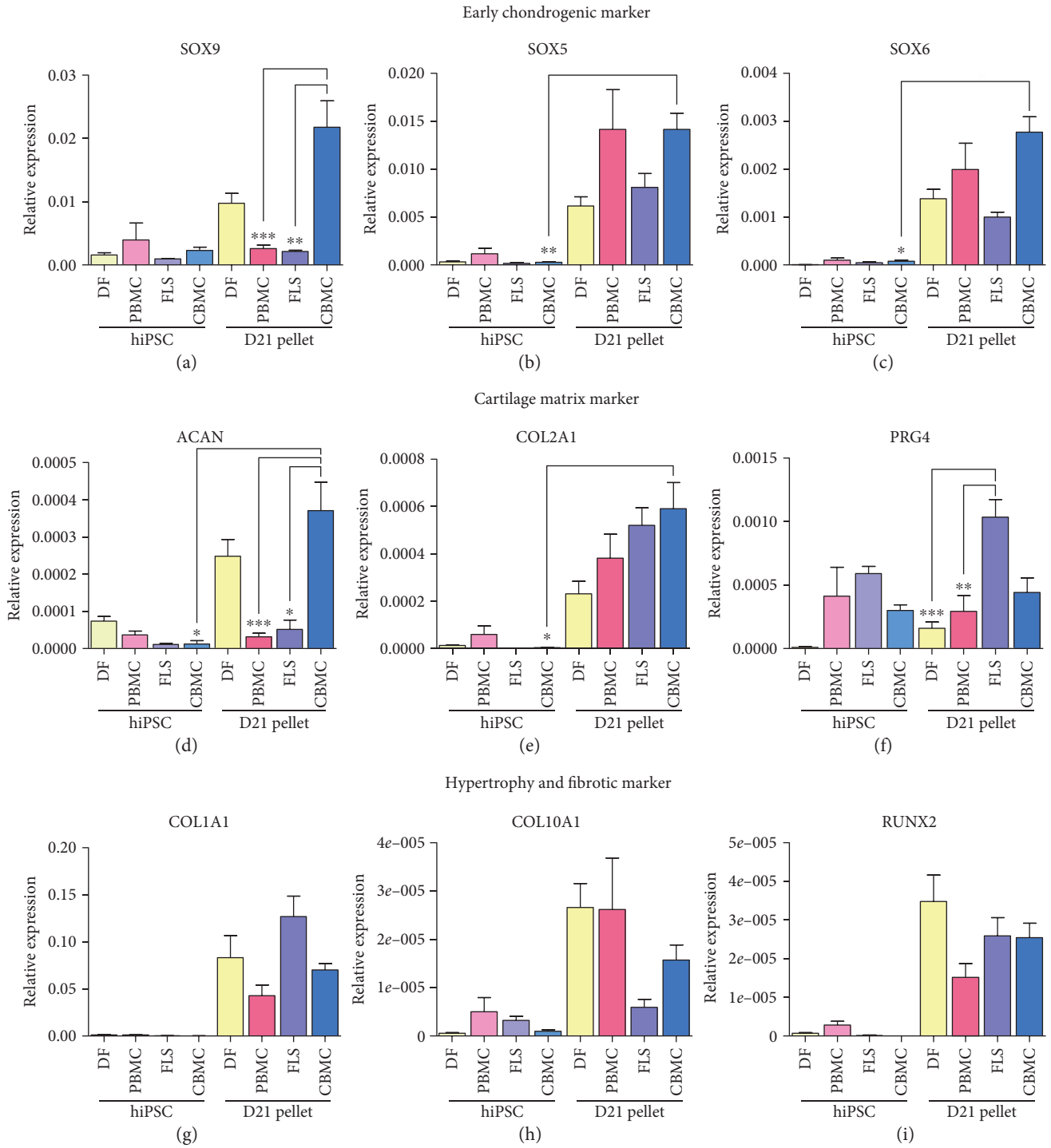

Figure 4: Gene expression in chondrogenic pellets. The expression of transcriptional activators or early chondrogenesis markers (a) SOX9, (b) SOX5, and (c) SOX6 was confirmed. The expression of matrix proteins, (d) aggrecan (ACAN), (e) collagen type II (COL2A1), and lubricin (PRG4) was confirmed. Also, hypertrophy or fibrotic markers such as (g) collagen type I (COL1A1), (h) collagen type X (COL10A1), and (i) RUNX2 was confirmed as well. To confirm the gene expression in each cell type, $n = 9$–18 pellets were used in this experiment ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$).

type-derived chondrogenic pellets and it also lacks significance between all samples. The levels of pluripotent markers were measured in the generated chondrogenic pellets (Figure S3). The expression levels of OCT4, SOX2, and NANOG were all decreased in the generated pellets. The most important hiPSC marker, NANOG, was most significantly decreased in the CBMC-derived chondrogenic pellets. However, KLF4 expression was increased in all cases. Through these results, CBMC-hiPSC-derived pellets had a fairly superior quality than the other three cell sources.

Yet, there was not much morphological difference between CBMC-, PBMC-, and DF-derived cell lines. The expression of pluripotent markers was also confirmed in chondrogenic pellets (Figure S3). The expression of hiPSC markers (i.e., OCT4, SOX2, and NANOG) was downregulated in differentiated chondrogenic pellets. However, the expression of KLF4 was increased in the chondrogenic pellets.

3.4. Gene Expression in Pellets Generated from hiPSCs Reprogrammed from Cells Sharing the Same Genetic Identity. Two of each of PBMC- and DF-hiPSCs were obtained from the same donor. To compare the chondrogenic potential from different cell sources (DFs and PBMCs) obtained from the same donor, we profiled the chondrogenic and other marker expression. Similar to the results of Figure 4(a), the expression of SOX9 was significantly higher in DF-hiPSC-derived pellets even in genetically identical conditions (Figure 5(a)). The expression of SOX5 turned out to be the opposite of the earlier data (Figure 4(b)); however, it was shown that the difference was not significant (Figure 5(b)). The expression of SOX6 was higher in PBMC-hiPSC-derived pellets as predicted; however, there was no significance as well (Figure 5(c)). The expression of ACAN was significantly higher in DF-hiPSC-derived chondrogenic pellets (Figure 5(d)). This was identical to the results shown in Figure 4(d). COL2A1 and PRG4 expression also showed a similar pattern as shown in Figure 4 (Figures 5(e) and 5(f)). The expression of COL1A1 was higher in DF-hiPSC-derived pellets (Figure 5(g)). COL10A1 expression was higher in PBMC-hiPSC-derived pellets (Figure 5(h)); however, the expression of RUNX2 was lower than DF-hiPSC-derived pellets. Taken all together, DF-hiPSC-derived pellets showed higher expression of the early transcription marker SOX9 and proteoglycan gene ACAN. Most of the markers had no significant difference in the chondrogenic pellets generated from PBMC- and DF-hiPSCs sharing the same genetic identity.

4. Discussion

In this present study, the chondrogenic differentiation potential of hiPSCs generated from different donor cell sources was investigated. Chondrogenic pellets were generated using several cell sources: CBMC, PBMC, DF, and FLSs. Between these samples, two of each of PBMCs and DFs were genetically identical. FLSs were obtained from an osteoarthritis patient's synovial tissue. The somatic cell source seemed to affect the chondrogenecity of hiPSCs. Our group previously showed the chondrogenic differentiation potential of CBMC-derived hiPSCs [15]. Compared to other cell sources, CBMC-hiPSC-derived pellets showed relatively higher expression of early and late (proteoglycan) markers of chondrogenesis. This data suggest that CBMC-hiPSCs can be used as a comparable cell source for in vitro chondrogenesis or cartilage regeneration.

In vitro chondrogenesis is an important issue, since the original tissue has limited capacity to regenerate or heal. Successful generation of hyaline cartilage in tissue engineering and in vitro cultivation systems can lead to tremendous therapeutic benefits in the related fields. Early in vitro chondrogenesis was done using MSCs or adipocyte-derived stem cells [20, 28]. These two adult stem cells are also actually used in current clinics along with autologous chondrocytes for the regeneration of the damaged cartilage [29–31]. Despite their high chondrogenic potential, the low coverage of the cells was the limit of these cell sources. Also, long in vitro cultures to proliferate these cells have the possibility of hypertrophy and dedifferentiation of cells [32, 33]. The low cellularity in elderly or patients may also impair the therapeutic effects of these cells [34]. Embryonic stem cells (ESCs) were mentioned for cartilage regeneration as an alternative; however, the ethical issues related to the embryo destructions are hard to overcome in several institutes. The emergence of hiPSCs was a breakthrough because of these reasons. The unlimited proliferation by self-renewal and high pluripotency was similar to that of ESCs, and the ethical issues were removed [35]. The spontaneous differentiation of hiPSCs also resulted in cartilage-like tissues, indicating the potential ability of hiPSCs in chondrogenesis.

The generation of hiPSCs can be theoretically done using any adult somatic cells. Various cell types (i.e., dermal fibroblast, blood cell, urine cell, and keratinocyte) were shown to reprogram successfully into hiPSCs [1, 3, 36–42]. Because of these reasons, hiPSCs are now widely used in the fields of disease modeling and drug screening that can replace the use of diseased animal models [43]. It is also the cutting edge material for tissue regeneration. However, the cell source used in reprogramming is now on the issue, since it might affect the outcome of reprogramming and regeneration. We generated hiPSCs from DFs, PBMCs, FLSs, and CBMCs. The generated hiPSCs had similar protein expression levels of pluripotent markers (i.e., OCT4 and TRA-1-60) (Figure 1), yet the genetic expression rather differed. The genetic expression levels of pluripotent markers such as OCT4, SOX2, NANOG, and KLF4 were highly expressed in DF-derived hiPSCs. PBMC-derived hiPSCs had the second highest expression and FLS-derived hiPSCs had the lowest. Interestingly, the expression of pluripotent markers was higher in the primary blood cells (PBMCs and CBMCs). This is thought to be caused by the effect of the maintenance media of suspension cells which can increase the hematopoietic stem cell population. Also, the expression of KLF4 in FLS-derived hiPSCs was highly expressed (Figure 1(h)). This fact was already confirmed in our previous report [13]. This finding is still being investigated in our other projects; however, a recent study reported that KLF4 is an inflammatory regulator in FLSs via IL-6 signaling [44].

While improved understandings and several protocols were developed in chondrogenic differentiation using hiPSCs, the selection of the donor somatic cell source used in reprogramming has become an important issue [25]. In this study, genetically identical PBMC- and DF-hiPSC were differentiated into chondrogenic pellets (Figure 5). Our results suggested that the two cell sources eventually had no significant difference in chondrogenic potential under the same genetic circumstances. The expression of SOX9 and ACAN correlated and were the only markers that shown

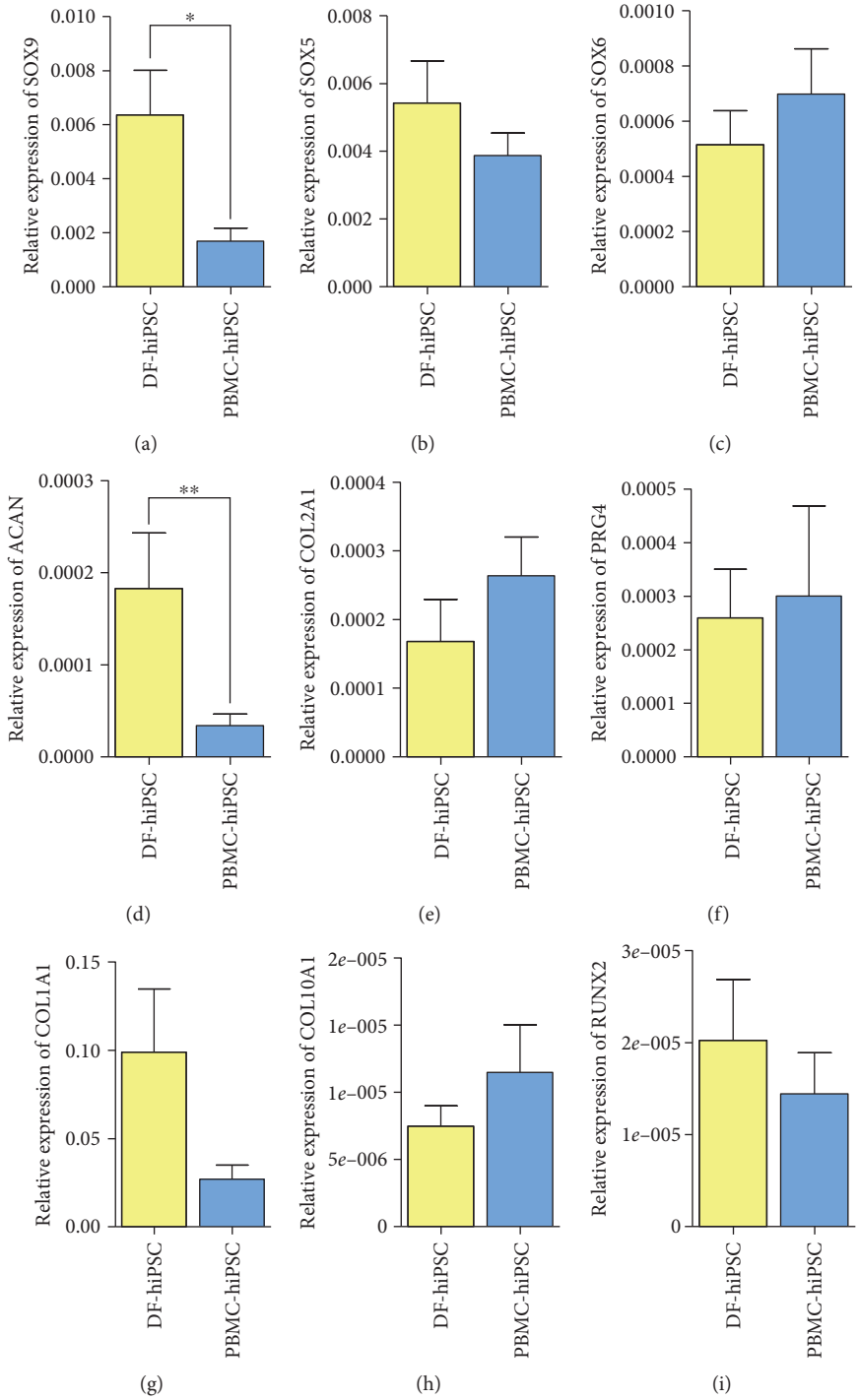

Figure 5: The expression of DF- and PBMC-hiPSC-derived chondrogenic pellets generated from the same donor. The expression of early transcription markers: (a) SOX9, (b) SOX5, and (c) SOX6. The expression of proteoglycan matrix proteins: (d) ACAN, (e) COL2A1, and (f) PRG4. The expression fibrotic and hypertrophic markers: (g) COL1A1, (h) COL10A1, and (i) RUNX2 ($^{*}P < 0.05$; $^{**}P < 0.01$).

significant difference between the two differently originated hiPSCs. Both SOX9 and ACAN were significantly higher in DF-hiPSC-derived pellets. We concluded that some somatic cell sources might not have different outcomes in chondrogenesis. Yet, further examination and more cell lines that share the same genetic profiles to confirm chondrogenesis might be required before we assert these results.

The expression of pluripotent markers were examined in the chondrogenic pellets (Figure S3). The expression of OCT4, SOX2, and NANOG was decreased in chondrogenic pellets compared to hiPSCs. Interestingly, KLF4 expression was increased in all chondrogenic pellets generated from different cell types. This data can suggest that KLF4 might play an important role in chondrogenesis. The relation

between KLF4 and chondrogenesis is not fully understood at this point; however, Outani and colleagues previously reported induction of chondrogenesis using KLF4 with other factors [45, 46]. Chondrogenic cells were directly induced from mouse dermal fibroblasts by transducing only SOX9, c-Myc, and KLF4. This might explain the reason of the increased levels of KLF4 in cells undergoing chondrogenesis. Also, based on the fact that FLS-hiPSCs had high expression of KLF4 (Figure 1(h)), it might be a characteristic of the pathology of OA. Previous reports such as the one reported by Liu et al. suggest that KLF4 inhibit the expression of IL-1β, which is the main cytokine that is thought to be closely related to OA. Further research on this subject may suggest another clue about the epigenetic memory of hiPSCs.

The inheritance of the initial epigenomes and characteristics of the primary cell source to the hiPSCs are mentioned as "epigenetic memory" [47]. This phenomenon makes hiPSC a reasonable source for disease modeling. It is thought that using patient-specific hiPSCs may reflect the pathological characteristics of the diseased somatic cell or individual. We also differentiated OA FLS-derived hiPSCs into chondrogenic pellets in this study. The pellets generated using FLS-derived hiPSCs showed low viability in the inner region, even though it had a relatively small size (Figure S2). The gene expression of OA FLS-hiPSC-derived chondrogenic pellets was also interesting. The expression of SOX9 was significantly lower in FLS-hiPSC-derived chondrogenic pellets (Figure 4(a)). The expression of the other two transcription factors, SOX5 and SOX6, was also low, compared to that of CBMC-hiPSC-derived chondrogenic pellets (Figures 4(b) and 4(c)). The expression of ACAN was also significantly lower than that DF- and CBMC-hiPSC-derived pellets. While the expression of COL2A1 and ACAN are usually known to have decreased expression in OA, however, COL2A1 expression was not that low than any other cell sources except CBMC-hiPSCs. Lubricin, or PRG4, is a protein in the cartilage ECM and synovial fluid that participates in the lubrication of the joint articular cartilage [48]. Interestingly, the expression of PRG4 was significantly higher than the other cell types. The expression of PRG4 was predicted to be decreased in OA; however, increased expression of PRG4 was also confirmed in OA patient human anterior cruciate ligament-MSCs [48]. Correlating with the previous reports, the high expression of PRG4 in OA might be an interesting subject to investigate. As other studies suggest that mechanical motions promote the expression of PRG4 in the articular cartilage, the high expression of PRG4 might be related to OA caused by external trauma or mechanical forces [49].

5. Conclusions

In conclusion, our CBMC-hiPSCs generated chondrogenic pellets with fair quality. However, no significant morphological difference was shown in normal hiPSC-derived pellets. With additional well-known benefits of the CBMCs (i.e., cell banking systems, stored HLA-typing information, and homozygous HLA types), the use of HLA-homozygous CBMC-hiPSCs have several advantages for future use of hiPSCs in clinics and tissue transplantation [50]. Our study suggests CBMC-hiPSCs as an ideal source for future application in cartilage regeneration. Also, we suggest hiPSCs generated from different somatic cells with different genetic and epigenetic backgrounds as a tool to assess and understand chondrogenesis and the cartilage in vitro.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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Supplementary Materials

Supplementary 1. Figure S1: Confirmation of vector-free hiPSCs. The elimination of viral vectors was confirmed in generated hiPSCs. The expression was confirmed in clone #1 of each hiPS cell line.
Supplementary 2. Figure S2: Size of chondrogenic pellets generated from hiPSCs. Chondrogenic pellets generated from CBMC-derived hiPSCs had the largest size.
Supplementary 3. Figure S3: The expression of pluripotent markers (OCT4, SOX2, NANOG, and KLF4) in day 21 chondrogenic pellets. The expression was compared to that of hiPSCs.

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Research Article

Successful Low-Cost Scaffold-Free Cartilage Tissue Engineering Using Human Cartilage Progenitor Cell Spheroids Formed by Micromolded Nonadhesive Hydrogel

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The scaffold-free tissue engineering using spheroids is pointed out as an approach for optimizing the delivery system of cartilage construct. In this study, we aimed to evaluate the micromolded nonadhesive hydrogel (MicroTissues®) for spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture) using cartilage progenitors cells (CPCs) from human nasal septum without chondrogenic stimulus. CPC spheroids showed diameter stability ($486\,\mu m \pm 65$), high percentage of viable cells (88.1 ± 2.1), and low percentage of apoptotic cells (2.3%). After spheroid compaction, the synthesis of TGF-β1, TGF-β2, and TGF-β3 was significantly higher compared to monolayer ($p < 0.005$). Biomechanical assay revealed that the maximum forces applied to spheroids after chondrogenesis were 2.6 times higher than for those cultured for 2 days. After spontaneous chondrogenesis, CPC spheroids were entirely positive for N-cadherin, collagen type II and type VI, and aggrecan and chondroitin sulfate. Comparing to monolayer, the expression of SOX5 and SOX6 genes analyzed by qPCR was significantly upregulated ($p < 0.01$). Finally, we observed the capacity of CPC spheroids starting to fuse. To the best of our knowledge, this is the first time in the scientific literature that human CPC spheroids were formed by micromolded nonadhesive hydrogel, achieving a successful scaffold-free cartilage engineering without chondrogenic stimulus (low cost).

1. Introduction

The classical tissue engineering relies on scaffold-based approaches in which the scaffold serves as a substitute for extracellular matrix. Despite the reasonable success reached by classical tissue engineering over the past decades [1], some remarkable issues are still unsolved, especially that related to the optimal delivery system for a better retention and

integration with surrounding tissue. As an alternative, 3D tissue constructs can be produced in the absence of scaffolds [2]. This strategy is named scaffold-free tissue engineering, pointed out as having superior results since cells are responsible for synthesizing their own extracellular matrix optimizing cell-matrix and cell-cell interactions, recreating their native tissue microenvironment and recapitulating tissue morphogenesis [3]. Furthermore, scaffold-free 3D constructs have showed long-term retention in the implantation site [4].

The pellet culture, hanging-drop, and 96-well plate have been notably used for 3D cartilage constructs in scaffold-free tissue engineering [5]. In pellet culture, the cellular self-organization is responsible for cell aggregate formation by applying an external force. The main limitation of aggregates is related to their uncontrolled and nonhomogeneous shape [6]. On the other hand, the spheroids are morphologically different from aggregates especially due to their distinct dimensions and gross appearances related to size, thickness, and shape. In contrast to aggregates formed by external forces, spheroids are formed by self-assembling process using nonadherent hydrogel molds or platforms such as hanging-drop, 96-well plate, and recently micromolded nonadhesive hydrogels [7, 8]. The main advantage of recent platforms of micromolded nonadhesive hydrogel is seeding cell suspension with single pipetting, reducing substantially technical errors, and allowing automation [3].

The first step of cartilage development in developing embryo is mesenchymal cell condensation [9]. The morphogenic proteins of the transforming growth factor-beta (TGF-beta) superfamily initiate the condensation phase by increasing expression of N-cadherins. In fact, this phase is mimic by cell-driven spheroid formation (self-assembling process), since cell-cell interaction is mediated by N-cadherins leading to spheroid compaction [10]. On the other hand, hydrogels, commonly used in scaffold-based cartilage tissue engineering, are responsible to impair cell-cell interaction [11]. Also at condensation phase, the SOX5 and SOX6 proteins form a complex with SOX9 in a close cooperation to regulate positively the col2a1 (collagen type 2a1) gene expression [12]. Thus, extracellular matrix-specific proteins such as collagen type II as well as aggrecan appear concomitant with condensation [13].

Several studies have been published using scaffold-free approaches for cartilage tissue engineering [10, 14], most of them using cell aggregates instead of spheroids [15]. Besides issues about their uncontrolled and nonhomogeneous shape as mentioned before, the 3D cell culture techniques commonly applied for aggregate formation are susceptible to external forces, jeopardizing the condensation phase [2]. In respect to stem/progenitor cell human source, although mesenchymal stem/stromal cells show chondrogenic capacity, they usually progress to hypertrophic chondrocyte phenotype [16]. A population of progenitor cells dwelling on the surface of articular cartilage has been described by several research groups. This source of cells shares similarities with mesenchymal stem/stromal cells mainly related to surface marker expression and multipotentiality [17]. Besides articular cartilage, it is also possible to isolate progenitor cells in cartilage from human nasal septum [18].

In this study, we aimed to evaluate the micromolded nonadhesive hydrogel for cell spheroid formation and compaction with a homogeneous size and shape and subsequent spontaneous chondrogenesis using cartilage progenitor cells (CPCs) from human nasal septum, firstly described by our research group [19]. The spontaneous chondrogenic capacity of CPCs was already proven in 3D culture system [19, 20]. Our analysis points refer to spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture). From our knowledge, this is the first time in scientific literature that micromolded nonadhesive hydrogel is tested for cartilage tissue engineering. Furthermore, our source of CPCs is advantageous since the cell culture medium for chondrogenic induction is totally free of induction factors such as TGF-β1 or TGF-β3 [20] substantially reducing the costs for an optimized delivery system using spheroids.

2. Material and Methods

2.1. Human Cartilage Sampling. Cartilage fragments from nasal septum were obtained from healthy donors ($n = 3$, age from 18 to 40 years) who underwent plastic surgery. This study was approved by the Research Ethics Committee of the Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, Brazil (Protocol 145-09), and informed consent was obtained from all donors included in the study. The samples were stored at 4°C after the surgery, and the cartilage progenitor cell isolation was performed within 18 hours.

2.2. Isolation and In Vitro Expansion of Cartilage Progenitor Cells (CPCs). CPCs were isolated as previously described [19]. Briefly, cartilage fragments from nasal septum were incubated with collagenase IA 1 mg/ml for 1 hour. Cells were harvested by centrifugation and plated in culture flasks. The culture flasks were maintained at 37°C in a humid atmosphere with 5% carbon dioxide with low-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). The medium was changed every 3 to 5 days until the cell monolayer reached confluence. At confluence, the cells were harvested with 0.125% trypsin (GIBCO) and 0.78 mM ethylenediaminetetraacetic acid (GIBCO).

2.3. Cartilage Progenitor Cell Spheroid Culture. For spheroid culture, silicone molds were used in order to produce the micromolded nonadhesive agarose hydrogel (agarose 2%—Ultrapure Agarose, Invitrogen, Carlsbad, CA, USA—in NaCl 0.9% solution) with 300 μm or 800 μm diameter in each circular recesses (MicroTissues 3D Petri Dish, Sigma) according to manufacturer's protocol (Figure 1). For cell seeding, a suspension of 2×10^6 cells was prepared in 190 ul of DMEM supplemented with 50 μg/ml ascorbic acid (Sigma), 1.25 μg/ml human albumin (FarmaBiagini SPA), 6.25 μg/ml insulin (Novo Nordisk), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma) and Insulin-Transferrin-Selenium, ITS 1X (Lonza). Cell density was chosen according to recommendations of the silicone mold manufacturer

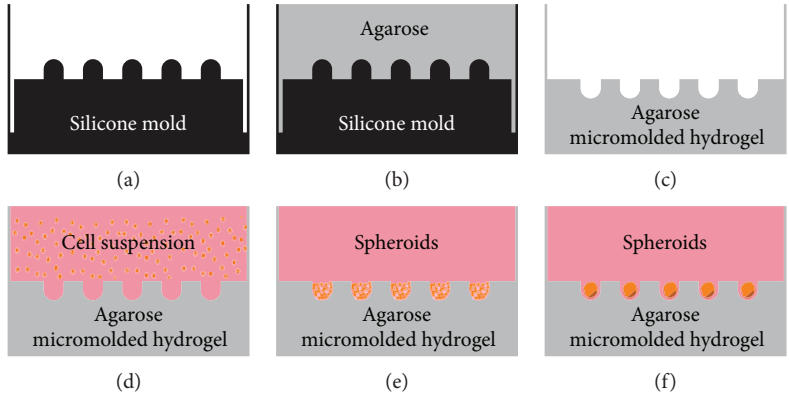

Figure 1: Scheme of cartilage progenitor cell spheroid culture. (a) Silicone molds were used from MicroTissues 3D Petri Dish. (b) The agarose solution was dispensed into silicone molds, resulting in the micromolded nonadhesive agarose hydrogel (c). (d) The cell suspension was carefully seeded into cell seeding chamber with single pipetting. (e) The cell suspension decanted into circular recesses after few minutes resulting in spheroids after 18 hours (f).

(MicroTissues 3D Petri Dish, Sigma). The cell suspension was carefully seeded into the hydrogel-cell seeding chamber. The micromolded nonadhesive agarose hydrogel was maintained for at least one hour at 37°C in a humid atmosphere with 5% carbon dioxide for cell sedimentation inside circular recesses. After that, 2.5 ml of medium were added to the outside of the cell seeding chamber. The spheroids were formed after 18 hours and the spheroid culture was maintained at 37°C in a humid atmosphere with 5% carbon dioxide until 21 days. The medium was changed twice a week. All the analysis points refer to spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture).

2.4. Measurement of the Spheroid Diameter. The spheroids cultivated in micromolded nonadhesive agarose hydrogel with 300 μm or 800 μm diameter in each circular recesses were examined after 2-day culture and 21-day culture under an optical microscope (Leica DMI 6000 B) equipped with Leica DF 500 digital camera. The diameter of the spheroids was determined using AxioVision software (AxioVs40 V4.8.2.0) with the bar size of each image as a reference for the measurements, which were expressed in micrometers. Results were expressed as the mean ± standard error. A total of 45 spheroids from 2 or 3 micromolded nonadhesive hydrogels were measured randomly. Both assays were performed in triplicate from three different cell samples ($n = 3$).

2.5. Cell Viability and Apoptosis Analysis. For cell viability analysis, the spheroids cultivated in micromolded nonadhesive agarose hydrogel with 800 μm diameter in each circular recesses were collected after spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture) and incubated with 1 mg/ml collagenase type I (Sigma Chemical), 0.125% trypsin (Gibco), and 0.78 mM ethylenediaminetetraacetic acid (Gibco) in a shaking water bath at 37°C for 40 minutes mixing well by pipetting up and down every 2-3 min [21]. After centrifugation, the cell suspension resulting from the spheroids was washed with phosphate-buffered saline and incubated for 30 minutes at 4°C with CD44-phycoerythrin (BD Biosciences). Subsequently, the cells were washed again with phosphate-buffered saline and incubated with 7-actinomycin D (7AAD, BD Biosciences, Franklin Lakes, NJ, USA) for 10 minutes. Cell apoptosis was evaluated by staining cells with Annexin V and PI (propidium iodide) according to the manufacturer's recommendations (BD Biosciences). For each tube, a total of 20,000 events of both samples were acquired in a FACSAria III (BD Biosciences). The flow cytometry analysis was performed using the software FACS Diva 8.0 (BD Biosciences). The viable cells identified by 7AAD exclusion were expressed as a percentage of total cells. The assays were performed from three different cell samples ($n = 3$).

2.6. Multiplex Analysis of Secreted Products. The culture medium was changed after spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture) in micromolded nonadhesive agarose hydrogel with 800 μm diameter in each circular recesses. For the monolayer, the culture medium was changed after 2 days of cell culture. After 24 hours of culture medium change, the supernatant of all samples was collected and frozen at −80°C. The determination of proteins in the supernatant was carried out using the LuminexxMAP technology based on a magnetic bead panel for recognition of human TGF-β1, TGF-β2, and TGF-β3. The Bio-Plex Magpix apparatus (Biorad Laboratories Inc., Hercules, CA, USA) was calibrated and validated, reagents reconstituted, and standard curve prepared. Experimental procedures were preceded by washing steps with automated Bio-Plex Pro wash station (Biorad Laboratories Inc., Hercules, CA, USA). The concentration of each secreted product was quantified by the xPONENT software version 4.2 (Biorad Laboratories Inc., Hercules, CA, USA). Results were expressed as picograms per milliliter (ρg/ml). The assay was performed from three different cell samples ($n = 3$) using three replicates from each sample.

2.7. Biomechanical Assay. Spheroid resistance to compression was evaluated using the Microsquisher (CellScale) equipment. Spheroids cultured for spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture) in micromolded nonadhesive agarose hydrogel with 800 μm diameter in each circular recesses were

collected and disposed in a platform inside a PBS bath at 37°C. A parallel plate exerted cycles of compression that consisted of a vertical force with amplitudes of 25% from spheroid diameter. Each cycle consisted of a load phase (20 s) followed by a recovery one (10 s). The maximum forces needed to reach 25% of spheroids compression were determined. Five cycles were performed in triplicate from three different cell samples ($n = 3$).

2.8. RNA Isolation and Quantification. After 21 days of cell culture, the monolayer and the spheroids (spontaneous chondrogenesis) cultivated in micromolded nonadhesive agarose hydrogel with 800 μm diameter in each circular recesses were incubated with RLT buffer (Qiagen, Sweden). RNA extraction was performed with RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Sweden). The protocol established for purification of total RNA from Animal Tissues was used to isolate the material for the spheroids. For the monolayer, the RNA was extracted from 1×10^6 cells using the protocol established for animal cells. RNA integrity was evaluated by electrophoresis on a 1.5% denaturing agarose gel. The RNA purity and yield were determined by spectrophotometry (Nanodrop 2000C, Thermo Scientific) at 260 nm and 280 nm. Samples presenting A260/A280 ratio of ~2.0 were considered for gene expression analysis. The RNA isolation was performed from three different cell samples ($n = 3$).

2.9. Quantitative Real-Time PCR (qPCR). The expression levels of SOX5, SOX6, and SOX9 genes, involved in the regulation of chondrogenesis, were evaluated in cells cultured as monolayer and as spheroids by quantitative polymerase chain reaction (qPCR). All reagents were purchased from Applied Biosystems, USA. The qPCR was performed using the AgPath-ID™ one-step RT-PCR kit. Briefly, 1 μl of purified RNA (50 ng/μl) was reverse transcribed and amplified in a 10 μl reaction mixture containing 5 μl of 2x RT-PCR buffer, 0.4 μl of 25x RT-PCR enzyme mix, and 1.25 μl yeast RNA 5 mg/ml (Ambion). Specific primers and TaqMan® probe sets for each gene were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). The RNA samples were run in triplicate for each gene. CASC3 (cancer susceptibility candidate gene 3) was used as a reference gene to normalize the expression of target gene samples in both monolayer and spheroids. The thermal cycling conditions comprised 10 min RT step at 45°C, a 10 min initial PCR activation step at 95°C (AmpliTaq Gold activation) followed by 40 cycles of 95°C for 15 s, and 60°C for 45 s each (ABI 7500, Applied Biosystems). Relative expression levels of SOX genes were calculated for each sample after normalization against the average of the geometric mean of CASC3 gene (internal control) using the $\Delta\Delta$Ct method for comparing relative fold expression differences between cells cultured as monolayer and cells cultured as spheroids.

2.10. Histology and Immunohistochemistry. For histological preparations, the spheroids cultivated in nonadhesive agarose hydrogel with 800 μm diameter in each circular recesses for 21 days (spontaneous chondrogenesis) were collected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 1 h at room temperature. After fixation, tissues were dehydrated in graded ethanol, cleared in xylol, and embedded in paraffin at 60°C. Sections of 5 μm were prepared using the Slee Medical microtome (CUT 5062). Sections were collected onto 0.01% poly-L-lysine-coated slides (Sigma Chemica) and stained by hematoxylin and eosin (H/E).

For immunohistochemistry analysis, paraffin sections were collected onto silano-treated slides (Starfrost®). After dewaxing, sections were hydrated and incubated with the protein blocking solution for 15 min. Antigen unmasking was performed only for collagen type II using the enzyme chondroitinase ABC (Sigma Chemical) at 37°C for 30 min. Endogenous peroxidase was blocked using the peroxidase blocking solution for 15 min followed by PBS-Tween wash. The following primary antibodies (Abcam, Cambridge, UK) were incubated for 1 h at room temperature: collagen type II (1 : 50), collagen type VI (1 : 100), aggrecan (1 : 100), chondroitin sulfate (1 : 300), and N-cadherin (1 : 800). All reactions were done in the same moment. After washing in PBS-Tween, secondary antibody staining was performed using the Reveal-Polyvalent HRP kit (Code SPB 125, Spring). Peroxidase was revealed with diaminobenzidine (DAB liquid + substrate—chromogen system DAB-125, Spring®). Sections were then counterstained with hematoxylin, dehydrated, mounted in Entellan® (Merck), and examined under Leica DM-2500 optical microscope. Nonspecific binding of secondary antibody was monitored by carrying out the immune reaction in the absence of the primary antibody.

2.11. Fusion Assay. Spheroids cultured for spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture) in micromolded nonadhesive agarose hydrogel with 800 μm diameter in each circular recesses were collected and transferred to 96-well flat bottom plates previously coated with agarose (agarose 2%—UltrapureAgarose, Invitrogen, Carlsbad, CA, USA—in NaCl 0.9% solution) in order to avoid adhesion of spheroids in well surface. Four spheroids were seeded per well in close contact and maintained in culture for until 7 days. Each well was examined under Zeiss 37,081 phase contrast microscope after 1 hour, 1 and 7 days.

2.12. Statistical Analysis. Nonparametric and unpaired Student's t-test (Mann–Whitney test) was performed to compare data concerning cell viability and mechanical tests of spheroids cultivated for 2 days with those cultivated for 21 days. One-way analysis of variance test was used for comparisons among the monolayer, spheroids cultivated for 2 days, and spheroids cultivated for 21 days in the TGF-β secretion assay. The results in the graphs are expressed as the mean ± standard deviation, and p values less than 0.05 were considered statistically significant. All analyses were performed using the software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Statistical analysis of the qPCR data was performed using the web-based RT2 Profiler™ PCR Array Data Analysis software (SABiosciences, http://www.SABiosciences.com/pcrarraydataanalysis.php).

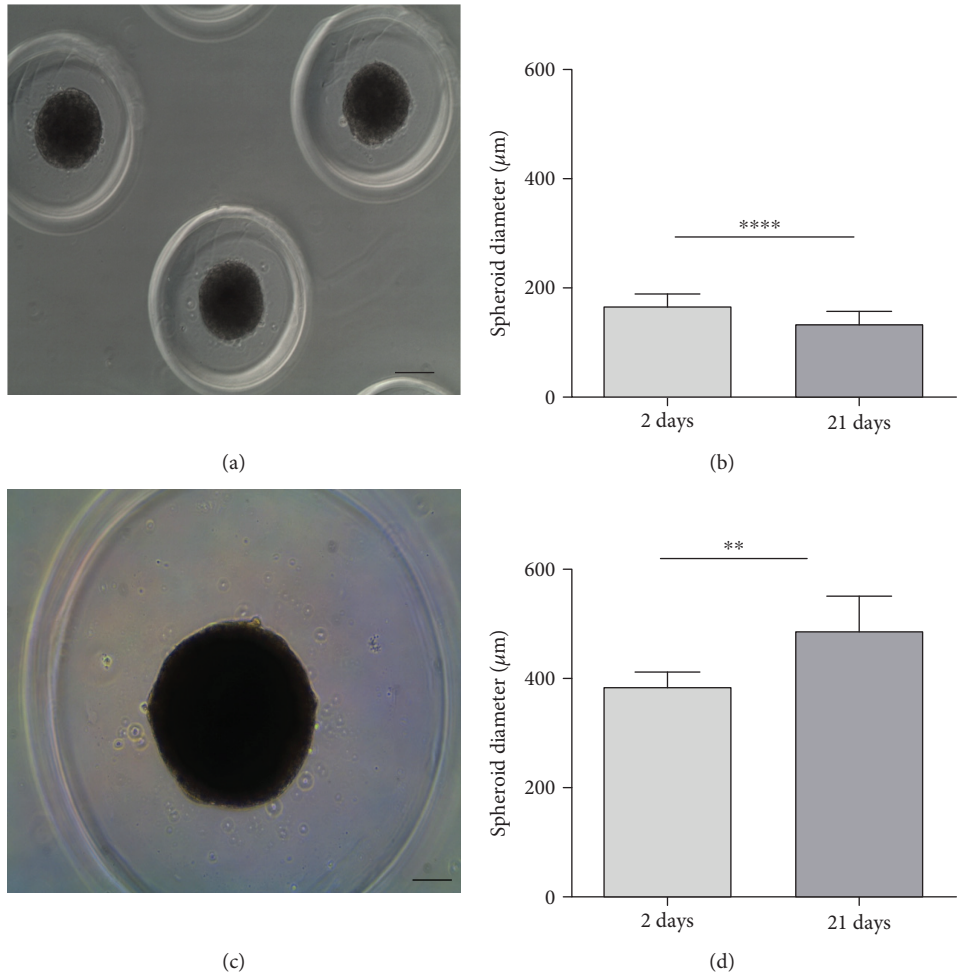

Figure 2: The CPC spheroids formed in the micromolded nonadhesive hydrogel with 800 μm diameter in each circular recesses showed stability in their diameter along 21 days of cell spheroid culture. (a, c) Representative images of CPC spheroids formed in micromolded nonadhesive hydrogel with 300 μm diameter (a) and with 800 μm diameter in each circular recesses (c). Phase contrast microscopy. Bar size: 100 μm. (b, d) The graphs represent the mean ± standard error of the spheroid diameter in micromolded nonadhesive hydrogel with 300 μm diameter (b) and with 800 μm diameter in each circular recesses (d). Light gray bar represents 2 days of cell spheroid culture. Dark gray bar represents 21 days of cell spheroid culture. $^{****}p < 0.0001$; $^{**}p < 0.01$.

3. Results

CPC spheroids with homogenous shape (Figures 2(a) and 2(c)) and size (Figures 2(b) and 2(d)) were produced by both nonadhesive hydrogel with 300 μm or 800 μm diameter in each circular recesses. Only 1 spheroid per circular recesses was observed. As expected, the micromolded nonadhesive hydrogel with 300 μm diameter formed CPC spheroids with a reduced diameter, approximately twice smaller compared to 800 μm circular recess counterpart (Figure 2(b) and 2(d)). After 21 days of cell culture, CPC spheroids formed by the micromolded nonadhesive hydrogel with 300 μm diameter in each recesses have reduced their diameter. Conversely, the CPC spheroids formed by the micromolded nonadhesive hydrogel with 800 μm circular recesses have increased their diameter in about 100 micrometers (Figure 2(d)).

Due to the diameter stability of CPC spheroids exclusively found in nonadhesive hydrogel with 800 μm diameter in each circular recesses, all the subsequent experiments were carried out using this type of molded hydrogel. The viability assay performed by flow cytometry revealed a high percentage of viable cells in the digested mass of CPC spheroids after spheroid compaction (2-day culture) and spontaneous chondrogenesis (21-day culture) (Figures 3(a)–3(c)). The CPC cells were identified in digested mass of CPC spheroids by CD44 positivity (Figures 3(a) and 3(b)). Furthermore, apoptotic cells (annexin V positive cells) were rare (Figures 3(d)–3(f)).

The dosage of the three isoforms of TGF-β was performed since this superfamily initiate the chondrogenic differentiation. After spheroid compaction (2-day culture), the synthesis of TGF-β1, TGF-β2, and TGF-β3 was significantly higher compared to those observed in monolayer (Figures 4(a)–4(c)) ($p < 0.005$). The levels of the three TGF-β proteins evaluated in this study presented a decrease after spontaneous chondrogenesis (21-day culture) with TGF-β3 exhibiting the lowest levels in cell spheroid culture (Figure 4(b)). Despite the decrease

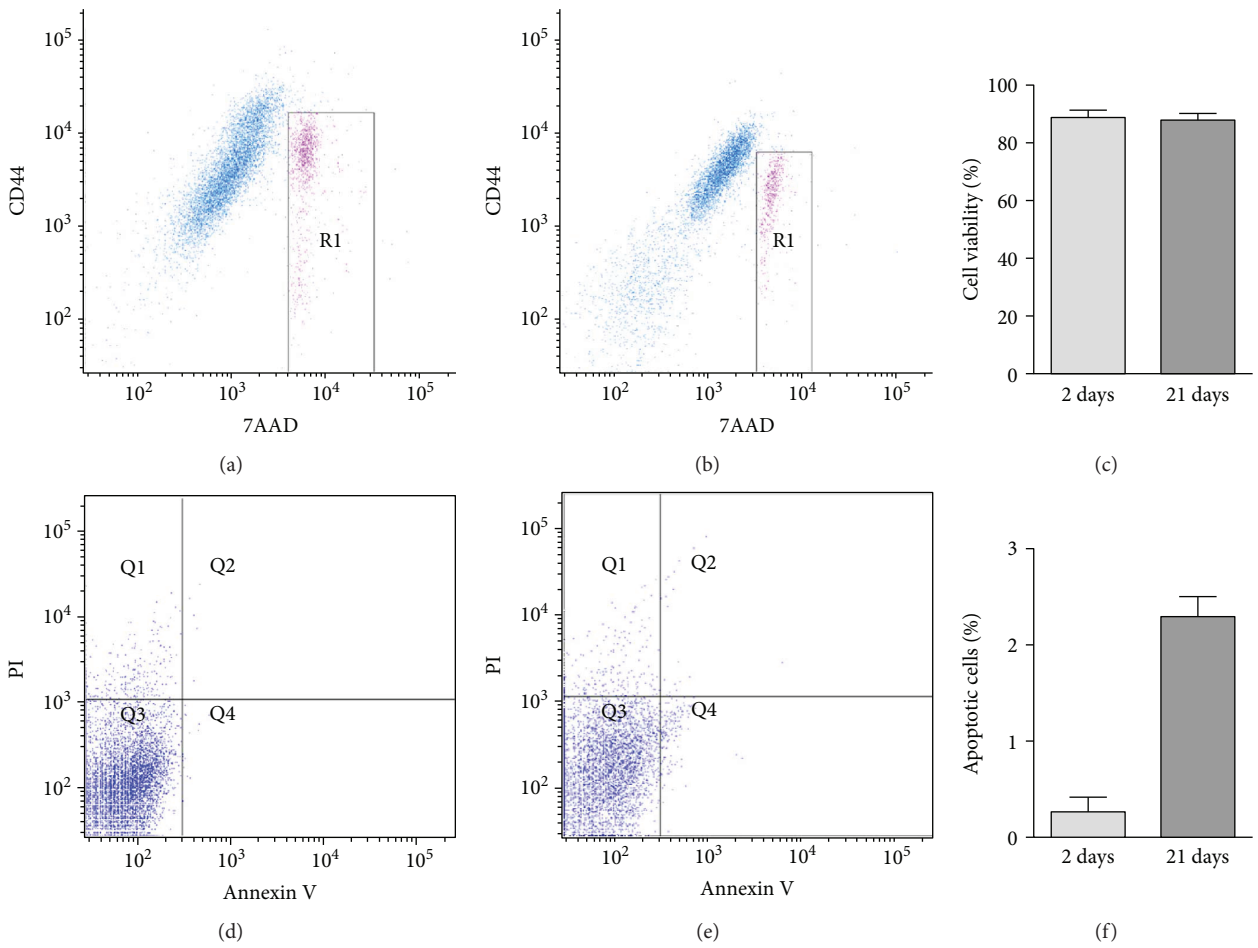

Figure 3: The CPC spheroids formed in the micromolded nonadhesive hydrogel with 800 μm diameter in each circular recesses showed a high percentage of viability after spontaneous chondrogenesis. (a, b) Dot-plot graphs showing CPC positive for only CD44 in blue and double positive for CD44 and 7AAD in pink, after digestion of spheroids cultivated for 2 (a) and 21 (b) days. The viable cells were identified by 7AAD exclusion, outside the region R1. (c) The graph represents the percentage of viable cells in three different samples ($n = 3$). (d, e) Dot-plot graphs of annexin V and PI evaluation showing CPC positive for annexin V in Q2 and Q4, after digestion of spheroids cultivated for 2 (d) and 21 (e) days. (f) The graph represents the percentage of apoptotic cells (annexin V positive cells) in three different samples ($n = 3$). 7AAD: 7-actinomycin D; CD: cell cluster of differentiation; PI: propidium iodide.

observed in the synthesis of TGF-β1 after 21 days of cell culture, the levels of TGF-β1 is still higher compared to TGF-β3 levels (Figures 4(a) and 4(c)).

As a typical functional assay for cartilage tissue, compressive tests were performed to compare the biomechanical properties of spheroids after spontaneous chondrogenesis (21-day culture) with those after compaction (2-day culture). CPC spheroids cultured for 21 days showed an increase in their force (μN) up to 4 times compared with those cultured for 2 days (Figure 5(d)), leading to a maximum deformation of 25% of their original diameter (Figures 5(b) and 5(c)).

Histological sections stained by Hematoxylin and Eosin showed a fibroblastic morphology in the external layer of CPC spheroids in contrast to a rounded shape inside (Figure 6(a)). The CPC spheroids were completely positive for N-cadherin (Figure 6(b)). Considering the extracellular matrix components, CPC spheroids also were positive for collagen type II and type VI (Figures 6(c) and 6(d)) and for the sulfated glycosaminoglycan aggrecan and chondroitin sulfate (Figures 6(e) and 6(f)) after spontaneous chondrogenesis (21-day culture).

The expression levels of SOX genes involved in the chondrogenic process were compared between cells cultivated as monolayer and as spheroids. The SOX5 and 6 genes were upregulated after spontaneous chondrogenesis (21-day culture) ($p < 0.001$). As shown in Figure 6(h), SOX5 and SOX6 genes were more than a hundred times more expressed in spheroids than in the monolayer counterpart used as control. Although SOX9 presented a fold-change higher than 30 times in spheroid samples compared to controls (monolayer), this difference in expression levels was not statistically significant ($p = 0.05293$).

Comparing to CPC spheroids after compaction (2-day culture), spheroids after spontaneous chondrogenesis (21-day culture) showed a reduced capacity of fusion. The spheroids were plated in close contact and after 1 hour, 1 and 7 days, a progressive increase in such proximity was noticed (Figures 7(a)–7(f)). A complete fusion was observed

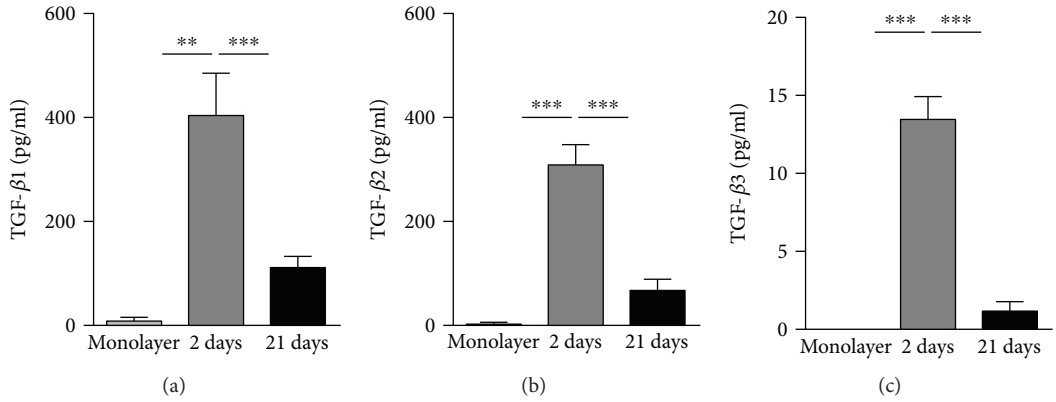

Figure 4: The CPC spheroids formed in the micromolded nonadhesive hydrogel with 800 μm diameter in each circular recesses showed an increase in TGF-β1, TGF-β2, and TGF-β3 synthesis comparing to CPCs monolayer. The CPC spheroids maintained for 2 days (spheroid compaction) in cell spheroid culture showed the highest synthesis of TGF-β1 (a), TGF-β2 (b), and TGF-β3 (c) comparing to the CPC spheroids maintained for 21 days (spontaneous chondrogenesis) and to monolayer ($^{**}p < 0.01$, $^{***}p < 0.005$). TGF-beta: transforming growth factor-beta.

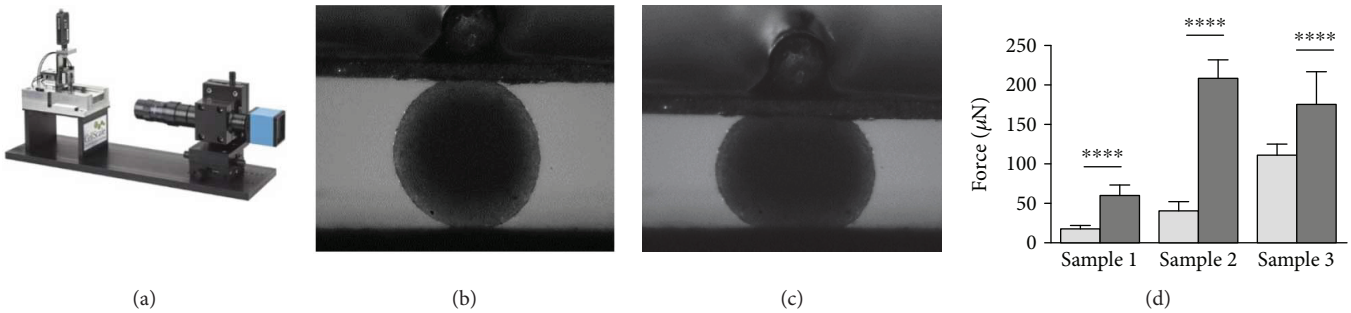

Figure 5: The CPC spheroids formed in the hydrogel with 800 μm diameter in each circular recesses show enhanced resistance to compression after spontaneous chondrogenesis (21-day culture) compared to those cultured for 2 days (spheroids compaction). (a) Equipment used to perform the loading tests. (b, c) One CPC spheroid between 2 plates before and after compression, respectively. Note spheroid deformation of 25% of the original diameter in (c). Five compression cycles were performed, with a load phase duration of 20 seconds and a recovery phase of 10 seconds. (d) Data collected from 3 spheroids of three different samples after spheroid compaction (2 days, light gray column) and after spontaneous chondrogenesis (21 days, dark gray column) are expressed as mean ± SD. $^{****}p < 0.0001$.

after 7 days in only CPC spheroids after compaction (2-day culture) (Figure 7(c)).

4. Discussion

The scaffold-free 3D cartilage constructs from human CPCs from nasal septum were successfully low-cost produced using the micromolded nonadhesive hydrogel technology. The spheroids showed homogeneity in their sizes and shapes, crucial for an efficient delivery of scaffold-free cartilage constructs for regenerative medicine approaches [22]. The CPC spheroids were established based on the self-assembling principle and recapitulated the chondrogenic differentiation pathway evaluated by TGF-β1, TGF-β2, and TGF-β3 synthesis, increasing force module, presented cartilage extracellular matrix components, and overexpressed SOX5 and SOX6 genes. In addition, we showed the *in vitro* capacity of CPC spheroids close to each other and even to fuse that could be interesting for successful *in vivo* implantation and long-term retention.

The commonly scaffold-free 3D technologies employed for cartilage tissue engineering are based on pellet culture [20], hanging-drop [23], and 96-well plate [10, 24], with the last two methodologies responsible for spheroid formation. Hanging-drop is a scalable technology; however, spheroids can be maintained in culture only for few days [23]. Recently, micromolded hydrogels have emerged as an alternative for spheroid culture due to the automation capacity and long-term culture for differentiation assays [25]. In contrast to 96-well culture plate, the micromolded nonadhesive hydrogel allowing to seed cell suspension with single pipetting, reducing substantially technical errors reflected in a homogeneity in spheroids size and shape. Besides, compared to 96-well culture plate, it is possible to obtain 10 times more spheroids per plate [3].

In this study, CPC spheroids showed homogeneity and stability in their size and shapes only in the micromolded nonadhesive hydrogel with 800 μm in each recesses. The difference between the two types of micromolded used in this study dwells on the diameter of each circular recesses, since

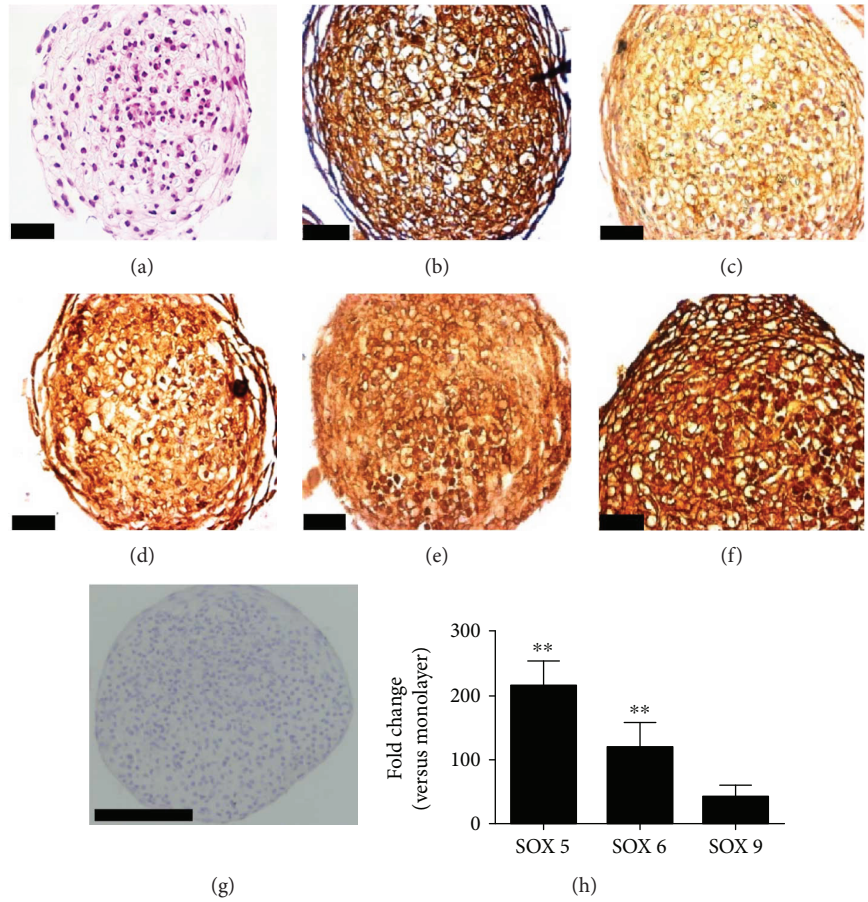

Figure 6: The CPC spheroids after spontaneous chondrogenesis are strongly positive for typical markers of cartilage tissue. (a) Hematoxylin and Eosin staining revealed rather rounded cells inside the spheroid. (b) N-cadherin is strongly present; (c–f) extracellular matrix typical markers of cartilage are shown throughout the spheroid area; (c) collagen type II; (d) collagen type VI; (e) aggrecan; (f) chondroitin sulfate. Bar size: 20 μm. (g) Control of nonspecific binding of secondary antibody (immune reaction carried out in the absence of the primary antibody). All reactions were done in the same moment. Bar size: 100 μm; μm: micrometers. (h) qPCR analysis of CPC spheroids showing upregulation of the SOX5 and SOX6 genes comparing to monolayer after 21 days of cell culture ($^{**}p < 0.01$). The expression of SOX9 gene was not statistically significant ($p = 0.05293$). Standard errors are shown.

both of them present 800 μm depth in each recesses (http://www.microtissues.com/3d-cell-culture-products.html). We hypothesize that the diameter found in micromolded with 300 μm in each recesses was not large enough to allow a precise self-assembly process in the entire cell population confined in each recesses.

The CPC spheroids established with the 800 μm micromold presented the diameter ranging from 486 μm $\pm$ 65. Usually, the diameter of CPC spheroids is smaller than that observed for the recesses due to the compaction phenomenon driven by adhesion molecules, mainly N-cadherins, similar to condensation phase in developing embryo [5, 9]. In this study, the compaction phenomenon was observed after 2 days of CPC spheroid culture.

In addition to homogeneity and stability in size and shape along spheroid culture, the CPC spheroids produced with micromolded with 800 μm in each recesses showed a high percentage (88.1 $\pm$ 2.1) of viable cells even after spontaneous chondrogenesis (21-day culture). A high percentage of viability is mandatory for cell differentiation and subsequent *in vivo* implantation of the scaffold-free 3D construct [26]. Viable cells were quantified by flow cytometry after the digestion of spheroids [21]. Besides 7AAD staining exclusion strategy (death cells), we used the surface marker CD44 to identify CPC cells [20] in the digested mass of cell spheroids. Only 7AAD-negative cells and CD44-positive cells were considered in our analyses. More importantly, cells in the core of spheroids can be dead by apoptosis. Recently, Murphy et al. showed considerable rate of apoptosis only in mesenchymal stem cell spheroids ranging from 700 μm [27]. In our study, apoptosis was a rare event even after spontaneous chondrogenesis (21-day culture).

After compaction phenomenon (2-day culture), the synthesis of TGF-β1, TGF-β2, and TGF-β3 by the CPC spheroids increases considerably comparing to monolayer. This increase may be related to the first step of chondrogenesis, known as the condensation phenomenon [5], similar to compaction phenomenon observed in spheroids driven by cadherins. Interestingly, the synthesis of all TGF-β isoforms decreases after spontaneous chondrogenesis (21-day culture). Our result supporting the hypothesis that TGF-β is crucial for the first phase of chondrogenesis [28].

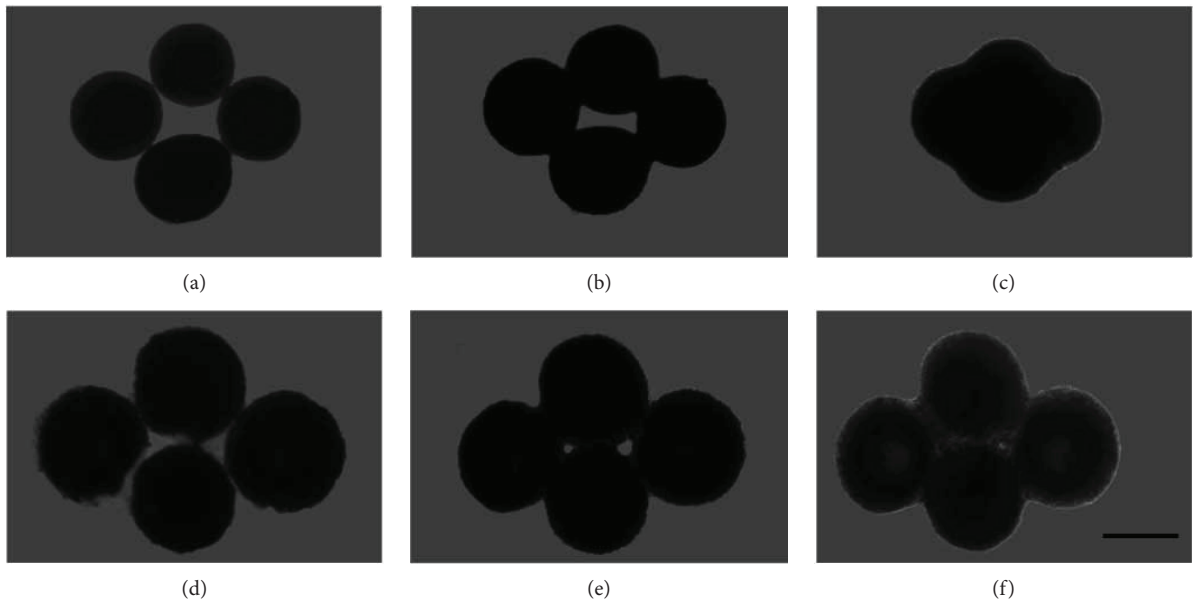

Figure 7: The CPC spheroids retained their fusion capacity even after spontaneous chondrogenesis. The CPC spheroids maintained for 2 days (a–c) and 21 days (d–f) in cell spheroid culture retained their fusion capacity. The CPC spheroids in close contact after 1 hour (a, d), 1 day (b, e) and 7 days (c, f). Note a complete fusion in c. Phase contrast microscopy. Bar size: 400 μm.

The TGF-β1 and TGF-β3 isoforms are attributed to chondrogenesis process, with the isoform β3 with strong *in vitro* evidence for successful chondrogenesis in human mesenchymal stem cells [28]. In chondrocytes, TGF-β1 induces extracellular matrix synthesis [29]. Because our source of cells represents a progenitor cell derived from cartilage, it is reasonable to attribute the high synthesis of TGF-β1 to the extracellular matrix synthesis evaluated at 21 days of CPC spheroid culture. Besides, TGF-β1 synthesis supports our hypothesis of spontaneous chondrogenesis of CPC spheroids. Furthermore, at 21-day culture, the CPC spheroids showed an increase in resistance to compression over time of spheroid culture as part of the successfully chondrogenic differentiation process. Our CPC spheroids have supported a deformation of 25% of their original diameter, reaching a maximum force of approximately 200 μN, possibly due to their extracellular matrix components—collagen type II and aggrecan [30].

Immunohistochemistry analysis revealed a mature extracellular matrix of cartilage tissue after spontaneous chondrogenesis (21-day culture). We noticed a strong and homogeneous staining for collagen type II, collagen type VI, aggrecan, and chondroitin sulfate, all of them are typical markers of cartilage tissue. Curiously, the positivity for N-cadherin was maintained until 21 days of CPC spheroid culture. During embryogenesis, this adhesion molecule is crucial for the mesenchymal condensation, the first step of chondrogenic differentiation [5].

Some studies have shown that N-cadherin cleavage is necessary for the progress of chondrogenesis [31]. Nevertheless, other studies showed a positive influence of N-cadherin not only in the condensation step but also in the extracellular matrix synthesis after *in vivo* implantation [32]. Besides cell-extracellular matrix adhesion, the cadherin-mediated adhesion is also involved in mechanotransduction [33]. In this study, the positivity for N-cadherin maintained after spontaneous chondrogenesis (21-day culture) may take account to the increase in resistance to compression over time of spheroid culture. Also at 21-day culture, the huge overexpression of SOX trio, especially SOX5 and SOX6, supports the successful differentiation of CPC spheroids towards chondrogenic pathway [8]. Likewise, just like N-cadherin, the SOX trio acts together to increase the expression of proteins related to extracellular matrix synthesis, such as COL2A1 [34], the main collagen of cartilage tissue.

As a preliminary step for *in vivo* assays, we have tested the ability of CPC spheroids to fuse with each other. Interestingly, despite the high content of extracellular matrix observed in CPC spheroids after spontaneous chondrogenesis (21-day culture), they were able to start to fuse with each other. However, the complete fusion was observed exclusively in CPC spheroids after compaction phenomenon (2-day culture). The capacity of fusion can be inversely correlated with the increase in resistance to compression over time of spheroid culture. Since spheroids follow the laws of fluid mechanics [35], it is reasonable to postulate that the increase in superficial tension can lead to a resistance in spheroids to fusion. On the other hand, this increase in superficial tension reflects a stability in spheroids and may indicate a tissue maturity. Although scaffold-free approaches seem to be well integrated with surrounding cartilage tissue, the integration boundary is still fragile [4]. The intrinsic capacity of fusion from CPC spheroids after compaction phenomenon (2-day culture) could be crucial for a long-term retention in implantation site.

The micromolded nonadhesive hydrogel is a promise technology for automatized biofabrication scaffold-free 3D constructs. In this study, spheroids of CPCs were

responsible for successful low-cost cartilage scaffold-free tissue engineering without exogenous stimulus (spontaneous differentiation), supporting the use of CPC spheroids for tissue engineering and regenerative medicine approaches. Our next step will be delivery CPC spheroids in a preclinical *in vivo* assay. Furthermore, the development of automation protocols is in progress, aiming the scaling of cell spheroid culture and scaffold-free 3D cartilage large construct biofabrication by bioprinting approaches [36].

5. Conclusion

The fabrication of 3D scaffold-free cartilage constructs using micromolded nonadhesive hydrogel was responsible for cartilage constructs with homogeneous size and shape and high cell viability together with the possibility of scaling cell spheroid culture using automation. To the best of our knowledge, this is the first time in the scientific literature that spheroids of CPCs from human nasal septum were produced using micromolded nonadhesive hydrogel, achieving a successful cartilage low-cost scaffold-free tissue engineering without exogenous stimulus. Delivering CPC spheroids introduce a great promise to improve retention in cartilage tissue implantation site.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

José M. Granjeiro, Vladimir Mironov, and Leandra S. Baptista made the concept/design. Mellannie P. Stuart, Renata A. M. Matsui, Matheus F. S. Santos, Isis Côrtes, Mayra S. Azevedo, Karina R. Silva, Anderson Beatrici, Paulo Emílio C. Leite, and Priscila Falagan-Lotsch made the methodology. Mellannie P. Stuart, Karina R. Silva, Anderson Beatrici, Paulo Emílio C. Leite, and Priscila Falagan-Lotsch did data analysis/interpretation. Mellannie P. Stuart, Karina R. Silva, Priscila Falagan-Lotsch, and Leandra S. Baptista were assigned for the drafting of the article. Leandra S. Baptista, José M. Granjeiro, and Vladimir Mironov were assigned for the critical revision of the article. Leandra S. Baptista was assigned for the approval of the article.

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Review Article

The Application of Stem Cells from Different Tissues to Cartilage Repair

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The degeneration of articular cartilage represents an ongoing challenge at the clinical and basic level. Tissue engineering and regenerative medicine using stem/progenitor cells have emerged as valid alternatives to classical reparative techniques. This review offers a brief introduction and overview of the field, highlighting a number of tissue sources for stem/progenitor cell populations. Emphasis is given to recent developments in both clinical and basic sciences. The relative strengths and weaknesses of each tissue type are discussed.

1. Introduction

Articular cartilage has a poor self-healing potential, mainly due to the lack of vascularisation and the paucity of undifferentiated cells [1]. Thus, if focal cartilage lesions are left untreated, they can progress to more extensive defects and may ultimately require treatment with joint replacement surgery if conservative options fail. The aim of this review is to describe in detail recent findings in both basic and clinical studies that have adapted cells from a variety of cell sources to cartilage repair strategies.

Current treatments for cartilage repair are mainly focused on bone marrow stimulation techniques: such as abrasive chondroplasty, subchondral drilling, microfracture and, more recently, nanofractures [2]. The aim of these techniques is to allow migration to the damaged area and the subsequent chondrogenic differentiation of multipotent bone marrow-derived stromal/stem cells (BMSCs). However, often, the regenerated tissue does not possess the same biochemical and biomechanical properties of the native cartilage; therefore, it is not able to resist the continuous stresses placed upon it, and it quickly degenerates [3]. Hence, new treatment options for articular cartilage lesions have grown in recent decades, due to promising results obtained with the development of new therapeutic options.

Tissue engineering strategies aim to regenerate the damaged tissue and restore a biologically and biomechanically valid articular surface. This requires three components, which may be alternately combined. The first is a suitable cell source which can differentiate into, and maintain, the specific cell phenotype; research in this area forms the body of this review (see Table 1). Additionally, signalling molecules such as growth factors, cytokines, or hormones stimulate cell growth and differentiation, and traditionally, a scaffold is used to provide an adequate three-dimensional environment [4, 5], although scaffold-free techniques have also proven successful (reviewed in Shimomura et al. [6]).

Growth factors, cytokines, and hormones are used to stimulate cell proliferation (owing to the low number of endogenous progenitors) and induce chondrocytic differentiation (without inducing hypertrophy or causing transformation) leading to the secretion of a collagen-rich extracellular matrix (ECM). Growth factors exert their effects by binding to, and activating, specific membrane-bound (usually transmembrane) receptors. Ligand binding typically leads to the activation of an intracellular signalling cascade (such as

Table 1: Summary of recent results in the application of stem and progenitor cells to cartilage repair and regeneration.

(a)

Author	Year	Cell source	Model	Experimental study	Adverse effects	Key findings
Shafiee et al.	2011	Cartilage	Mice	Proliferation, tumourigenesis, and multipotency of nasal septum-derived adult cells	None	NCs retained chondrogenic potential until passage 35. Markers suggest chondrogenic ability equal to that of BMSCs
do Amaral et al.	2012	Cartilage	*In vitro*	Proliferation and multipotency of nasal septal cartilage surface zone cells within the context of cartilage repair	NA	Cells in pellet culture resulted in chondrogenesis without TGF-β or BMPs. NCs were $CD105^+$, $CD73^+$, $CD44^+$, and $CD146^-$
Pelttari et al.	2014	Cartilage	Humans (10), mice, goats	Suitability of adult human neuroectoderm-derived nasal chondrocytes for articular cartilage repair	None	NCs proliferated faster and were more chondrogenic than Acs *in vitro*. *In vivo*, defect filling was observed after 4 months
Jiang et al.	2016	Cartilage	Humans (15), mice	Cartilage repair potential of resident cartilage stem/progenitor cells	None	ACs became $CD146^+$ in high-density 2D culture, and their chondrogenic potential is similar to that of BMSCs. *In vivo* results were promising
Embree et al.	2016	Cartilage	Rats, rabbits	Potential of single resident fibrocartilage stem cells (FCSC) to regenerate cartilage, bone, and haematopoietic compartment	None	FCSCs spontaneously produced cartilage anlage *in vivo* which was then remodeled into trabecular bone. Addition of sclerostin maintained the FCSC pool and led to chondrocyte differentiation and cartilage repair *in vivo*
Fellows et al.	2017	Cartilage	*In vitro*	Senescence of healthy versus diseased human knee articular cartilage rather than regenerative potential per se	NA	The number of progenitor cells was greater (2x, $P < 0.001$) in OA tissue than in healthy cartilage. Subpopulation of OA-derived cells had reduced proliferative potential and underwent early senescence *in vitro*. An increase in senescent cells may contribute to the disease phenotype
Pittenger et al.	1999	BM	*In vitro*	Maintenance of multipotency in individual adult BMSCs	NA	Adult stem cells can be induced to differentiate exclusively into adipocytic, chondrocytic, and osteogenic lineages
Wakitani et al.	2004	BM	Humans (2)	Effectiveness of autologous BMSC transplantation for the repair of full-thickness articular cartilage defects in the patellae of 2 individuals	None	Clinical symptoms (pain & walking impediment) were significantly reduced 6 months postop. Benefits remained for 4-5 years. Arthroscopy revealed defects filled with fibrocartilage
Wakitani et al.	2011	BM	Humans (41)	Safety of autologous BMSC implantation for cartilage defects	None	No tumour or infections reported in any patient. Five had total knee replacement due to progression to OA
Wong et al.	2013	BM	Humans (56)	Autologous BMSC i.a. injections with microfracture and tibial osteotomy	None	The experimental group showed significantly better IKDC ($P = 0.001$), Tegner ($P = 0.021$), MOCART ($P < 0.001$), and Lysholm ($P = 0.016$) scores

(b)

Author	Year	Cell source	Model	What was examined	Adverse effects	Key findings
Vangsness et al.	2014	BM	Humans (55)	Safety and effects on OA changes in the knee following intra-articular injection of allogeneic human BMSCs	None	Evidence of meniscus regeneration and improvement in knee pain following treatment with allogeneic human mesenchymal stem cells

Table 1: Continued.

Author	Year	Cell source	Model	What was examined	Adverse effects	Key findings
Gobbi et al.	2014	BM	Humans (25)	BMAC (BM aspirate concentrate) for the repair of large full-thickness knee cartilage defects	None	Significant improvement in Tegner, Marx, Lysholm, VAS, IKDC subjective, and KOOS scores at the final follow-up compared with their respective preoperative scores ($P < 0.001$); MRI analysis at the final follow-up showed stable implantation and complete filling of the defect in 20 of 25 patients
Vega et al.	2015	BM	Humans (30)	Effects of i.a. injection of allogeneic BMSC versus hyaluronic acid for the treatment of knee OA	None	At 1-year follow-up, cartilage formation in cell-treated defects was significantly improved over control (HA)-treated defects
Nakagawa et al.	2016	BM	Rats	Lubricin expression and chondrogenesis in BMSCs using pellets & hanging-drop cultures *in vitro* and *in vivo*	NS	The treatment group scored significantly higher than the control group when assessed histologically at 8 and 12 weeks
Chen et al.	2016	BM	Rabbits	PTH-treated versus untreated BMSCs embedded in fibrin glue for the repair of induced articular cartilage injury in rabbits	None	The ICRS score significantly increased ($P < 0.05$) in PTH-treated versus non-PTH and untreated groups. Significantly increased levels of type II collagen and aggrecan mRNA and protein in PTH versus non-PTH groups ($P < 0.05$)
Shapiro et al.	2017	BM	Humans (25)	BMAC for the treatment of knee pain from bilateral osteoarthritis	None	Knee pain decreased in all groups, although no significant difference between BMAC and saline groups ($P > 0.9$)
Koga et al.	2008	Synovium	Rabbits	"Local adherent technique" whereby an i.a. injection of synovium stem/progenitor cells adheres to the defect site within 10 minutes	NA	Increased cell attachment correlated with improved cartilage repair at 24 weeks. It was reported that 60% of injected cells adhered at the site
Nakamura et al.	2012	Synovium	Pigs	Adherence of synovium-derived cells to cartilage defects and effects on cartilage	None	The cartilage matrix detected in all treated defects versus none in the control group. Wakitani and ICRS scores were significantly higher in treatment groups ($P < 0.05$). Higher chondrogenic potential in synovial cells versus BM, adipose, muscle, or periosteum-derived cells
Sekiya et al.	2015	Synovium	Humans (10)	"Local adherent technique" using autologous synovium-derived stem/progenitor cells	1 patient had fibrillation of repaired cartilage	Transplantation of synovial cells was deemed effective: Lysholm and MRI-based scores increased over 3 years + follow-up period (both $P = 0.005$)
Mak et al.	2016	Synovium	Mice	Chondrogenic potential of synovium-derived sca-1-positive stem/progenitor cells injected into injured joint	NS	Intra-articular injection of Sca-1^+ GFP^+ synovial cells from C57BL6 or MRL/MpJ "super-healer" mice to C57BL6 mice following cartilage injury

Table 1: Continued.

Author	Year	Cell source	Model	What was examined	Adverse effects	Key findings
						led to similar levels of cartilage repair. Treatment with cells resulted in cartilage repair that was significantly greater than that of untreated defects
Baboolal et al.	2016	Synovium	Dogs	Role of HA on MSC attachment to cartilage	NS	It was confirmed that HA inhibits MSC-cartilage attachment
Diekman et al.	2010	Adipose	*In vitro*	Differences in chondrogenic potential of ADSC and BMSC in different culture conditions	NA	ADSCs and BMSCs require different *in vitro* culture conditions to achieve optimal chondrogenic outcomes. While both ADSC and BMSC underwent chondrogenic differentiation in all conditions tested, BMSCs produced a more matrix over a wider range of conditions
Koh et al.	2013	Adipose	Humans (18)	Outcome of i.a. injections of autologous ADSCs for the treatment of knee OA	One case of pain and swelling	Significant reduction in WOMAC scores ($P > 0.001$) relative to preop levels. The Lysholm score increased from 40.1 points to 73.4 points ($P > 0.001$), and the mean VAS score decreased over the period of the study from 4.8 to 2.0 ($P > 0.005$)
Jo et al.	2014	Adipose	Humans (18)	Safety and efficacy of i.a. injections of autologous ADSC for knee OA	None	Improvements were seen in the high-dose group (improvement in WOMAC & VAS at 6 months). Significant decreases in cartilage defect size paralleled by an increase in cartilage volume at some defect sites at 6 months
Koh et al.	2015	Adipose	Humans (30)	Injection of ADSCs and arthroscopic lavage for knee OA	Slight knee pain, resolved with medication	The technique appears to be effective in cartilage healing, reducing pain, and improving function
Koh et al.	2016	Adipose	Humans (80)	ADSCs with fibrin glue and microfracture (MFX) versus MFX alone in patients with symptomatic knee cartilage defects	NS	Both treatment groups saw improvement in multiple clinical outcomes; however, the degree of improvement was greater in patients who received ADSC in addition to MFX
Pers et al.	2017	Adipose	Humans (18)	Intra-articular injections of different doses of ADSCs	Unstable angina pectoris reported in 1 patient, 5 minor AEs reported by four patients potentially related to the procedure	All dose groups saw an overall negative trend in WOMAC (pain, stiffness, and function), VAS, and SAS, although these data were significant only in the low-dose group
Saw et al.	2013	Peripheral blood	Humans (50)	Postoperative i.a. injections of hyaluronic acid with and without PBSC	None	A nonsignificant ($P = 0.8$) increase in the IKDC score for the PBSC group at 24 months. A significant ($P = 0.013$) increase in the MRI score in the PBSC group at 18 months

Table 1: Continued.

Author	Year	Cell source	Model	What was examined	Adverse effects	Key findings
Fu et al.	2014	Peripheral blood	Rabbits	Mobilised rabbit PBSCs versus rabbit BMSCs for *in vivo* chondrogenesis	None	PBSCs showed greater chondrogenic potential than BMSCs *in vitro*, although both cell types performed equally well in *in vivo* assays for cartilage repair
Fu et al.	2014	Peripheral blood	Humans (1)	Injection of autologous activated PBSCs + autologous periosteum flap in a chondral lesion	None	Second-look arthroscopy showed a smooth surface at 8 months postoperation. CT and MRI evaluations showed a significant improvement compared to preoperation
Saw et al.	2015	Peripheral blood	Humans (8)	Autologous PBSCs and HA with concomitant medial open-wedge high tibial osteotomy	None	At 25-month follow-up, arthroscopy and biopsy revealed smooth, well-integrated regenerated tissue rich in type II collagen and proteoglycan, with some type I collagen present
Ha et al.	2015	Umbilical cord	Minipigs	Ability of human UBSC cell lines in HA hydrogel (versus empty defects) to repair osteochondral defects	None	Defects which received cells + HA had more safranin-O-positive staining, more regenerated cartilage, and better integration with the surrounding tissue. The IRCS score was better in cell transplant defects than in empty defects
Li et al.	2016	Umbilical cord	*In vitro*	It was determined whether coculture of human ACs could increase chondrogenic potential of human UBSCs	NA	Indirect coculture increased expression of chondrogenic markers. However, qPCR, WB, and some 2D IHC data contain inconsistencies
Gomez-Leduc et al.	2016	Umbilical cord	Mice	Chondrogenic potential of human UCBSCs seeded on type I/III collagen sponges ± chondrogenic factors	NS	UBSCs cultured *in vitro* with TGF-β1 and BMP-2 were implanted in nude mice. Cells exposed to growth factors in an *in vitro* phase produced a cartilaginous matrix rich in type II collagen. No scaffolds progressed to calcification but instead deposited type II collagen-rich ECM
Park et al.	2017	Umbilical cord	Humans (1)	Transplanted human UCBSCs in a 4% HA hydrogel into a rabbit trochlea defect	None	VAS, IKDC, & WOMAC improved. At 1-year follow-up, second-look arthroscopy and biopsy showed smooth safranin-O-positive hyaline-cartilage with excellent peripheral integration. MRI showed defect filling, abundant repair tissue, and good integration with the surrounding tissue
Park et al.	2017	Umbilical cord	Humans (7)	Treatment of a large osteochondral defect by autologous UCBSCs in a HA hydrogel	None	Regenerated tissue was thick, smooth, and glossy white with good integration with the surrounding tissue and

Table 1: Continued.

Author	Year	Cell source	Model	What was examined	Adverse effects	Key findings
Park et al.	2017	Umbilical cord	Rabbits	Efficacy of human autologous UCBSCs and HA hydrogels for cartilage regeneration	None	resembled hyaline-like cartilage with abundant GAG content. No bone formation or overgrowth was observed Macroscopically, cells + hydrogel produced better cartilage formation than hydrogel only or untreated controls. Regenerated tissue was smooth and type II collagen rich

NA: not applicable; NS: not stated.

MEK/ERK, protein kinase C, and PI3K/AKT) and/or transcription factors, resulting in altered gene expression. Genes related to proliferation and differentiation are common targets of fibroblast growth factor 2 (FGF-2), which has been utilized for BMSC expansion [7], while insulin-like growth factor 1 has been applied to chondrogenic differentiation of peripheral blood (PB) cells [8] and to the repair of cartilage defects in rabbits [9]. Members of the TGF-β superfamily, which include TGF-β1 and BMPs 2, 4, and 7, have been shown to influence the development of cartilage [10] but may skew differentiation towards hypertrophic chondrogenesis and endochondral ossification [11], and TGF-β has been linked to cancerous progression in humans [12]. Alternatively, growth differentiation factor 5 has been shown to regulate the differentiation of articular chondrocytes [11] at least in part through inhibition of the BMP4 pathway [13].

Methods for the isolation and preparation of stromal cell populations are not standardized. Indeed, the method of isolation and preparation and the degree of *ex vivo* manipulation vary widely between laboratories and donor tissue source. Generally, tissue samples are harvested in sterile conditions, and cells are isolated with by different methods (enzymatic digestion or direct culture). Subsequently, cells are cultured *in vitro* with different conditions. The most popular method to induce chondrogenesis consists in of pellet culture with conditioned medium, which is enriched with insulin, dexamethasone, ascorbic acid, sodium pyruvate, and growth factors, such as TGF-βs or BMPs [14]. Chondrogenesis is then confirmed by the analysis of the extracellular matrix (production of GAGs) and by gene expression of cartilaginous markers (i.e., collagen type II, Sox-9).

The choice of scaffold material is significant as the 3D microenvironment is important for the correct growth and differentiation of cells [5, 15–18]. This microenvironment includes not only the materials which constitute the scaffold and their characteristics (such as porosity [19], rigidity [18], and biodegradability [16]) but also the *in vitro* culture conditions (media formulations, as well as both hydrostatic and mechanical forces [20, 21] and oxygen levels [22] that cells are exposed to).

Thus, growth factors and scaffolds are often combined with cells for regenerative purposes. For cartilage repair, several cell sources are already available and others are rapidly emerging; the aim of this manuscript is to provide an overview of recent developments in the field, with a particular focus on stem cells.

2. Terminally Differentiated Cells or Multipotent Cells for Cartilage Repair

Articular chondrocytes have been extensively used in the past years for autologous chondrocyte transplantation. However, the use of articular chondrocytes is limited by several factors: morbidity at the harvest site, the requirement of a second surgical procedure, and cell dedifferentiation due to *in vitro* expansion [4, 23–25], necessitated by the limited number of harvestable cells. Alternative sources of differentiated chondrocytes have been investigated, and recently, in a first-in-human trial, autologous nasal septum chondrocytes were used for the repair of full-thickness articular cartilage defects of the knee [26]. At 2-year follow-up, the changes in a range of clinical scores (IKDC, KOOS pain, KOOS symptoms, KOOS function in daily living, KOOS sport, and KOOS quality of life, relative to preintervention) were positive and the safety of the procedure was confirmed [26]. A phase II clinical trial (NCT01605201) is currently underway to confirm these data.

Stem cells are a cell source of vast potential, which can be isolated from a range of different tissues. These cells constitute a self-renewing population, which can undergo multilineage differentiation [27]. Pluripotent embryonic stem cells derive from the fertilization of the egg, and they can differentiate into any of the three germ layers (endoderm, mesoderm, or ectoderm); thus, they possess the potential to differentiate into any cell lineage. The role of these cells for tissue engineering has been investigated since the late '90s; however, along with induced pluripotent stem cells (iPSCs), the potential tumourigenicity and ethical issues have limited their use in clinical practice (with the notable exception of umbilical cord-derived stem cells [28, 29]). Conversely, adult postnatal stem cells can be more easily utilized for tissue engineering. These cells have a limited self-renewal and multilineage potential [27, 30, 31], but they can be isolated from individuals of any age without the ethical dilemmas of embryonic stem cells. The term "mesenchymal stem cell' (MSC) [32] describes a specific subpopulation of adult stem cells on the

basis of established "minimal criteria" identified by the International Society for Cellular Therapy (ISCT) [33, 34] including several cell-surface markers, adherence to plastic culture dishes, and the potential to differentiate into chondrogenic, osteogenic, myogenic, adipogenic, and tenogenic lineages. Cell populations conforming to these criteria can be isolated from several tissues: bone marrow, synovium, adipose tissue, periosteum, peripheral blood, and umbilical cord blood, as well as from the inner part of cartilage of the knee. It must, however, be pointed out that often, these cell populations which are labelled "stem cells" would be more accurately described as stem cell-containing populations. Frequently, the multipotency and self-renewal capacity of these cells are not reported despite the existence of simple tests to do so, such as the colony-forming assay (CFU). The number of actual stem cells isolated from tissue can vary enormously depending on the age of the patient, the technique used for isolation, and the source tissue [31]. The omission of this data makes it challenging to assess the true role of the stem cell as opposed to stromal cells in these studies and to make meaningful comparisons between different studies [35]. Finally, the term "MSC" is sometimes used with no additional information as to the tissue of origin, while stromal cell populations isolated from the bone marrow or adipose tissue, for example, may both conform to the ISCT criteria for "MSCs"; they differ at the epigenetic [36] and phenotypic levels [37, 38] making the inclusion of this information crucial.

The chondrogenic potential of numerous stem cells has been analysed with regard to their possible use in tissue engineering. Probably, the most obvious source of stem cells to regenerate cartilage tissue is cartilage itself, and many studies have sought to isolate and harness the regenerative power of cartilage-resident stem/progenitor cells, some with great success [39–41] (see Table 1). Early studies followed from the illustration of the multilineage potential of BMSCs [27] culminate at the end of the last century with the demonstration of the exclusive and stable differentiation of clonal BMSC populations into chondrocytes [41]. Since then, researchers have capitalised the diversity of tissues from which stem/progenitor cells can be extracted.

Hereafter, we will singularly describe the different tissue sources of stem cells (Figure 1).

2.1. Cartilage. Tissue engineering strategies utilizing autologous cartilage-derived stem/progenitor cells have been attempted since the late 1980s [42–45]. The largely acellular character of cartilage [44] combined with the scarcity of progenitors has been a hurdle to its use; however, some success has been seen using the cells resident in the articular cartilage of the knee [42, 45] and the jaw [39]. The advantage of chondrocytes and cartilage-resident cells is their ability to survive in the hypoxic environment found in the wound/implant. Successful results have also been reported using cells taken from the nasal septum [26, 46–48].

The articular cartilage of the knee is a thin layer of largely acellular connective tissue that protects and facilitates the movement of the joints [44]. Due to the low number of resident progenitor cells and challenges in defining the characteristics of the cartilage stem/progenitor cell [1], cartilage isolates have proven unconducive to *in vitro* cartilage production and any *in vitro* manipulation must be checked for unintended subsequent osteogenesis or tumourigenesis after implantation.

Recently, resident cartilage progenitor cells isolated from autologous cartilage tissue were shown to form tissue with the characteristics of hyaline cartilage when implanted ectopically in a mouse model; this was supported by data from high-density 2D cultures [39]. These cells were expanded *in vitro* and implanted in the knees of patients on a collagen scaffold. Patients reported significant improvements (using both IKDC and Lysholm scoring systems); importantly, MRI indicated that the implants covered the defect site and that no sign of hypertrophy was present; histological examination of a subset of implants showed no calcification, inflammation, or vascularisation. In addition to the improved clinical scores, 14 of 15 patients resumed sports activities within 1 year of the intervention, indicative of the practical value of this technique for improving patient quality of life.

Another study overcame the paucity of resident fibrocartilage stem cells (FCSC) within the jaw articular cartilage through prolonged *in vitro* culture [37]. Animal studies showed that a single FCSC was capable of generating a cartilage template that was remodelled into bone and a bone marrow space, including the haematopoietic microenvironment, without exogenous stimulation from osteogenic scaffolds (such as hydroxyapatite), Matrigel or factors, such as BMPs. This would seem to be great news for bone tissue engineers, but not so great for cartilage regeneration as formation of bone within the articulation is hardly an ideal outcome. However, the authors describe the mechanism by which the pool of resident FCSCs is maintained, though the inhibition of WNT signalling by sclerostin. Indeed, application of sclerostin favoured the differentiation of FCSCs into mature chondrocytes and aided cartilage repair in a rabbit model of cartilage injury [37].

The potential of cells from the nasal septum cartilage for tissue engineering applications was hinted at by basic studies from 2011 to 2012 [46, 48]. Through *in vitro* experiments and mouse studies, the inherent chondrogenic potential of nasal chondrocytes (NCs) was shown to be similar to that of matched BMSCs with NCs undergoing chondrogenesis in pellet culture without stimulation from either TGF-β or BMPs [46]. Significantly, NCs retained their chondrogenic abilities for far longer, until passage 35, in line with observations that NCs displayed lower levels of senescence markers than BMSCs [48] which would indicate that NCs could be advantageous for tissue engineering strategies that call for multiple rounds of ex vivo expansion. Dedifferentiated NCs have been shown to have greater clonogenic potential (over 3-fold more) and to proliferate faster that articular chondrocytes [38]. Unlike BMSCs, NCs were not susceptible to adipogenic induction [46, 48], possibly due to the significantly higher levels of BMP2 mRNA in NCs [48]. *In vivo*, NCs displayed no tumourigenicity or signs of metastasis in mice after 4 months, and clinical data show that autologous *ex vivo*-expanded NCs filled the defect and had no signs of delamination after a similar period of time [38].

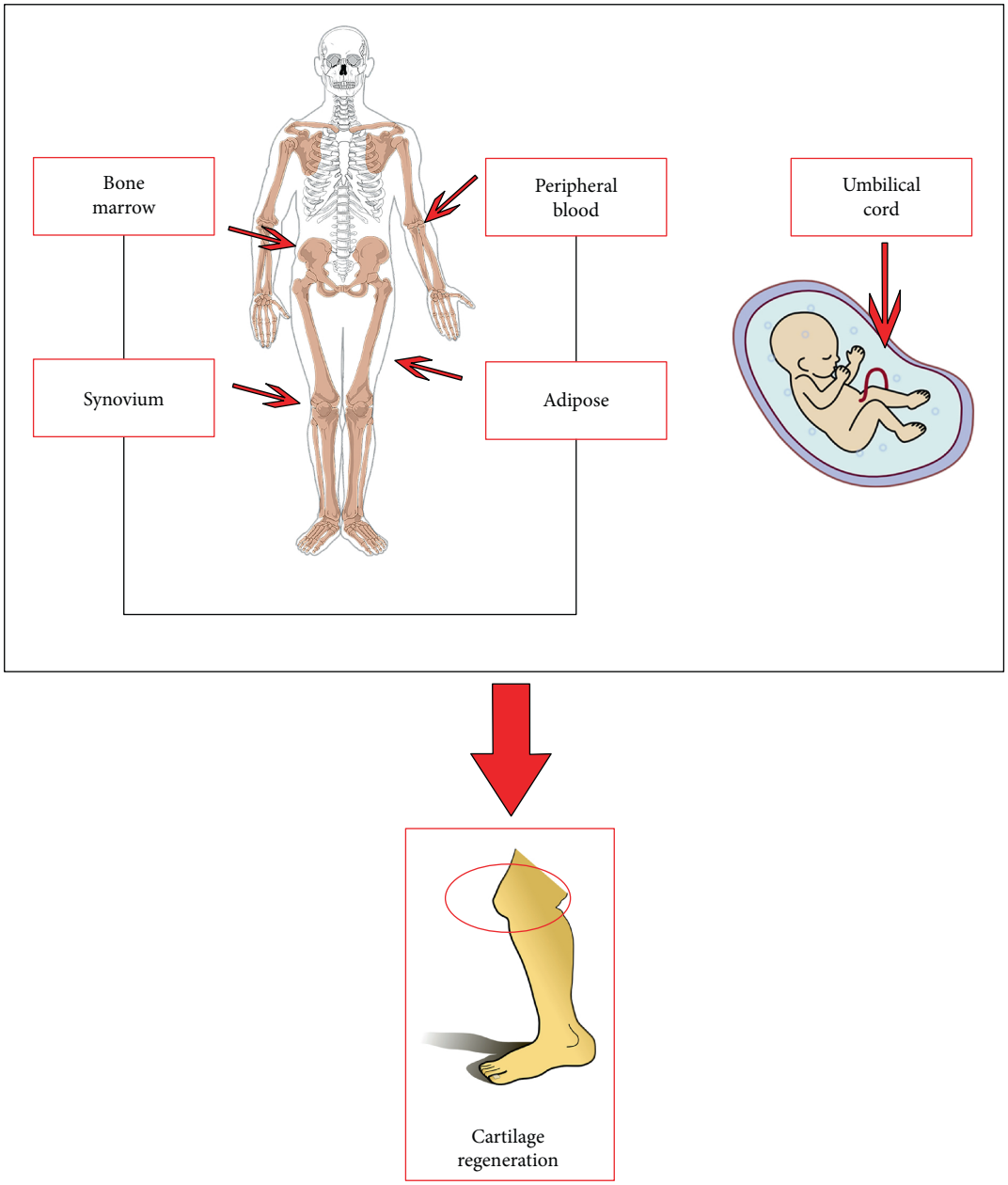

Figure 1: Stem cell sources for cartilage repair.

2.2. Bone Marrow. Substantial clinical information is available on the suitability of bone marrow stromal cells (BMSCs) for cartilage tissue engineering. From initial results showing the potential for cartilage repair [41] to multiple clinical trials [45, 49–54], there is ample evidence to illustrate the applicability of BMSCs to cartilage defect repair. The majority of studies have focused on the use of autologous cells [45, 49–51, 53, 54]; however, there are also instances of successful application of allogeneic stem cell preparations to cartilage repair [52].

Removal of the bone marrow is usually achieved by aspiration from the iliac crest of the pelvis. While this is less invasive than some other methods of harvesting cells, the number of stem/progenitor cells obtained is not high and some form of expansion is often performed to obtain sufficient cell numbers. Aside from the concerns about the loss of cell multipotency during 2D cell culture [55], this remains the standard method for expansion of BM stromal cell samples. In some cases, the 2D expanded cell population is then embedded or seeded on a scaffold which provides rigidity and form, before being implanted at the defect site: the choice of scaffold material is not trivial and may influence the differentiation of the embedded cells [56]. The efficacy of 2D expansion followed by implantation on a cartilage-based scaffold has been demonstrated in the lab and in the clinic [45, 49, 51, 53, 54] with follow-up times up to 11 years [53]. Clinically scaffold-based BMSC implantation resulted in significant improvements in various indicators of quality of life and joint function, including increased mobility and reduction of pain. Although not all patients are willing to undergo second-look

arthroscopy to assess cartilage formation and coverage, some data exists which shows that some defects were filled with fibrocartilage [49]. Scaffold-free administration of BMSCs expanded *in vitro* to form a "cell sheet" has also been shown to be effective in an animal model of cartilage defects at 12 weeks [7]. Here, FGF-2, in combination with chondrogenic factors, was noted to increase chondrogenic differentiation as well as cell growth [7]. Intra-articular (i.a.) injection of BMSCs alone [57, 60, 61] or with additional materials (such as hyaluronic acid (HA)) [59] has been applied to cartilage repair in clinical studies. The results were mostly positive, with an improvement in articular cartilage and meniscal repair noted in patients treated with BMSCs as opposed to controls (when analysed by IKDC, Tegner, and Lysholm scores, as well as MRI and MOCART scores in addition to evaluations of pain and quality of life) [50, 54].

Owing to the low frequency of stem/progenitor cells within the BM and the period of time required for *in vitro* expansion (typically several weeks), an alternate approach has been used for bone marrow aspirate concentrate (BMAC) [49, 50]. This technique has produced mixed results for the treatment of both osteoarthritis (OA) and cartilage defects in the knee. Gobbi and colleagues describe a case series with significant improvements in multiple scoring matrices (Tegner, Marx, Lysholm, VAS, IKDC subjective, and KOOS scores: $P > 0.001$) at 41 months postoperation (postop), relative to the same tests prior to the intervention [49]. On the other hand, Shapiro et al., in a randomised controlled trial for the treatment of bilateral OA, injected patients with either saline or BMAC, with follow-up at 6 months only to find that the level of pain relief afforded was similar in both treatment and control groups [50]. An additional technique involves the *in vitro* use of FGF-2 to rapidly expand autologous BMSCs to the point where it is feasible to generate a scaffold-free osteochondral implant thus partially overcoming the often limiting number of BMSCs obtainable from patients [7].

Caution must be exercised when using cells derived from the BM for cartilage repair, as the cells that generate hyaline cartilage are distinct from the growth plate chondrocytes found in the BM which form hypertrophic cartilage that is then remodelled into bone [13, 57–60]. Also, there is evidence suggesting that the differentiation and colony-forming potential of BMSCs decrease with donor age, a potential hurdle for autologous use in the elderly [61].

Future prospects for the use of BMSCs for cartilage engineering include the application of 3D printing technologies to tissue engineering with various groups reporting on the fabrication of 3D scaffold materials [62–64]. Recently, the concept has been taken to the next logical step, and a mixture of viable BMSCs and various polymers was used to create a 3D ECM containing live cells which survived *in vivo* and expressed markers of chondrocytic differentiation [62].

2.3. Synovium. It has been shown, via lineage tracing, that articular chondrocytes derive from synovial joint progenitors, or interzone cells [57], which do not contribute to the growth plate and thus to the formation of bone though endochondral ossification. This represents an advantage in the field of cartilage tissue engineering as heterotopic ossification is to be avoided. The development of articular chondrocytes, as opposed to hypertrophic chondrocytes, has been shown to be influenced by the TGF-β pathway, as opposed to signalling through BMP4 [13].

Synovial cells have been assessed for their use in cartilage repair, although few clinical data are published. Basic studies in animal models however are promising showing that synovium-derived cells represent a valid option for continuous study. Mak et al. found a population of synovial sca-1^+ progenitor cells with inherent chondrogenic potential which were shown to increase cartilage repair 4 weeks after i.a. injection in a mouse model [65], while Baboolal and colleagues present results suggesting that HA present in the synovial fluid inhibits the initial interaction between stromal cells and cartilage [66]. These last results may be significant as inhibition of early binding events could be deleterious for the repairing potential of injected cells. Indeed, a series of studies from researchers at Tokyo Medical and Dental University in Japan have illustrated the significance of early cell attachment through the use of their "local adherent technique" whereby a short period (10 minutes) of joint immobility is sufficient for improved attachment of synovium-derived stem cell populations and results in significantly improved healing in both nonhuman animals and clinical studies [67–69].

2.4. Adipose Tissue. In 2001, Zuk et al. demonstrated that adipose-derived stromal/stem cells (ADSCs) can be differentiated into chondrocytes, adipocytes, and osteoblasts [70] paving the way for a host of studies into the application of autologous ADSCs in regenerative medicine [71–73]. As in the neighbouring field of bone regenerative medicine, where proponents of BMSC-based or ADSC-based cell therapies cite the merits of either tissue versus the other [40], the same is true for cartilage engineering. There are parallels between the fields, and the various merits are somewhat overlapping. On the one hand, the accessibility and abundance of adipose tissue are an obvious advantage over the limited volumes that can be collected from the bone marrow, with less comorbidity to boot. ADSCs were shown to have a higher clonogenic potential and lower tendency towards osteogenic differentiation [74]. On the other hand, the regenerative potential of ADSCs versus BMSCs, millilitre for millilitre, appears to favour the use of the less abundant, harder to access, BMSCs. Indeed, *in vitro* comparisons of the chondrogenic potential of human BMSCs and ADSCs have concluded that BMSCs possess a greater chondrogenic potential than matched ADSCs [75, 76]. An important paper from 2010 highlighted the potential pitfalls of comparing BMSC and ADSC for cartilage regeneration using *in vitro* culture conditions that were optimised to one cell type, at the expense of the other [71]. Nevertheless, the same authors concluded that while both ADSCs and BMSCs underwent chondrogenic differentiation, it was the latter that produced the greater amount of matrix over a greater range of culture conditions.

In recent years, a number of clinical studies have focused on the chondrogenic potential of ADSCs [71, 74–77]. Jo et al. compared various doses of autologous ADSCs administered

via i.a. injection in both phase I and II trials and concluded that better results positively correlated with higher numbers of ADSCs [77]. The highest dose (100 million cells) produced smooth glossy white cartilage that was well integrated with the subchondral bone, comparable to native cartilage and free of calcification at 6 months postinjection. Importantly, in the highest dose, the defect underwent significant reduction in volume paralleled by an increase in cartilage volume in some cases at 6-month follow-up; lower doses of ADSCs did not produce such positive results. In contrast to these findings, a recent clinical trial (NCT01585857) reported that the lowest dose (2 million cells) of autologous ADSCs injected i.a. for knee OA produced the greatest improvement in pain and function tests using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), although this seems to be primarily due to the differences in baseline pain and function seen in the low-dose group [78]. Indeed, little difference is seen at later time points [78].

Further positive results were reported using the autologous stromal vascular fraction (SVF) harvested from the buttocks of 30 patients which were then reinjected intraoperatively to assess the clinical effect on elderly patients with knee OA [72]. Assessment at 2 years showed improvements in motor function, cartilage healing, and reduced pain. After 2 years, the average Lysholm score increased (from 54 to 74), the VAS pain score decreased (from 4.7 to 1.7), and the KOOS increased in all categories at all postoperative time points. The same group followed up the previous study with a level II, prospective comparative study to compare the clinical and radiologic efficacy of ADSCs harvested from the SVF, with fibrin glue and microfractures (MFX) versus MFX alone in 80 patients with knee cartilage defects [73]. The outcomes at 24 months suggest that the addition of ADSCs to MFX protocols could significantly reduce OA pain (reflected in improved MOCART and KOOS scores). The authors reported no significant effects of ADSCs on other matrices measuring daily activity and quality of life.

2.5. Peripheral Blood. Cells isolated from the peripheral blood and activated using a combination of the CXCR4 antagonist, AMD3100, and granulocyte colony-stimulating factor have been noted to conform to the criteria defining "MSCs" [79, 80] as laid out by the ISCT. *In vitro* studies using rabbit peripheral blood cells (PBSCs) have shown that not only are these cells substantially more accessible than the corresponding BM-derived cells but that they also possess a greater chondrogenic and adipogenic differentiation potential in *in vitro* assays [79]. In the same *in vitro* tests, BMSCs had a greater osteogenic and proliferative capacity while implantation of both BMSCs and PBSCs produced similar chondrogenic results in an *in vivo* cartilage defect model.

In humans, PBSCs have produced different results when applied to cartilage repair. In a trial comparing 5 weekly injections of HA only or HA plus PBSCs after arthroscopic subchondral drilling for chondral lesions, improvements were noted at 24 months using the IKDC score ($P = 0.8$), using MRI inspection ($P = 0.013$), and using the ICRS score (109-point increase, $P = 0.022$) [81]. It would be interesting to see further studies expanding an essential "cells versus no cells" experiment to include the effects of other stem cells, such as ADSCs or BMSCs. An extension of the above study by the same group repeated the i.a. injections of HA ± PBSCs weekly for 5 weeks and again at 6, 12, and 18 months after arthroscopic subchondral drilling in addition to open-wedge high tibial osteotomy [82]. Assessment of cartilage repair was carried out by histology (ICRS II scoring system) and by second-look arthroscopy indicating that the technique including PBSCs produced cartilage rich in proteoglycans and collagen which closely resembled native cartilage with no adverse effects reported.

2.6. Umbilical Cord/Umbilical Cord Blood. Another emerging source of stem cells for tissue regeneration is the umbilical cord; with specific regard to cartilage repair and regeneration, several recent reports have highlighted the potential for these cells in the clinic [83–85]. In a recent case report, autologous umbilical cord blood cells (UBSCs) in a HA hydrogel were implanted in 5 mm diameter and 5 mm deep drilled holes in the lateral femoral condyle. Assessment was at 1 and 5.5 years and showed improvements in VAS (from 46 preop to 8 and 12 at 1 and 5.5 years postop, resp.), IKDC (63.22 preop to 85.02 and 85.5 at 1 and 5 years postop), and WOMAC scores (25 preop to 2 and 4 at 1 and 5 years postop) [86]. Encouragingly at 1 year, second-look arthroscopy revealed no bone formation or bone exposure at the articular surface which was covered with smooth firm hyaline cartilage. MRI at 1 and 5.5 years showed that the defect was filled, that there was smooth integration with the surrounding tissue, and that the repair was maintained over time. In a larger cohort of patients ($n = 7$) assessed at 1, 3, and 7 years postintervention, human allogeneic UBSCs mixed with a HA hydrogel were evaluated for cartilage repair in the femoral condyle [87]. Human UBSCs as with stem cells from other sources have been used allogeneically on the basis of their reputed immunomodulatory properties [84, 88]. Ha et al. used human UBSCs in a hydrogel to examine the repair potential of osteochondral defects in a minipig model and noted no adverse effects, no infection, and no rejection after 12 weeks [83]. Additionally, the UBSC-treated defects, in contrast to the untreated controls, contained GAG-rich cartilage with better integration with the surrounding tissue; the defects which received human UBSCs also did better on the ICRS scoring system.

3. Conclusions

The issue of degenerated cartilage will remain a pressing medical need as the world population ages. Tissue engineering represents a valid alternative to current techniques, which can offer temporary or partial relief, but is far from ideal. As illustrated in this review, a wide variety of tissues have been examined for their potential suitability for cartilage regeneration or replacement. Each tissue has different advantages in terms of invasiveness, cell yield, cell proliferation, and chondrogenic potential. Thus, the choice of the best cell source depends on several factors: the intrinsic chondrogenicity, the ease of harvest, and the available cell number. In general terms, it seems that more accessible tissues such as

adipose, blood, and umbilical cord tissues have the advantage despite their noncartilage origins. An alternative approach involves the use of allogeneic cells or implants made using allogeneic cells which are subsequently decellularised to overcome this blockade.

This would permit the use of allogeneic cells for cartilage repair, but at the expense of the potentially anti-inflammatory effects of live MSCs [89]. Indeed, downregulation of inflammatory cytokines has been reported in cocultures of ADSCs and osteoarthritic chondrocytes or synoviocytes [90]. Moreover, reductions in reported pain following injection of stromal cell populations from BM [91] and adipose tissue [77, 92] have been reported. Thus, live MSCs may have a central role in pain reduction following a cartilage repair procedure.

Existing techniques, such as autologous or allogeneic chondrocyte implantation, can be optimised by drawing upon fresh insights from basic science and by continuing to experiment with new cell populations. Excitement over basic findings must as always be tempered with caution regarding the safety of treating cells with growth factors and hormones prior to implantation. A balance between guiding cells down the desired lineage path and pushing them over the edge towards malignant transformation is crucial; however, there are few reported instances.

The issue of premature differentiation during 2D *in vitro* expansion is especially salient when discussing explanted articular chondrocytes, which seem to have limited capacity in this regard. Advances in cell culture techniques such as the application of hypoxic growth chambers, as well as 3D perfusion culture utilizing bioreactors that recapitulate not only the 3D in vivo environment but also both hydrostatic and compressive loading [93] found in joints, will be vital to compensate for the low number of progenitors found in articular cartilage. Research into alternate sources of cartilage-forming cells is developing, as illustrated by the range of cell sources covered in this review. Nowadays, autologous mesenchymal cells can already be applied in the clinical settings; in particular, BMSCs or ADSCs can be injected i.a. in case of moderate osteoarthritis with the aim to reduce inflammation and, therefore, pain. In case of focal cartilage lesions, BMSCs can also be used in combination with collagenic membranes to repair the defect. However, the use of MSCs in clinical practice is still limited due to all the issues described above.

Clearly, current methods can generate cartilage *in vivo* with a great promise for future clinical applications. We report on more than 15 case studies or clinical trials in humans with the majority describing positive findings and no adverse effects with follow-up times extending to double-digit figures. This is enormously encouraging, and as we continue to learn more about the nature of progenitor and stem cell populations, we anticipate that improvements in the production of regenerated cartilage will see increased clinical translation and patient benefit.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Abbreviations

AC: Articular chondrocyte
ADSC: Adipose tissue-derived stromal/stem cell
BM: Bone marrow
BMAC: Bone marrow aspirate concentrate
BMSC: Bone marrow stromal/stem cell
BMP: Bone morphogenetic protein
CFU: Colony-forming unit
ECM: Extracellular matrix
FCSC: Fibrocartilage stem cell
FGF-2: Fibroblast growth factor 2
GAG: Glycosaminoglycan
HA: Hyaluronic acid
i.a.: Intra-articular
iPSC: Induced pluripotent stem cell
IKDC: International Knee Documentation Committee
ISCT: International Society for Cellular Therapy
KOOS: Knee Injury and Osteoarthritis Outcome Score
MFX: Microfracture
MOCART: Magnetic resonance observation of cartilage repair tissue
MRI: Magnetic resonance imaging
MSC: Mesenchymal/medicinal stem/stromal cell
NC: Nasal chondrocyte
OA: Osteoarthritis
PB: Peripheral blood
PBSC: Peripheral blood stromal/stem cell
RA: Rheumatoid arthritis
SVF: Stromal vascular fraction
TGF-β: Transforming growth factor-beta
WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index
VAS: Visual analogue scale.

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Review Article

Mesenchymal Stem Cells for Cartilage Regeneration of TMJ Osteoarthritis

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Temporomandibular joint osteoarthritis (TMJ OA) is a degenerative disease, characterized by progressive cartilage degradation, subchondral bone remodeling, synovitis, and chronic pain. Due to the limited self-healing capacity in condylar cartilage, traditional clinical treatments have limited symptom-modifying and structure-modifying effects to restore impaired cartilage as well as other TMJ tissues. In recent years, stem cell-based therapy has raised much attention as an alternative approach towards tissue repair and regeneration. Mesenchymal stem cells (MSCs), derived from the bone marrow, synovium, and even umbilical cord, play a role as seed cells for the cartilage regeneration of TMJ OA. MSCs possess multilineage differentiation potential, including chondrogenic differentiation as well as osteogenic differentiation. In addition, the trophic modulations of MSCs exert anti-inflammatory and immunomodulatory effects under aberrant conditions. Furthermore, MSCs combined with appropriate scaffolds can form cartilaginous or even osseous compartments to repair damaged tissue and impaired function of TMJ. In this review, we will briefly discuss the pathogenesis of cartilage degeneration in TMJ OA and emphasize the potential sources of MSCs and novel approaches for the cartilage regeneration of TMJ OA, particularly focusing on the MSC-based therapy and tissue engineering.

1. Introduction

The temporomandibular joint (TMJ) is a hinge and gliding joint that connects the mandibular condyle with the temporal articular surface. It is one of the most frequently used joints in the human body [1]. Osteoarthritis (OA) is a group of degenerative diseases primarily affecting the joint, characterized by progressive cartilage degradation, subchondral bone remodeling, synovitis, and chronic pain [2, 3]. Osteoarthritis that happened in TMJ often involves degenerations of both hard and soft tissues of TMJ, and patients with TMJ OA usually have joint pain and dysfunction with reduced quality of life. It is estimated that approximately 15% of populations in the world suffer from OA [4]. Epidemiologic studies on the prevalence of TMJ OA differ due to variations in diagnostic criteria, and clinical evidence occurs in 8–16% of populations with symptoms of joint pain, limited occlusion motion, or TMJ sound [5]. Moreover, women have increased susceptibility to the initiation of TMJ OA and induced pain, which occurs mainly after puberty during the reproductive years [6].

The pathogenesis and underlying molecular mechanisms involved in TMJ OA development remain elusive and largely understudied [7, 8]. The treatment strategy for TMJ OA aims at preventing the progressive destruction of cartilage and the subchondral bone, relieving joint pain and restoring TMJ function. The traditional clinical treatments mainly include nonsurgical options, such as physical therapies, occlusal splints, nonsteroidal anti-inflammatory drugs (NSAIDs), and arthrocentesis [9, 10], while surgical intervention is applied to patients with severe symptoms. Although those abovementioned treatments can prevent disease progression

to a certain degree, they are unable to completely restore degraded cartilage or subchondral bone lesions, as well as disc deteriorations.

Recent years, stem cell-based therapy has aroused a great attention. As a subpopulation of stem cells, mesenchymal stem cells (MSCs) have become vital seed cells for tissue regeneration due to their easy obtainment and multilineage differentiation potential. In addition, the trophic modulations of MSCs exert a biologic function in injured tissues and inflammatory diseases [11, 12]. Combined with appropriate scaffolds via transplantation *in vivo*, MSCs could restore tissue impairments to form cartilaginous or even osseous compartment in TMJ OA animal models [13, 14]. All these data indicate the capacity of MSCs for the cartilage regeneration in TMJ OA disease.

In this review, we will briefly summarize the pathogenesis of TMJ OA and emphasize the potential of novel approaches for the cartilage regeneration of TMJ OA, particularly focusing on the MSC-based therapy.

2. Pathogenesis of Cartilage Degeneration in TMJ OA

TMJ OA is a highly prevalent degenerative disease affecting articular cartilage as well as other TMJ tissues under pathological conditions and aging process [5]. TMJ condylar cartilage is an avascular, compressible tissue comprised of dense collagen fibres and extracellular proteoglycans, which protect the joint from damage during mechanical loading. The collagen fibres are mainly composed of type I and type II collagen (Col1/Col2) and are well organized to align in an anteroposterior orientation for the resistance to shear stress [15–17]. Chondrocytes embedded in the condylar cartilage are generally separately localized into three layers. Cells in the superficial and middle layers are considered to be progenitor cells with high proliferation capability and differentiation potential. Descending to the deep layer, chondrocytes undergo terminal differentiation with enlarged, swollen, and vacuolated morphology leading to apoptosis process. Since TMJ OA is a multifactorial disorder [18], understanding of the pathogenesis of cartilage degeneration in TMJ OA could help to identify potential therapeutic targets and interventions.

2.1. Chondrocyte Apoptosis. During TMJ OA progression, articular chondrocytes with low metabolism usually in advance undergo hypertrophy and apoptosis, accompanied with cartilage fibrillation and progressive loss. In cultured TMJ-derived chondrocytes, oxidative stress induced by H_2O_2 could elevate intracellular reactive oxygen species (ROS) and subsequently induce chondrocyte apoptosis and function impairment [19]. Intra-articular injection of monosodium iodoacetate (MIA) can effectively induce TMJ OA-like phenotype in rats and chondrocyte apoptosis as the most apparent characteristic in cartilage was observed starting from the early stage [20]. Surgical malocclusion induction in another TMJ OA rat models increased chondrocyte autophagy with a reduced activity of mitogen-activated protein kinase kinase kinase kinase-3 (MAP4K3) and mammalian target of rapamycin (mTOR) [21]. Moreover, chondrocyte apoptosis was also illustrated in the initiation stage of TMJ OA in a senescence-accelerated mouse model [22]. Collectively, all these data elucidated the involvement of chondrocyte apoptosis during the initiation and progression of TMJ OA.

2.2. Catabolic Enzymes. In adult healthy cartilage, chondrocytes are in a quiescent state characterized by a fine balance between anabolic and catabolic activities. However, during the disease progression, the condylar chondrocytes demonstrate the progressively decreased synthesis of anabolic components, such as Col2 and aggrecan, the upregulated expression of hypertrophic marker genes, such as Runx2 and ColX, and the increased catabolic enzymes synthesis, such as matrix metalloproteinases (MMPs) [23] and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [24].

The MMPs belong to a family of proteases and function to degrade extracellular matrix proteins. Among these, MMP-13 is the most important catabolic enzymes involved in cartilage degradation during TMJ OA development. Studies have detected a higher expression of MMP-13 in late-stage OA patients compared with these in the early stage with a lower disease degree [25]. In addition, overexpression of *Mmp-13* in transgenic mice has led to degenerative cartilage with excessive Col2 cleavage and aggrecan degradation [23]. Furthermore, mice with the knockout of *Mmp-13* rescued surgically induced OA phenotype in knee joint, indicating that the cartilage damage in OA mice model is dependent on MMP-13 activity [26].

The ADAMTS family, particularly ADAMTS4 and ADAMTS5, contributes to the proteoglycan/aggrecan degradation during TMJ arthritis [27]. Inhibition of these enzymes in cultured chondrocytes effectively reduced aggrecan degradation *in vitro* [28]. The ablation of Adamts5 activity in transgenic mice prevented aggrecan loss and cartilage erosion in inflammation-induced OA models [29]. Similarly, in surgically induced OA mice models, depletion of Adamts5 or double knockout of both Adamts4 and Adamts5 protected against proteoglycan degradation *in vitro* and decreased the severity of OA progression [30]. Besides, in genetic TMJ OA mouse models, such as β-catenin(ex3)^{Co12ER} mice, rescue effects were also observed by deletion of the either the Mmp-13 or Adamts5 gene [31]. All these evidence suggest that the catabolic enzymes play a key role in the development of TMJ OA.

2.3. Subchondral Bone Remodeling. The abnormal remodeling of the subchondral bone is another pathogenic change contributed to OA in TMJ. Recent studies revealed the interaction between chondrocyte and adjacent osteoclast or osteoblast to regulate the bone-remodeling process [32]. In Camurati-Engelman disease (CED) mice with the systemic skeletal disease, the activated TGF-β signals in the bone marrow developed phenotypes of abnormal bone remodeling, as well as obvious cartilage degradation accompanied by an upregulation of *Mmp-9* and *Mmp-13* in condylar chondrocytes [33–35]. Besides, several lines of evidence suggested

that chondrocytes can regulate bone remodeling via RANKL/osteoprotegerin (OPG) signaling. A thinner articular cartilage layer with severe destruction of growth plate cartilage was observed in OPG-deficient mice, indicating that OPG is related to the regulation of cartilage metabolism [36]. Moreover, articular chondrocytes can produce both RANKL and OPG proteins, and different RANKL/OPG ratios may adjust cartilage degradation and subchondral bone remodeling in OA [37, 38]. In malocclusion-induced TMJ OA models, the loss of subchondral bone was observed after 12 weeks, followed by increased expression of osteoclastic factors, such as M-CSF, VEGF, and RUNX, as well as the upregulated RANKL/OPG ratio [37]. To mimic OA environment *in vivo*, articular chondrocytes were cocultured with peripheral blood mononuclear cells (PBMCs) *in vitro*. Chondrocytes under the prostaglandin E2 (PGE2) stimulation paracrine secreted RANKL protein to induce osteoclastic activity of monocytes [39]. On the contrary, the β-catenin(ex3)^{Col2CreER} mice developed an OA-like phenotype in the knee joint characterized by subchondral bone erosion and osteophyte formation. The activation of β-catenin signals in chondrocytes produced OPG protein to further inhibit osteoclast differentiation by completely binding with RANK on the osteoclasts [40]. Further investigations are needed to elucidate mechanisms governing the interactions between chondrocytes and local bone remodeling; nonetheless, chondrocytes are demonstrated to serve as a link with bone changes in the OA-related process.

Apart from chondrocytes, other pathogenic factors, such as immune cells, cytokines, and hormones, lead to the pathogenic changes in the subchondral bone. Increasing evidence has demonstrated that synovium inflammation is involved in the progression of OA and associated with joint pain and dysfunction [41, 42]. Various types of immune cells are infiltrated in the inflamed synovium of OA patients. Among these, macrophages are the most abundant cells present in the synovial tissue [43, 44]. In a surgery-induced OA mouse model by intra-articular injections of collagenase into joints, synovial lining macrophages are shown to play a pivotal role in mediating the osteophyte formation during the progression of OA [45]. In cultured synovial cells derived from OA patients, synovial macrophages were specifically depleted from digested synovium using anti-CD14-conjugated magnetic beads. The macrophage depletion resulted in the downregulation of proinflammatory cytokines, such as interleukin- (IL-) 6 and IL-8 and MMPs, such as MMP-1 and MMP-3, and it indicated the important role of macrophages in promoting the production of inflammatory and degradative mediators in the OA synovium [46]. Some other studies provided clues about the inhibitory influence on the chondrogenic differentiation of MSCs under the activation of synovial macrophages [47, 48]. Despite the abundance of macrophages, natural killer cells have also been isolated from synovial tissues of OA patients; however, the mechanism involved in the pathogenesis needs to be further elucidated in detail [49].

Along with the infiltration of immune cells, inflammatory cytokines are isolated from the inflamed synovial fluid of patients with TMJ OA, such as IL-1β and tumor necrosis factor- (TNF-) α [50, 51]. In the experimental chronic inflammation of rodent TMJ induced by intra-articular injections of complete Freund's adjuvant, increased expressions of IL-1β and TNF-α were detected and supposed to be one cause for the TMJ degenerative changes [52]. Although the administration of TNF inhibitors did not show significant improvement in radiographic scores of patients, individuals have some benefits on joint pain relief and the trends suggested possible targets for the intervention of OA [53]. What is more is that several other cytokines, such as IL-6, have also been shown to be implicated in the progression of TMJ OA [51].

Females especially during the reproductive period have susceptibility to the occurrence of TMJ OA, suggesting that female hormones have a possible involvement in the pathologic changes of condylar cartilage and the subchondral bone. In an iodoacetate-induced TMJ OA rat model, estrogen can aggravate the disease progression by upregulating Fas- and caspase 3-related proapoptotic genes [54]. On the contrary, estrogen could inhibit the expression of nitric oxide to protect TMJ chondrocyte from apoptosis [55]. Thus, the role of estrogen in TMJ OA progression is with controversy and needed more investigations.

3. Mesenchymal Stem Cell-Based Therapy for Cartilage Regeneration of TMJ OA

The treatment for TMJ OA focuses on preventing the destruction of cartilage and subchondral bone, relieving pain, and eventually restoring TMJ function. There are different treatment strategies according to clinical stages of TMJ degeneration, including noninvasive options, such as physical therapies, occlusal splints, NSAIDs, arthrocentesis [9], and surgical intervention, such as joint replacement [56]. Although all these therapies have symptom-modifying and/or structure-modifying effects to some extent, they rarely reverse the disease process to restore the degenerative cartilage and reestablish joint functions.

In recent years, stem cells have been extensively applied to fields of tissue engineering and regenerative medicine [57–59], mainly due to their self-renewal ability and multiple differentiation potentials. Among alternative cell sources for OA treatment, MSCs have raised particular concerns to play a role of seed cells, based on their ease of collection, the potential of chondrogenic differentiation, response to tissue damage, and contributions to tissue turnover. Therefore, extensive progress has been made in the investigation of differentiation potentials and functional modulations of MSCs for cartilage regeneration in TMJ OA treatment.

3.1. Potential of Mesenchymal Stem Cells. MSCs have been identified from various tissues, such as skeletal muscle [60], adipose tissue [61], the placenta [62], the bone [63], the deciduous teeth [64], and the synovium [65]. As the most widely studied sources of MSCs for cartilage regeneration, bone marrow-derived MSCs, synovium-derived MSCs, and umbilical cord-derived MSCs are mainly discussed below.

3.1.1. Bone Marrow-Derived MSCs. MSCs were first identified in the bone marrow via the formation of colonies, represented as colony-forming unit fibroblasts (CFU-F) [66], and bone marrow-derived MSCs (BMSCs) have multilineage differentiation potentials [63], including chondrogenic and osteogenic differentiation.

Studies found that cultured BMSCs preconditioned in osteogenic and chondrogenic media *in vitro* can form bone-like and cartilage-like structures, respectively, mimicking a primordial joint-like structure when seeded in opposite portions of a hyperhydrated collagen gel via ultrarapid tissue engineering techniques [67]. Some clinical trials have proposed the approaches for OA treatment, which involve the intra-articular injection to deliver BMSCs directly into the synovial fluid compartment [68]. Although most clinical trials participated in the intervention of OA in knee joints, studies on the cartilage regeneration of BMSCs in TMJ OA have been largely investigated. Chen et al. [69] conducted intra-articular injections of both undifferentiated and pre-chondrogenic differentiated BMSCs for cartilage regeneration in TMJ OA rabbit models. They also compared that the differentiated MSC-treated group gained better histological scores than the undifferentiated MSC-treated group at an observation period of 4 and 12 weeks, along with decreased expression of MMP-13 and upregulation of Sox9, Col2, and aggrecan. However, the rescue effect displayed no difference in both groups until 24 weeks. Collectively, local delivery of chondrogenic differentiated BMSCs may enhance the regenerative process of cartilage repair at the early stage of TMJ OA through key mediators involved in chondrogenesis. In addition, the implanted cells could be traced by the label of adenoviral vectors containing the LacZ gene, and implanted MSCs were detected within cartilage, subchondral bone, and synovium lasting at least 4 weeks, indicating the involvement of BMSCs in the cartilage repair [69].

Although predifferentiated BMSCs appear to enhance the cartilage regeneration, they do not maintain their proliferative capability and differentiation potentials after prolonged expansion *in vitro* [70]. In order to attain a better therapeutic outcome, applicable strategies of pretreatment and/or preconditioning of BMSCs are essential to improve long-term effect in OA. Further studies have proved that pretreatment of BMSCs with fibroblast growth factor-2 (FGF-2) [71] and hypoxic preconditioning of BMSCs [72] are two attractive approaches to enhance cell proliferation and chondrogenic differentiation. Whether there are other biophysical approaches needs more investigations to expand the applications of BMSCs in cell-based treatment. Therefore, BMSCs provide an alternative approach for cartilage regeneration; meanwhile, a better application of TMJ OA treatment could be achieved with the pretreatment of BMSCs.

3.1.2. Synovium-Derived MSCs. A number of studies have isolated cells from synovial fluid and synovium in TMJ [73–75], which are able to differentiate into different lineages, such as osteoblasts, chondrocytes, adipocytes, and neurons [73, 76]. These synovium-derived MSCs express MSC markers such as CD90, CD105, and CD73 and negatively express CD11b, CD19, CD34, CD45, and HLA-DR [75], compliant with the widely adopted criteria stipulated by the International Society for Cellular Therapy (ISCT) [77]. Compared with MSCs from other tissues, synovium-derived MSCs possess a greater proliferative rate and superior chondrogenic differentiation potential [78–80]. Particularly, the combination treatment of TGF-βs, dexamethasone, and BMP-2 became the optimum for chondrogenic differentiation of synovium-derived MSCs *in vitro* [81]. Based on the properties mentioned above, synovium-derived MSCs have been utilized to test the chondrogenic potential for cartilage repair in animal models. In rabbit OA models with defects in whole cartilage layers, synovium-derived MSCs were embedded in collagen gel and transplanted into the injury site. The cartilage defects were repaired with productions of cartilage matrix [82]. The *in vivo* chondrogenic differentiation of synovium-derived MSCs on cartilage repair have been reported in many other studies [83, 84], leading to a general acceptance that synovium-derived MSCs have the ability to repair cartilage defects to some extent.

The perforation of TMJ disc tissue is usually happening in the late stage of TMJ OA, leading to severe degeneration of condylar cartilage. The application of synovium-derived MSCs for the TMJ disc repair has aroused great attention. A recent study [85] has cultured synovium-derived MSCs on fibrin/chitosan hybrid scaffold under chondrogenic induction combined with TGF-β3 *in vitro*. In order to evaluate the *in vivo* repair ability, the construct was inserted into the punched TMJ disc explants of rats, which can mimic TMJ disc perforation in human. After 4 weeks of operation, distinct fibrocartilage formation with deposition of Col1 and Col2 was observed at the implantation site. Thus, synovium-derived MSCs are able to repair the defective cartilage in TMJ disc.

3.1.3. Umbilical Cord-Derived MSCs. Apart from adult tissue-derived MSCs, MSCs could also be isolated from the umbilical cord (UC) [86, 87]. Compared with BMSCs, UC-derived MSCs show a more similar gene expression profile to that of embryonic stem cells [88]. They possess a faster proliferative rate and a larger number of CFU-F [89, 90]. The differentiation capacity of UC-derived MSCs into adipogenic, chondrogenic, and osteogenic lineages has been extensively studied [87, 90]. For cartilage tissue engineering, UC-derived MSCs and BMSCs embedded in the polyglycolic acid (PGA) scaffolds and cultured under chondrogenic differentiation medium *in vitro*. After 3 to 6 weeks, UC-derived MSCs produced more glycosaminoglycans (GAGs) and Col1 than BMSCs, indicating the superior capability of fibrochondrogenesis of UC-derived MSCs [91]. Above all, UC-derived MSCs might be an alternative cell source for the cartilage regeneration.

3.2. Trophic Modulations of MSCs for Cartilage Tissue Regeneration of TMJ. It is well known that MSCs are able to secrete a broad range of bioactive molecules, such as growth factors, cytokines, and chemokines, which constitutes their biological role under injury conditions [92–94]. These trophic factors produced in the MSC-conditioned medium are collectively described as the MSC secretome. Various

MSC-based clinical trials have revealed that transplanted MSCs exert biological functions through trophic modulations rather than differentiation potential [11, 12]. This paradigm shift in the use of MSC-based therapy is becoming a hot issue attracting various studies for better applications to tissue regeneration.

Although TMJ OA is classified as a "low-grade-inflammatory arthritic condition" [9], the involvement of inflammation has been concerned to play a role in disease progression. Several inflammatory cytokines are increased in the synovial fluid of TMJ OA patients, such as IL-12, IL-1β, and TNF-α [51]. Also, the increased expressions of IL-1β and TNF-α were detected in rat TMJ OA models. Particularly, the biomechanical properties of TMJ discs were significantly decreased and the disc ultrastructures were impaired in rodent TMJ OA [52], implying that the chronic inflammation in TMJ OA deteriorates the adaptive ability of the joint. Since MSCs have been explored to regenerate damaged tissue and treat inflammation in many diseases, such as cardiovascular disease, neuron injury, stroke, diabetes, and bone regeneration [95, 96], further studies elucidated that the trophic factors secreted by MSCs exert an anti-inflammatory effect. van Buul et al. added TNF-α and interferon-γ (IFN-γ) to conditioned medium of MSCs to mimic the inflammatory environment of OA. It was found that the increased secretion of secretome decreased expression of the inflammatory gene *IL-1*β and collagenase genes *Mmp-1* and *Mmp-13* in response to inflammation [97]. Besides, human BMSCs were intra-articularly injected into rat's knee joint after hemimeniscectomy [97]. Despite the rapid decrease of cell numbers, the human BMSC injection enhanced Col2 expression in the articular cartilage, associated with increased expression of Indian hedgehog (Ihh), parathyroid hormone-like hormone (PTHLH), and bone morphogenetic protein 2 (BMP2), eventually contributing to the cartilage regeneration and inhibition of OA progression [98]. Furthermore, the periodontal ligament-derived MSCs could increase the cell proliferation and matrix biosynthesis of cocultured TMJ-derived fibrochondrocytes through paracrine secretion of trophic factors [99]. In addition, increased GAG deposition with enhanced expression of chondrogenic genes, such as aggrecan, Col1, and Col2, is also observed in this study.

As a component of secretome, exosomes are demonstrated to play a key role in mediating tissue repair in MSC-based therapy. Exosomes are cell-secreted nano-sized vesicles covered by the bilipid membrane and containing a myriad of regulatory components including microRNAs (miRNAs), mRNAs, and proteins [100–102]. Exosomes can be synthesized in many cells, such as lymphocytes, dendritic cells, and tumor cells, and they are found in most bodily fluids such as blood, urine, and saliva [103]. Diseased cells also secret exosomes as vehicles to transmit injurious signals, thereby exerting various pathological effects on both recipient and parent cells [104–106]. In MSC-based regenerative therapy, exosomes are widely found in the secretome of MSCs derived from the bone marrow [107, 108], fetal tissues [109], and umbilical cord [110]. To isolate exosomes from secretomes, the conventional culture medium is replaced by medium containing exosome-depleted fetal bovine serum. When BMSCs reach 60–80% confluence, cell culture media are collected and density gradient centrifugations are performed to obtain pellets containing exosomes. The pellets are then passed through a 0.22 μm filter to remove cell debris, and the purified exosomes are obtained [108].

Although the role of the individual components of exosome has not been elucidated, the combined functional complexity of MSC exosomes has therapeutic effects on tissue repair and regeneration in the heart [100], liver [111], skin [112], bone [113], and cartilage [114]. In an experimental rat model with critical-sized osteochondral defects on trochlear grooves of the distal femurs, human embryonic MSC-derived exosomes were intra-articularly administrated to investigate the efficacy of exosomes in osteochondral repair. The results showed that over the 12-week period of exosome injections, exosome-treated cartilage and subchondral bone defects were completely restored, characterized by the accelerated neotissue filling and enhanced matrix synthesis of Col2 and sulphated GAG, while only fibrous repair tissues were found in the PBS-treated defects [114]. A recent study conducted the destabilization of medial meniscus (DMM) surgery to induce OA in the knee joints of mice, and the intra-articular injections of exosomes isolated from embryonic MSC medium successfully impeded cartilage destruction in the DMM model. Further *in vitro* studies using cultured chondrocytes treated with IL-1β illustrated that these exosomes maintained the chondrocyte phenotype by increasing Col2 synthesis and decreasing ADAMTS5 expression, exerting a beneficial therapeutic effect on OA through balancing synthesis and degradation of cartilage extracellular matrix [115]. Among components in the exosome, miRNA might participate in mediating the efficacy of MSC exosome against OA. Exosomal miR-23b could inhibit protein kinase A (PKA) signaling to induce chondrogenic differentiation of human MSCs [116]. Moreover, overexpression of miR-125b in human OA chondrocytes can suppress the IL-1β-induced upregulation of ADAMTS4, and in silico analysis further predicted ADAMTS4 as a putative target gene of miR-125b can be directly regulated in chondrocytes during OA development [117].

All these results demonstrated that the trophic modulations of MSCs play an essential role in the cartilage regeneration in TMJ OA. However, more investigations are needed to figure out the exact effective components among various trophic factors produced by MSCs, which could not only fulfill the current understanding of MSC-based therapy under abberant environment but also enable allogenic transplantations in a more controlled manner for better applications of tissue regeneration.

3.3. Tissue Engineering Approaches for Cartilage Tissue of TMJ. Based on the rapid development of scaffold materials, the cartilage regeneration of TMJ has been extensively explored in tissue engineering investigations [118–120].

Polyglycolic acid (PGA) is one of the widely applied biocompatible materials in tissue engineering of TMJ cartilage. Studies have proved that PGA enables proliferation of porcine TMJ disc cells, and matrix deposition of Col1 was

detected in PGA nonwoven meshes [121, 122]. However, the rapid degradation of PGA is the major demerit leading to the loss of structural/mechanical integrity and construct contraction over time. Due to the defects of PGA, polylactic acid (PLA) has emerged as a more applicable biomaterial for TMJ cartilage regeneration. Porcine TMJ disc cells seeded in PLA had the increased cellularity, with more matrix deposition of collagen and GAGs compared with these in PGA construct [123]. Specifically, adverse construct contraction was not observed in PLA constructs compared to PGA. Another study developed a PLA construct with autologous adipose-derived MSCs predifferentiated in chondrogenic medium. After transplantation in rabbit TMJ, a regular and calcified surface of condyle cartilage was observed, accompanied by the increased expression of Col2 [124].

Except for these scaffolds, syntheses with synthetic polymers, such as PGA and PLA, natural biopolymers, such as fibrin and chitosan, are also applied to the TMJ cartilage regeneration based on their inherent advantage of biocompatibility. A recent study showed that fibrin could improve cell seeding, proliferation, and chondrogenic differentiation *in vitro* [85]. Moreover, a combination of fibrin/chitosan scaffolds can promote the reparative ability of synovium-derived MSCs, characterized by the fibrocartilage formation with extracellular matrix deposition of Col1, Col2, and GAGs in rat models with TMJ disc explants [85].

In addition to those preceding biopolymers, biologic scaffold materials consisting of extracellular matrix (ECM), such as decellularized urinary bladder matrix (UBM), have aroused great attention in the field of cartilage regeneration. These biologic scaffolds possess constructive remodeling properties by promoting the de novo formation of site-appropriate and functional host tissues, and they are already applied to various preclinical studies and clinical trials [125, 126]. In a canine model of TMJ discectomy, decellularized UBM scaffold acted as an effective interpositional material for the TMJ disc remodeling with no secondary pathologic changes. Of note, the UBM device was further replaced by native tissues, including fibrocartilage, muscle, and connective tissues. This remodeled device consists of elongated fibroblast-like cells within a highly aligned matrix of collagen and site-appropriate soft tissue attachments at the periphery of the implanted material [127].

Considering the pathological features of TMJ OA, emerging investigations have emphasized the pretreatments on the scaffold to construct the stratification of cartilage and the subchondral bone from a single source of MSCs [128]. BMSCs were induced into chondrogenic and osteogenic lineages, and then encapsulated in the stratified polyethylene glycol- (PEG-) based hydrogels to further photopolymerize into human-shaped condyle. After transplantation *in vivo*, distinct cartilaginous and osseous compartments of the mandibular condyle were formed, with the expression of cartilage-related Col2 and GAGs in the chondral layer and osteogenic markers alkaline phosphatase and osteonectin in the osteogenic layer [13].

Moreover, injectable biomaterial scaffolds have been designed to act as delivery systems containing both cells and biomolecules for more effectively modulating stem cell fate and functions [129, 130]. It has the potential to sustain stem cell survival and signal release [131]. The injectable scaffolds with a lower crosslinked degree and matrix stiffness can promote the chondrogenic differentiation of encapsulated MSCs and increase the matrix biosynthesis of Col2 and GAGs [132]. Furthermore, changes on the mechanical parameters of scaffolds could modulate the MSC differentiation to form different cartilage tissues. MSCs in scaffolds of higher cross-linking degree tend to differentiate into fibroblasts and subsequently form fibrous/osteochondral tissues in the OA rabbit model [133]. Collectively, the scaffold architecture can influence the lineage differentiation of MSCs and the mechanical parameters should be fully estimated to improve MSC-based therapy and tissue engineering.

In summary, the ideal scaffold could incorporate specific biomolecules and growth factors and enhance both chondrogenic and osteogenic differentiation potential of MSCs under different external stimuli, thus providing better applications in cartilage regeneration in TMJ OA via tissue engineering.

4. Conclusion

Given the limited self-healing potentials of avascular cartilage, little effective therapy is available for the repair of normal TMJ tissues in OA disease. Although the conventional nonsurgical or surgical treatments can relieve the joint pain to some extent, they cannot completely restore the TMJ function and reverse disease progression. MSCs, which have the multilineage differentiation potentials, may provide an alternative treatment for the cartilage degradation in TMJ OA. Combined with the trophic modulations of MSCs and various scaffold application, the formation of cartilaginous compartment or even stratified cartilaginous and osseous compartments has been accomplished in TMJ OA animal models. Furthermore, continuous investigations are required to detect the target efficiency and biocompatibility in the therapeutic intervention of TMJ OA, hopefully towards the preclinical and clinical researches like in OA treatment.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Dixin Cui and Hongyu Li contributed to work equally.

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Research Article

Polysaccharide Hydrogels Support the Long-Term Viability of Encapsulated Human Mesenchymal Stem Cells and Their Ability to Secrete Immunomodulatory Factors

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While therapeutically interesting, the injection of MSCs suffers major limitations including cell death upon injection and a massive leakage outside the injection site. We proposed to entrap MSCs within spherical particles derived from alginate, as a control, or from silanized hydroxypropyl methylcellulose (Si-HPMC). We developed water in an oil dispersion method to produce small Si-HPMC particles with an average size of about 68 μm. We evidenced a faster diffusion of fluorescein isothiocyanate-dextran in Si-HPMC particles than in alginate ones. Human adipose-derived MSCs (hASC) were encapsulated either in alginate or in Si-HPMC, and the cellularized particles were cultured for up to 1 month. Both alginate and Si-HPMC particles supported cell survival, and the average number of encapsulated hASC per alginate and Si-HPMC particle (7102 and 5100, resp.) did not significantly change. The stimulation of encapsulated hASC with proinflammatory cytokines resulted in the production of IDO, PGE_2, and HGF whose concentration was always higher when cells were encapsulated in Si-HPMC particles than in alginate ones. We have demonstrated that Si-HPMC and alginate particles support hASC viability and the maintenance of their ability to secrete therapeutic factors.

1. Introduction

Mesenchymal stem cells (MSCs) have generated significant medical consideration for tissue engineering since they have shown their ability to differentiate into a wide variety of cell types, including chondrocytes, osteocytes, and adipocytes in appropriate culture conditions [1]. MSCs can be isolated from a large panel of tissues including bone marrow, adipose tissue, and articular synovial fluid [2]. MSCs mainly exert their regenerative properties through the secretion of bioactive trophic factors that have potent immunomodulatory, proangiogenic, antiapoptotic, antifibrotic, and anti-inflammatory effects [3, 4]. Proinflammatory cytokines (IL-1β, TNF-α, and interferon-gamma (INF-γ)) or interaction with monocytes activates MSCs [5]. In response to those activating stimuli, MSCs exert their therapeutic properties by secreting immunomodulatory factors such as indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor (HGF), prostaglandin E_2 (PGE_2), and transforming growth factor-beta (TGF-β) [4, 6–8]. Unfortunately and despite promising results, direct injection of MSCs suffers from certain limitations including massive cell death upon

injection [9] and risk of cell leakage outside the site of injection due to the propensity of MSCs to migrate [10, 11].

To overcome both these limitations, several studies have proposed to encapsulate MSCs by using cytoprotective biomaterials [9, 12, 13]. The multipotency of MSCs after encapsulation, for example, has been exploited for the treatment of several pathologies including osteoarticular diseases, diabetes, cancer, cardiovascular pathologies, angiogenic diseases, and skin injury [14, 15]. However, their immunomodulatory properties after encapsulation have been little investigated.

Alginate is nowadays the most largely investigated and characterized hydrogel for the development of cell encapsulation technology [16]. However, alginate particles are sensitive toward nongelling agents such as sodium ions and in physiological solution, a calcium replacement occurs, leading to destabilization and rupture of the gel [17]. Furthermore, the calcium, generally used to cross-link alginate hydrogels, has been shown to exert an immunostimulatory effect *in vitro* and *in vivo* [18]. Our laboratory has developed an injectable and biocompatible cellulose-based hydrogel that can be used for the arthroscopic injection of MSCs in cartilage defects. The Si-HPMC hydrogel supports (i) the three-dimensional culture of MSCs and their controlled differentiation towards a chondrogenic phenotype [19], (ii) the transplantation of MSCs in subcutis of nude mice and the *in vivo* formation of chondrogenic nodules [20], and (iii) the repair of osteochondral lesions in rabbit [20]. Despite this large body of evidence suggesting that Si-HPMC exhibits the required properties, it has never been used to encapsulate MSCs in a particulate form, although it is well known that encapsulation in spherical particles improves injectability, biocompatibility, and stability.

The aims of this work were first to demonstrate the feasibility of generating particles with Si-HPMC hydrogel and to compare their mechanical and physicochemical properties with alginate particles and second to evaluate the *in vitro* viability and bioactivity of MSCs (human adipose-derived MSCs, hASC) encapsulated in Si-HPMC particles.

2. Materials and Methods

2.1. Materials. Sodium alginate (Protanal™LF10/60FT) and hydroxypropyl methylcellulose (HPMC) (Methocel™E4M) were purchased, respectively, from FMC Biopolymer and Colorcon-Dow chemical (Bougival, France). Glycidoxypropyltrimethoxysilane (GPTMS) was obtained from Acros (Geel, Belgium). Hank's balanced sodium salt (HBSS), Dulbecco's modified eagle medium (DMEM) high glucose (4.5 g/L), phosphate-buffered salt (PBS) without calcium chloride and magnesium chloride, penicillin/streptomycin, and trypsin/EDTA (0.05%/0.53 mM) were obtained from Invitrogen (Paisley, UK). Calcium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), olive oil, fluorescein isothiocyanate- (FITC-) dextrans, collagenase crude type I A, trypan blue, sodium citrate, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was purchased from Dominique Dutscher (Brumath, France). Live/Dead Viability/Cytotoxicity kit and Quant-iT PicoGreen dsDNA assay kit were, respectively, obtained from Molecular Probes (Leiden, The Netherlands) and Thermo Fisher (Waltham, MA, USA). PGE_2 EIA Kit-Monoclonal and Human HGF Duo Set ELISA were purchased from Cayman Chemical and R&D Systems, respectively.

2.2. Preparation of Particles. Alginate particles were obtained using a dropwise method in $CaCl_2$ as previously described [21]. First, sodium alginate was sterilized by steaming (134°C, 4 minutes) and then dissolved in sterile PBS (2% *w*/*v*). Particles were obtained by extruding this solution through a 31G needle into a stirred solution of 100 mM calcium chloride. After 15 minutes, alginate particles were collected by filtration, washed in HEPES buffer, and stored at room temperature. Synthesis of Si-HPMC was performed by grafting 14.24% (*w*/*w*) of GPTMS onto HPMC in heterogeneous medium as previously described [22]. Si-HPMC powder was solubilized (3% *w*/*v*) in 0.2 M NaOH under constant stirring for 48 h. The solution was then sterilized by steaming (121°C, 20 minutes). To initiate the formation of a cross-linked Si-HPMC, the solution was mixed with 0.5 volume of 0.26 M HEPES buffer (pH 3.6). To obtain Si-HPMC particles, an oil dispersion protocol was performed. The solution of Si-HPMC/HEPES was injected into olive oil under stirring. To optimize the encapsulation process, three dispersion parameters were studied: time (1 h or 1 h and 30 min), temperature (room temperature or 37°C), and stirring speed (250 rpm or 400 rpm). Si-HPMC particles were collected by filtration, washed in HEPES buffer, and stored at room temperature.

2.3. Particle Characterization

2.3.1. Shape and Size. Particles were observed under light microscopy (Leica confocal system, TCS NT/SP series, Germany) to investigate their shape. A Mastersizer 3000 Laser (Malvern Instruments, UK) was used for particle size and reproducibility analysis.

For the subsequent analyses, alginate and Si-HPMC particles of 1 ± 0.2 mm diameter were selected under light microscopy.

2.3.2. Particle Diffusion Properties. To study the diffusion properties, alginate and Si-HPMC particles were immobilized at the bottom of Lab-Tek chambers and incubated in 1 mg/mL solutions of fluorescently labeled dextran (Mw 20, 250, or 2000 kDa), for 2 h and 30 min at room temperature. At specific time points, particles were assessed by confocal microscopy (Nikon A1R Si, Champigny-sur-Marne, France; excitation 488 nm, emission 520 nm). Image analysis was performed with NIS-Elements software, and maximum fluorescence intensity was determined inside particles, as well as the fluorescence intensity of the FITC-dextran solution (outside particles). A ratio of internal to external fluorescence was then calculated, a ratio of 1 indicating that fluorescence intensity was identical in a particle and the surrounding solution. The results are presented as the ratio internal/external fluorescence as a function of time.

2.3.3. Particle Mechanical Properties. Mechanical properties of alginate and Si-HPMC particles were investigated by subjecting them to a compressive force between 2 parallel plates for 30 s (Microsquisher, CellScale). The force (μN) and the displacement (μm) were measured using a microscale test system equipped with an integrated image analysis module. The results are expressed as the force applied versus the recorded displacement. Young's modulus was calculated according to the manufacturer's recommendations and the standard expression $E = \text{stress/strain} = (F/A)/(\Delta l/l_0)$ where E is Young's modulus; F is the force applied on a particle; A is the area through which the force is applied; Δl is the displacement; and l_0 is the initial diameter of the particle.

2.4. Isolation and Culture of hASC. Human adipose-derived MSCs (hASC) were isolated from subcutaneous adipose tissue of patients undergoing liposuction after having given their informed consent as described elsewhere [23]. All protocols were approved by the French national ethical committee. Briefly, lipoaspirate was washed five times in HBSS and then digested for one hour at 37°C under constant stirring, in a solution of 0.025% collagenase in HBSS. The collagenase treatment was inactivated by adding an equal volume of DMEM high glucose containing 1% penicillin/streptomycin and 10% FCS (complete medium). After 5 minutes of centrifugation (260g, 4°C), the lower phase containing the stromal vascular fraction was collected, homogenized, filtered through a 70 μm cell strainer, and centrifuged for 8 minutes (260g, 4°C). Cells were suspended in complete medium, seeded at 5000 cells/cm^2, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 2-3 days of incubation, the nonadherent cells were removed by successive washes. Before encapsulation, hASC surface marker expression was characterized by flow cytometry (data not shown) as previously described [19].

2.5. hASC Encapsulation. Cells were tested at passage 5 (population doubling level (PDL) of 13.6). hASC were harvested using trypsin/EDTA, counted, and then loaded into alginate and Si-HPMC particles. For alginate, 2.10^6 hASC (100 μL) were suspended in 1 mL of a sterile solution of 2% sodium alginate. After homogenization, the mixture was extruded through a 31G needle into a sterile solution of calcium chloride. The particles were collected by filtration, washed with HEPES buffer, and cultured in complete medium. For Si-HPMC, 2.10^6 hASC (100 μL) were also mixed with 1 mL of Si-HPMC hydrogel, after induction of cross-linking by adding HEPES buffer as previously described [22]. The microparticles were obtained by introducing the mixture into olive oil under stirring at 250 rpm and at room temperature. After 1 h and 30 min of stirring, the microparticles were collected by filtration, washed with HEPES buffer, and incubated in complete medium.

The average number of encapsulated hASC per particle was assessed, every week, by using twenty alginate and Si-HPMC particles for each measure. Alginate particles were disrupted into a 60 mM sodium citrate for 15 minutes, and Si-HPMC particles were incubated in Tris/EDTA buffer for 30 seconds then disrupted by ultrasonication for 15 seconds (Misonix XL2000 Microson Ultrasonic Cell Disruptor). The average number of encapsulated hASC was estimated by DNA quantification which was performed on cell lysate using a Quant-iT PicoGreen dsDNA assay kit, following the manufacturer's instructions as previously described [24, 25].

2.6. hASC Viability and Biofunctionality. Encapsulated cells in alginate and Si-HPMC were cultured and incubated for up to 1 month at 37°C in a humidified atmosphere containing 5% CO_2. Complete medium was changed every 2 days.

hASC viability after encapsulation in alginate and Si-HPMC particles was followed from 24 h to 1 month of culture using a Live/Dead Viability/Cytotoxicity kit and determination of encapsulated cells. Alginate and Si-HPMC particles were recovered and washed in PBS for 20 seconds. Then, particles were incubated for 45 minutes in the combined Live/Dead assay reagents. Labeled cells were followed by confocal microscopy using an inverted fluorescence microscope (Nikon Eclipse TE 2000 E, Badhoevedorp, The Netherlands). The viability was calculated using the Volocity Software V6.2 (PerkinElmer, MA, USA).

In separate experiments, 1 week after encapsulation in alginate or Si-HPMC, hASC were stimulated for 72 hours with proinflammatory molecules (TNF-α + IFN-γ at 20 ng/mL) in complete medium, as previously described [26]. For each condition, 20 alginate or Si-HPMC particles were used. In order to quantitatively assess the production of soluble molecules (IDO, PGE_2, and HGF), commercially available kits were used. IDO enzymatic activity was measured by tryptophan-to-kynurenine conversion with photometric determination of kynurenine concentration in the supernatant. Briefly, 60 μL of cell supernatant (conditioned medium) was transferred to a 96-well culture plate, and 30 μL of 30% trichloroacetic acid solution was added for 30 minutes at 50°C. After centrifugation, 75 μL of samples was added to 75 μL of freshly prepared Ehrlich's solution, and absorbance was read at 450 nm. PGE_2 and HGF were measured in conditioned medium using ELISA kits according to the manufacturer's recommendations. The secretion of these biofactors in supernatant was normalized to the number of encapsulated hASC in both alginate and Si-HPMC particles.

2.7. Statistical Analysis. All experiments were performed with replicate samples from independent conditions ($n = 3$ for particle size, $n = 3$ for diffusion properties, $n = 4$ for mechanical testing, $n = 3$ for cell counts and viability, and $n = 2$ for biofunctionality). The results are presented as the mean of the independent replicates, and the error bars represent the standard error of the mean. The comparative studies of means were performed with GraphPad software by using one-way ANOVA followed by a post hoc test (Fisher's projected least significant difference) with a statistical significance at $p < 0.05$.

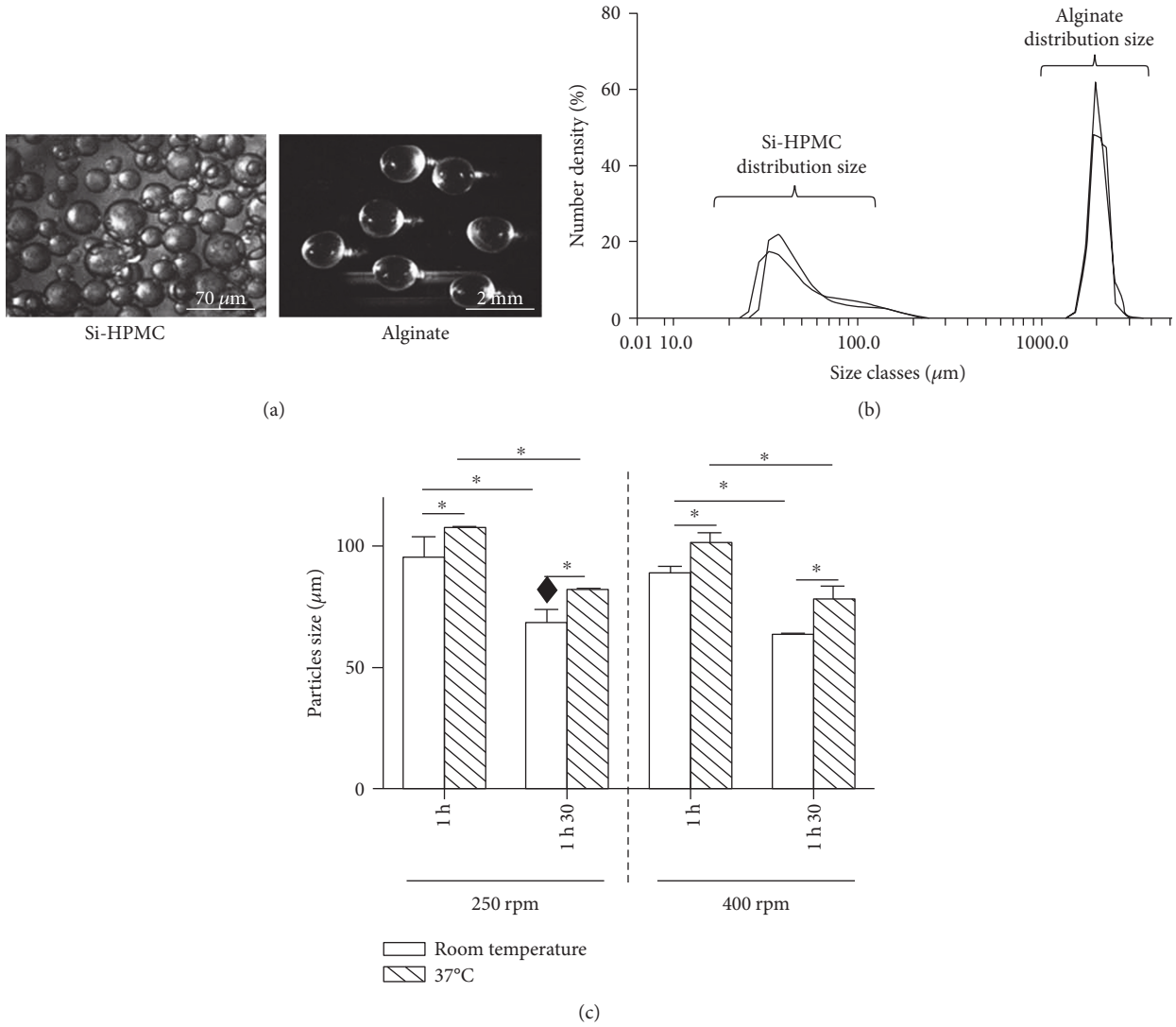

Figure 1: Particle characterization. (a) Representative observation of Si-HPMC and alginate particles obtained by oil dispersion and dripping method, respectively, and observed under light microscope. (b) Dispersity and size distribution of Si-HPMC and alginate particles investigated by laser diffraction technique. (c) Reproducibility and effect of dispersion parameters (time, temperature, and stirring speed) on Si-HPMC particle size. ✦The operating conditions selected to generate Si-HPMC particles for the size distribution investigation (Figure 1(b)) and for the following experiments. $^{*}p < 0.05$. Scale bars = 70 μm and 2 mm for Si-HPMC and alginate particles, respectively.

3. Results

3.1. Assessment of the Shape and Size of Particles. Si-HPMC and alginate particles were obtained using an oil dispersion and a dripping method, respectively, (Figure 1(a)).

Alginate and Si-HPMC particles possessed a shape uniformity. Prepared with a 31G needle, alginate particles exhibited an average size of nearly 2 mm (1.91 ± 0.2 mm). The laser diffraction size analyzer showed that more than 80% of alginate particles had a size distribution between 1600 and 2100 microns (Figure 1(b)). Moreover, some particles of about 1 mm were also detected. To optimize encapsulation process with Si-HPMC, three dispersion parameters were used: dispersion time (1 h or 1 h and 30 min), temperature (room temperature or 37°C), and stirring speed (250 rpm or 400 rpm). Figure 1(c) shows the influence of these parameters on Si-HPMC particle size. Increasing temperature from room temperature to 37°C induced a significant increase in particle size from 92 ± 10.1 μm to 108 ± 2 μm at 250 rpm for one hour. This effect of temperature on particle size was observed with all the other conditions. Increasing the dispersion time from 1 h to 1 h and 30 min decreased by approximately 25% the size of particles whatever the stirring speed applied. On the other hand, the variation of stirring speed had no significant effect on the Si-HPMC particle size. For the following experiments, we selected the subsequent dispersion parameters: dispersion time 1 h and 30 min and stirring 250 rpm at room temperature. Under these operating conditions, Si-HPMC particles presented an average

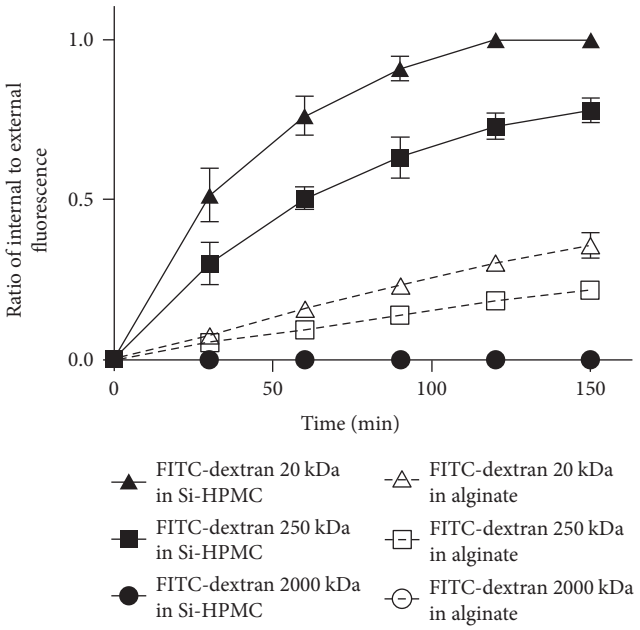

Figure 2: Alginate and Si-HPMC particle diffusion properties. Alginate and Si-HPMC particles were incubated in fluorescently labeled FITC-dextran (Mw 20, 250, and 2000 kDa) solutions for 2 h and 30 min. Fluorescence intensities of particles (inside) and FITC-dextran solutions (outside) were assessed by confocal microscopy. A ratio of internal to external fluorescence was then calculated, and a ratio of 1 indicating that fluorescence intensity was identical in a particle and the surrounding solution. Alginate and Si-HPMC particles of 1 mm diameter were selected for this study. Each test was performed for one particle at a time and repeated at least 3 times.

size of $68 \pm 28\,\mu$m with a good reproducibility. Laser analysis showed a size distribution larger for Si-HPMC particles than for alginate ones (Figure 1(b)). We also observed a predominant population from 31 ± 18 to $76 \pm 11\,\mu$m (69%) and a minor one from 76 ± 11 to $144 \pm 23\,\mu$m (11%). Furthermore, we detected some larger Si-HPMC particles greater than or equal to 1 mm.

3.2. Alginate and Si-HPMC Particle Characterization. Diffusion properties of alginate and Si-HPMC hydrogels were investigated after incubation of particles (1 mm in diameter) in 1 mg/mL solutions of FITC-dextran (Mw 20, 250, or 2000 kDa) for 2 h and 30 min. At the beginning of the experiment, particles, observed using confocal microscopy, were not fluorescent at all, and then we noted an increase in the fluorescence intensity in some particles as a function of time. To compare hydrogels, a ratio of internal (i.e., inside a particle) to external (i.e., dextran solution) fluorescence intensity was calculated. For 20 kDa and 250 kDa FITC-dextrans, we noted that both alginate and Si-HPMC particles became fluorescent (Figure 2) and that the intensity ratio increased as a function of time.

On the contrary, when incubated with high molecular weight-labeled dextran (2000 kDa), no fluorescence was detected in particles (circle symbols) and the calculated ratio remained equal to zero. In less than 2 hours, equilibrium of fluorescence intensity (i.e., ratio = 1) was reached for the 20 kDa dextran in Si-HPMC particles (black delta symbol) but not observed for 250 kDa (black square symbol) nor with alginate (white delta and square symbols), indicating that diffusion rate of dextrans was faster in Si-HPMC particles as compared to alginate ones.

To investigate their mechanical properties, particles of 1 mm size were subjected to a 30% compressive displacement over a period of 30 s (Figure 3).

While an alginate particle required a 14 mN force, a force as low as 0.4 mN was sufficient for Si-HPMC one. This is reflected in the significantly different Young's moduli of 16 ± 0.82 kPa for alginate particles and 0.22 ± 0.06 kPa for Si-HPMC ones. In addition, we noticed that alginate particles had a viscoelastic response as the unloading path was steeper than the loading curve while Si-HPMC particles presented a minimal hysteresis and returned to their original geometry when the compressive force was removed.

3.3. Estimation of the Average Number of Encapsulated hASC. To investigate the average number of encapsulated hASC, alginate and Si-HPMC particles were disrupted by using sodium citrate solution and ultrasonication, respectively. The average number of encapsulated hASC per alginate particle was significantly higher than the one in Si-HPMC particle (Figure 4(a)).

We counted about 7102 ± 514 hASC per alginate particle versus 5100 ± 407 hASC per Si-HPMC particle, 24 hours after encapsulation. It is worth noting that, with regard to a theoretical volume of 3.6 μL (based on a particle mean size), a particle could contain 7200 encapsulated cells, at most. Hence, the encapsulation efficiency, calculated as the ratio of the experimental number of encapsulated cells to the theoretical one, was 97% for alginate and 70% for Si-HPMC.

3.4. Evaluation of Encapsulated hASC Viability and Biofunctionality. Whatever the polymer used, the number of encapsulated cells did not significantly change during the 4-week study. For the duration of the study, the viability of encapsulated hASC in Si-HPMC and in alginate was $90.8 \pm 3.8\%$ and $90.5 \pm 2.4\%$, respectively (Figure 4(b)). No significant difference between the viability of hASC encapsulated in Si-HPMC and in alginate was observed. The distribution in cell viability along the radial direction of both alginate and Si-HPMC particles was uniform, and no accumulation of dead cells in the center of the particles was detected (Figure 4(b)). Furthermore, viable cells do not seem to physically interact among themselves and formation of cell clusters was not observed. As such, hydrogels may provide optimal conditions for cell survival, since clusters might limit nutrient availability and slow down secretion of biological factors.

To assess the secretion properties of encapsulated hASC, cells were treated for 72 hours with proinflammatory molecules (TNF-α + IFN-γ) as previously described [26]. In the control condition, when encapsulated hASC in alginate were not stimulated, the basal concentration of L-kynurenine in supernatant was $6.08 \pm 1.4 \times 10^{-5}\,\mu$M per encapsulated hASC.

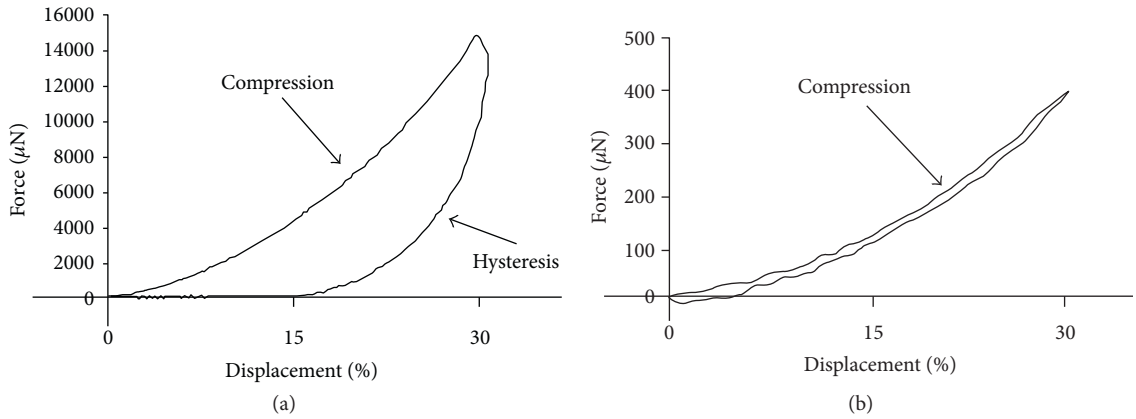

Figure 3: Mechanical properties of the particles. Compressive properties of alginate (a) and Si-HPMC (b) particles were investigated by subjecting them to a 30% compression for 30 seconds. The force (μN) and displacement (%) were recorded, and Young's modulus was determined. Alginate and Si-HPMC particles of 1 mm diameter were selected for this study.

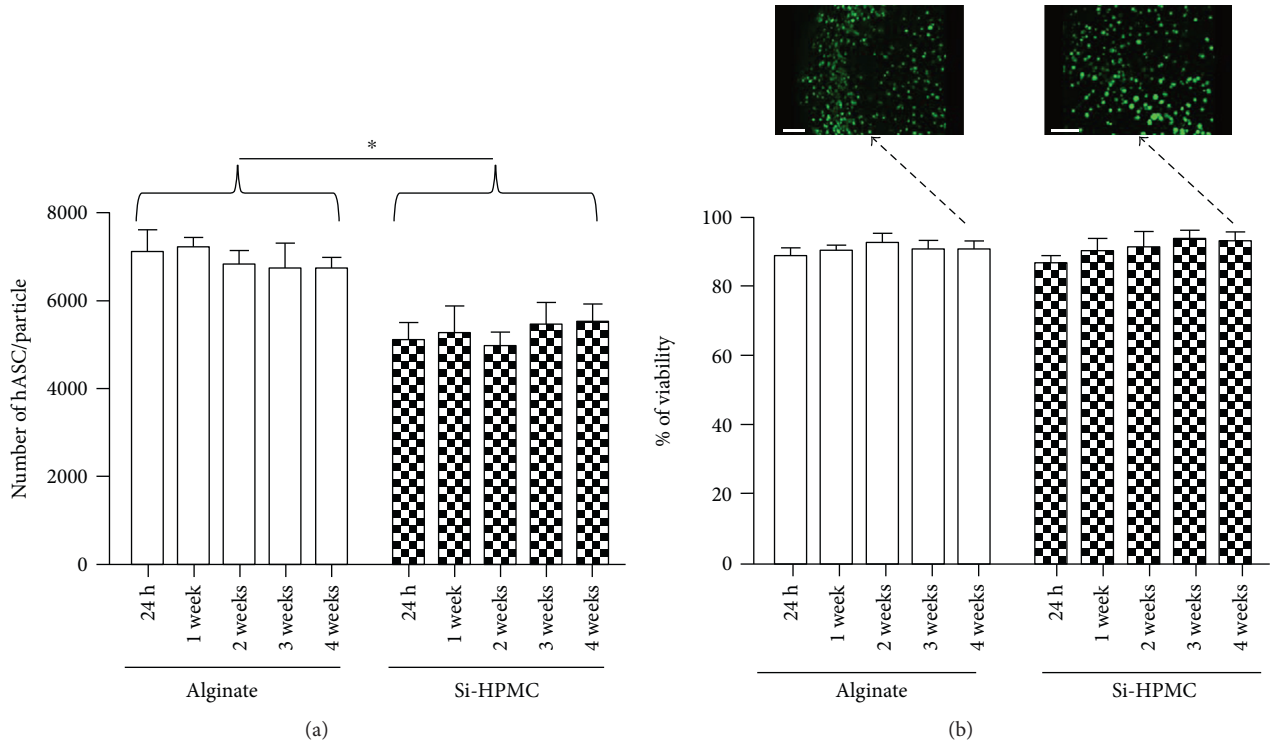

Figure 4: hASC viability after encapsulation in alginate and Si-HPMC. The average number of encapsulated hASC per alginate and Si-HPMC particles was evaluated from 24 h to 1 month. (a) Average number of cells per particle. Alginate particles were dissolved in sodium citrate solution, and Si-HPMC particles were disrupted by ultrasonication. A DNA quantification by PicoGreen has allowed us to estimate the cell number. (b) Cell viability. Using a Live/Dead Viability/Cytotoxicity kit, cells encapsulated in alginate and Si-HPMC particle were assessed from 24 h to 1 month of culture. Viable cells were imaged using a confocal microscope, and viability was calculated with Volocity software. Scale bars = 100 μm. $^*p < 0.05$.

The stimulation of hASC encapsulated in alginate and Si-HPMC with IFN-γ combined with TNF-α induced a respective 8-fold and 19.7-fold increase in the concentration of L-kynurenine in supernatant as compared to the control condition (unstimulated encapsulated hASC in alginate) (Figure 5(a)). Moreover, encapsulation of hASC in Si-HPMC showed a concentration of L-kynurenine significantly higher than in alginate.

For PGE_2, and compared to unstimulated hASC in alginate, we observed a 4.9-fold increase when encapsulated hASC in alginate were stimulated with TNF-α + INF-γ (Figure 5(b)). The increase was more prominent (7.67-fold

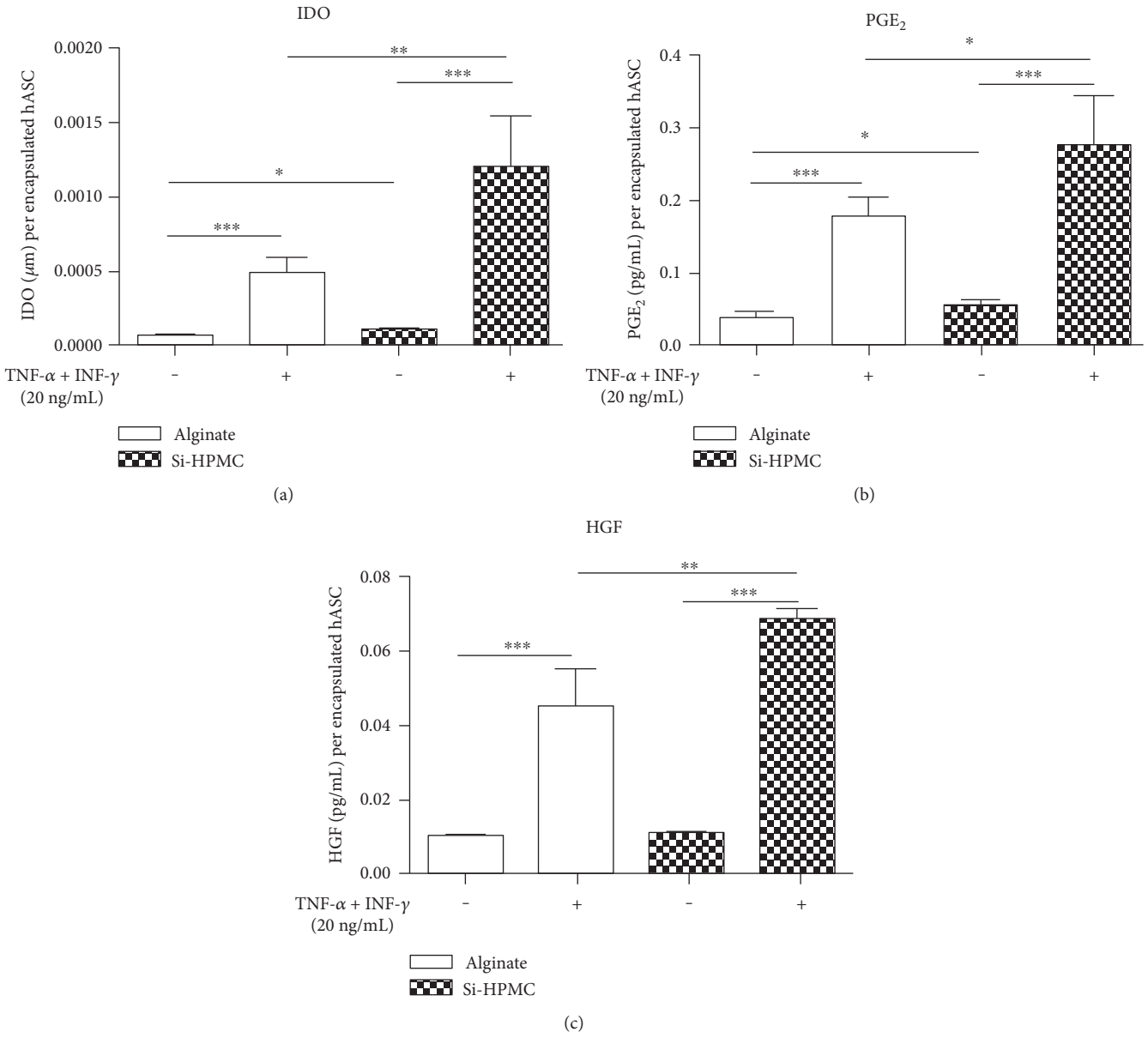

Figure 5: IDO enzyme activity and PGE_2 and HGF concentrations in supernatants of encapsulated hASC. One week after encapsulation in alginate and Si-HPMC, hASC were stimulated (or not) with TNF-α and IFN-γ (20 ng/mL each) for 72 h. For each condition, twenty alginate or Si-HPMC particles were then disrupted and encapsulated hASC were counted. IDO enzyme activity (a) was measured by tryptophan-to-kynurenine conversion with photometric determination of kynurenine concentration in the supernatant. PGE_2 (b) and HGF (c) were measured in cell supernatant using ELISA kits according to the manufacturer's recommendations. The secretion of these biofactors was normalized to the number of encapsulated hASC in both alginate and Si-HPMC particles. Alginate and Si-HPMC particles of 1 mm diameter were selected for this study. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

increase) when hASC were encapsulated in Si-HPMC. As already observed, encapsulated hASC in alginate or Si-HPMC were able to secrete PGE_2, and the concentration in supernatant was increased after stimulation with proinflammatory cytokines. hASC encapsulated in Si-HPMC also showed a significantly higher concentration of PGE_2 in supernatant than hASC encapsulated in alginate.

Compared to unstimulated hASC in alginate, the stimulation of encapsulated hASC in alginate and Si-HPMC with TNF-α and IFN-γ increased the supernatant concentration of HGF by 4.4 and 6.7, respectively (Figure 5(c)). As previously observed for L-kynurenine and PGE_2, after stimulation, HGF concentration in supernatant was significantly higher when hASC were encapsulated in Si-HPMC than in alginate.

All these results indicate that hASC encapsulated in alginate or Si-HPMC are sensitive to proinflammatory cytokines and respond to this stimulation by increasing their secretion of IDO, PGE_2, and HGF. Moreover, higher levels of L-kynurenine, PGE_2, and HGF were measured in the supernatant of Si-HPMC-encapsulated hASC than those encapsulated in alginate.

4. Discussion

MSCs mainly exert their regenerative properties *in vitro* and *in vivo* through the secretion of biofactors [3, 4]. However, the secretion of these therapeutic bioactive factors after encapsulation of MSCs has not yet been proven and investigated for a potential application in inflammatory disease treatment.

In this context, the aims of this study were to evaluate (i) the feasibility of generating particles with Si-HPMC hydrogel and (ii) the *in vitro* viability and bioactivity of hASC encapsulated in Si-HPMC particles. Si-HPMC is a semisynthetic polymer that undergoes condensation and cross-linking when the pH decreases by addition of an acidic HEPES buffer [22]. Unlike alginate which gelation in $CaCl_2$ solution is near instantaneous, Si-HPMC cross-linking requires about 40 minutes at room temperature. We verified previously that dropwise method is not appropriate to generate Si-HPMC particles. So we have developed an oil dispersion protocol to obtain Si-HPMC particles. Our results showed that this method is noncytotoxic and appears suitable for hydrophilic materials, as Si-HPMC.

The choice of particle size selection is governed by a compromise between the site of injection, the targeted number of cells to inject, and the desired quality of exchange between particles and their external environment. One of the limiting factors in the use of alginate for encapsulation is that the size of generated particles is dictated by the extrusion needle diameter. In practice, it is difficult to generate particles with needles of a diameter smaller than 31G (availability of needles and high viscosity of polymeric solutions). Extrusion through a 31G needle generated spherical alginate particles with a size of about 2 mm. This size is not compatible with injection in a small animal model like rodents. Theoretically, it would be possible to reduce the size of alginate particles by modifying the extrusion operating conditions (surface tension of alginate solution, concentration of calcium chloride, and curing time particle in gelation bath [27]) or by selecting another method of particle generation like microfluidic technologies. Another option is to select a different polymer to obtain smaller particles. In this study, we generated about 100 μm diameter Si-HPMC particles and we observed that this size was mainly influenced by 2 parameters: temperature and dispersion duration. The following dispersion parameters were chosen: 1 h and 30 min as dispersion time to allow sufficient particle gelation, stirring at 250 rpm to reduce as much as possible shear stress, and room temperature to obtain smaller particles. Under these operating conditions, Si-HPMC particles are smaller than alginate ones as expected but with an important size distribution.

Particle geometry is also a parameter which has an impact on the injectability and biocompatibility of hydrogels. Indeed, it was reported that nonspherical shape with acute angle particles does not promote their injectability (caused by their poor flow rate) and above all induce inflammation by producing important amount of foreign body responses *in vivo* [28, 29]. We observed that both alginate and Si-HPMC particles are spherical making their shape likely appropriate for *in vivo* application.

As the extrusion process of alginate generates particles of more than 1 mm in diameter, we decided, for further experiments, to select both alginate and Si-HPMC particles of about 1 mm size. This allows a consistent comparison of both materials for particle properties and hASC viability and biofunctionality.

This study relies on the diffusion of molecules of interest from outside to inside of the particles (proinflammatory cytokines as stimulants) and inversely (bioactive factors secreted by stimulated MSCs as therapeutic agents). For therapeutic effectiveness, encapsulation should allow a sustained delivery of biofactors and maintain a high local concentration of these molecules in the surrounding tissues. This diffusion is affected by the mechanical stress applied on the particle *in vivo* which strongly depends on elasticity, swelling, and charge density of the hydrogel. These factors and their interactions create a complex environment that determine the diffusion kinetics, rate of bioactive factor release through the particles, and duration [30]. Due to its wide use in diffusion studies as a result of its ease of use [31, 32], we selected FITC-dextran (Mw 20, 250, or 2000 kDa). A particular interest was focused on the 20 kDa FITC-dextran, since proinflammatory cytokines and biofactors secreted by stimulated MSCs have molecular weights ranging from about 10 to about 45 kDa [33–35]. Our results clearly show that Si-HPMC and alginate hydrogels have different diffusion properties. Indeed, FITC-dextrans diffuse faster in Si-HPMC particles than in alginate ones identically sized. This observation could be due to a larger mesh size (i.e., porosity) of Si-HPMC particles compared to alginate ones. This result suggests that MSC encapsulation in Si-HPMC would allow a faster reactivity to proinflammatory cytokines and a faster release of bioactive factors.

Mechanical properties of nonadhesive hydrogels dictate the behavior of encapsulated cells and might impact their viability and bioactivities. For example, high stiffness can decrease the viability of encapsulated cells as described by Wright et al. [36]. Mechanical tests and Young's modulus suggest that alginate particles were stiffer than Si-HPMC ones. Young's modulus value of alginate particles (16 kPa) was similar to previously reported literature values (ranging from 5 to 18 kPa, [37]). After compressive displacement, Si-HPMC particles recover their original shape faster than alginate ones. This difference suggests that Si-HPMC particles would allow a quicker relaxation during and after injection than alginate ones thereby allowing a better behavior of encapsulated cells [38].

Given that MSCs exert their therapeutic potential partly by the secretion of bioactive factors exhibiting immunomodulatory, antifibrotic, proangiogenic, or antiapoptotic properties, encapsulated MSCs must be viable and their average number per particle should be sufficient to obtain an efficient production of these bioactive factors. MSCs were isolated from human adipose tissue which can be obtained easily under local anesthesia. Furthermore, adipose tissue contains MSCs in larger numbers (5% of nucleated cells, [2]). Our results show that the viability of encapsulated hASC in alginate and Si-HPMC particles remained higher than 86% for over a month. The disruption of particles is essential for

estimating the average number of encapsulated hASC. Si-HPMC particles cannot be disrupted by enzymatic treatments (data not shown), and only ultrasonication is effective. The average number of hASC encapsulated per alginate particle was higher than in Si-HPMC particle identically sized. This result could be due to a loss of hASC (during their incorporation in precross-linked Si-HPMC hydrogel) or to their leakage from Si-HPMC droplets during the dispersion protocol (1 h and 30 min) since, unlike alginate, Si-HPMC cross-linking is not instantaneous and requires about 40 minutes at room temperature. The number of encapsulated hASC per alginate and Si-HPMC particles remained constant over time, which is consistent with numerous publications reporting a slow cell proliferation rate, often linked to the lack of bioactive molecules in alginate and Si-HPMC hydrogels [39–41]. This long-term *in vitro* viability study confirms that the particle matrix allows the diffusion of nutrients, oxygen, and glucose needed to maintain the viability of encapsulated hASC. Despite the use of large alginate and Si-HPMC particles (1 mm in diameter) for the cell cultures, a good viability of hASC at the center of particles was observed. This strongly suggests that if a suitable diffusion through the center of the particles is obtained, the particle size is not a limiting factor for cell viability. We also noted that the difference of FITC-dextran diffusion between alginate and Si-HPMC particles is not related to a difference in encapsulated hASC viability.

Since they exert anti-inflammatory effects, MSCs seem to be effective in preventing the evolution of various diseases, including Crohn's disease, GVHD (graft-versus-host disease), IBD (inflammatory bowel disease), and osteoarthritis [42–47]. However, there is no study investigating the secretion of therapeutic bioactive factors by encapsulated MSCs. Therefore, we investigated the secretion of 3 soluble factors by hASC after encapsulation. IDO is an enzyme of tryptophan catabolism through kynurenine pathway. IDO activity is directly reflected by the concentration of L-kynurenine in supernatant. The gradual decrease of tryptophan in the environment and kynurenine synthesis leads to the inhibition of T cell and NK cell proliferation and their apoptosis through c-Myc pathway [7]. PGE_2 inhibits activation, proliferation, and cytotoxicity of immune cells [6, 8, 43]. PGE_2 has also anti-inflammatory properties by activating the programming of macrophages in anti-inflammatory M2 phenotype which express IL-10 [48]. HGF has also an anti-inflammatory function on dendritic cells [49]. In addition, it is well known that secreted HGF by MSCs has antifibrotic properties [44].

Our results show that encapsulation does not impair the ability of hASC to secrete bioactive trophic factors in a proinflammatory environment. Indeed, after stimulation by IFN-γ and TNF-α, the concentrations of HGF, PGE_2, and L-kynurenine in supernatant were found to dramatically increase. Therefore, these results demonstrate that (i) proinflammatory cytokines diffuse within particles to stimulate hASC and (ii) stimulated hASC secrete soluble factors which are released through the particles. Compared to alginate, the lower number of encapsulated hASC in Si-HPMC does not cause any negative impact on their ability to secrete bioactive trophic factors. Moreover, kynurenine, PGE_2, and HGF concentrations in cell supernatant were always higher when hASC are encapsulated in Si-HPMC particles. This difference confirms that hASC encapsulation in Si-HPMC allows a faster release of bioactive trophic factors in supernatant. This could be due to differences in production of biofactors by encapsulated hASC. These results are promising for the use of encapsulated MSCs in the treatment of inflammatory diseases.

5. Conclusion

In this study, we propose a device allowing an extended release of biofactors that can be instrumented for reducing inflammation and fibrosis. MSCs can be encapsulated in spherical Si-HPMC particles and remain viable for a month *in vitro*. Moreover, we demonstrated that encapsulation allows the maintenance of hASC responsiveness to IFN-γ and TNF-α treatment in terms of IDO, PGE_2, and HGF secretion. Differences in mechanical and physicochemical properties, including diffusion and reactivity of encapsulated MSCs as compared to alginate ones, bring us to the conclusion that Si-HPMC may be more appropriate for the development of an injectable therapeutic cell encapsulation system for MSC delivery. These results are promising, and further *in vitro* and *in vivo* experiments are under investigation to determine whether encapsulated MSCs may be a relevant strategy to prevent inflammation in degenerative disease such as osteoarthritis.

Disclosure

Part of this study was previously presented as a poster at the 2016 OARSI World Congress (March 31–April 03, Amsterdam, The Netherlands).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Review Article

Infrapatellar Fat Pad Stem Cells: From Developmental Biology to Cell Therapy

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The ideal cell type to be used for cartilage therapy should possess a proven chondrogenic capacity, not cause donor-site morbidity, and should be readily expandable in culture without losing their phenotype. There are several cell sources being investigated to promote cartilage regeneration: mature articular chondrocytes, chondrocyte progenitors, and various stem cells. Most recently, stem cells isolated from joint tissue, such as chondrogenic stem/progenitors from cartilage itself, synovial fluid, synovial membrane, and infrapatellar fat pad (IFP) have gained great attention due to their increased chondrogenic capacity over the bone marrow and subcutaneous adipose-derived stem cells. In this review, we first describe the IFP anatomy and compare and contrast it with other adipose tissues, with a particular focus on the embryological and developmental aspects of the tissue. We then discuss the recent advances in IFP stem cells for regenerative medicine. We compare their properties with other stem cell types and discuss an ontogeny relationship with other joint cells and their role on *in vivo* cartilage repair. We conclude with a perspective for future clinical trials using IFP stem cells.

1. Introduction

Cell-based approaches are increasingly gaining attention in the development of treatments for articular cartilage defects [1–4], especially since the clinical application of autologous chondrocytes for articular cartilage repair in 1994 (autologous chondrocyte implantation, ACI) [5, 6]. However, the development of a regenerated cartilage that fully recapitulates the native tissue still eludes us. It is therefore unsurprising that a full consensus has not yet been reached on the optimum cell source for cartilage tissue regeneration [7, 8].

Some of the most frequently studied cells include mature chondrocytes, chondrocyte progenitors, embryonic stem cells (ESC), induced pluripotent stem cells (iPS), and mesenchymal stem cells (MSC). Mature chondrocytes, such as those currently used in ACI, have led to improved clinical outcomes [5], although there are challenges associated with their isolation, culture, donor-site morbidity, and dedifferentiation [9–11]. Tissue-specific progenitor cells found in the perichondrium [12, 13], periosteum [14], and in normal or osteoarthritic (OA) cartilage itself [15–17] are being actively explored as substitutes to mature chondrocytes. Studies on the chondrogenic differentiation of ESC and iPS have shown these cell types are emerging as potential future cell sources for cartilage repair [18]; however, ethical and/or safety issues remain (e.g., tumor formation) [19]. Given their availability

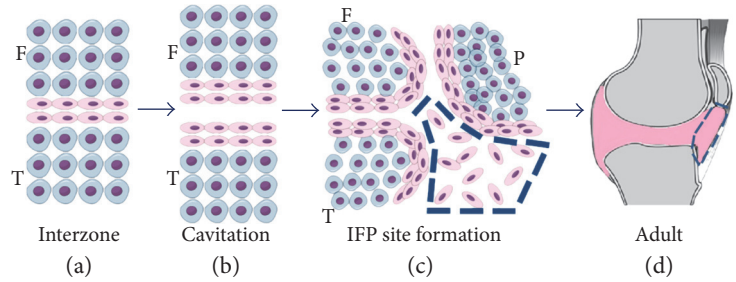

Figure 1: Ontogeny of the infrapatellar fat pad (IFP). During embryonic development, (a) a dense mesenchyme tissue arises between the chondrification of the femur (F) and tibia (T), the interzone (flattened cells in pink). (b) This is followed by a cavitation in between this region. (c) By the 9th week of human development, a triangular space composed of a mesenchymal tissue becomes visible below the patella (P) (highlighted by the blue dashed). (d) In adulthood, interzone cells will have contributed to several joint structures (pink), including the IFP (highlighted by the blue dashed). This image was made using https://MindTheGraph.com.

and chondrogenic potential, MSC—primarily from the bone marrow but also from adipose tissue—have emerged as the most promising cell source to regenerate articular cartilage [20–22].

Interestingly, MSC isolated from tissues within the articular joint possess superior chondrogenic capacity when compared to the bone marrow or subcutaneous adipose tissue-derived MSC [23]. Specifically, MSC can be isolated from the synovial fluid [24, 25], synovial membrane [26, 27], and the infrapatellar fat pad (IFP) [28–32]. MSC isolated from the synovial fluid or the synovial membrane have been previously discussed in another review paper [33], and the latter have already been investigated in a clinical study, where significant improvements in clinical outcomes were demonstrated including improved MRI scores (from 1.0 ± 0.3 to 5.0 ± 0.7, median $\pm$ 95% CI) which grade for "degree of defect repair and filling of the defect" [34], Lysholm knee scores (from 76 ± 7 to 95 ± 3, median $\pm$ 95% CI) which grade "patients' own opinion of function" [35] and histological qualitative assessments [27]. Although very few clinical trials have been reported so far employing IFP stem cells [36, 37], this review will outline how these cells could be a very promising source for cartilage regeneration. First, we will discuss IFP as a tissue source, anatomically and developmentally. Next, we will describe the latest advances in analyzing the therapeutic potential of IFP stem cells for cartilage regeneration. Finally, we will compare IFP stem cells to other cell types in the joint, suggesting their main role in the maintenance of joint homeostasis. In the conclusions and future perspectives section, we will motivate the use of IFP cells in future clinical trials.

2. The IFP Structure and Development

In order to put forward the IFP as a promising cell source for cartilage regeneration, it is important to understand its anatomical characteristics, as well as its developmental origin. As an adipose tissue within the joint, the IFP can be easily harvested arthroscopically or during open knee surgery [38]. The IFP is an intracapsular structure in the anterior knee compartment, composed of approximately 20 cm^3 of adipose tissue, or slightly larger in patellofemoral OA joints [39–41]. As it is lined on its deep surface by the synovial membrane, it is classified as an extrasynovial structure. The IFP lies inferior to the patella and posteriorly extends into the infrapatellar plica (IPP) (ligamentum mucosum), which inserts into the anterior border of the intercondylar notch [42]. The infrapatellar plica is, together with the suprapatellar and mediopatellar, one of the three plicas in the knee. These plicas are believed to be synovial fold remnants from the incomplete resorption of the synovial septa during the embryological development of the knee [43].

Although generally considered to participate in the biomechanics of the knee [44], the exact roles of IFP in articular physiology have not been fully elucidated [45]. In 1691, it was originally proposed by Havers et al. that synovial fat pads were responsible for the secretion of synovial fluid. However, it is now believed that they simply occupy space in the joint, maintaining the articular cavity, allowing the synovial fluid to circulate over the joint, and contributing to lubrication [46], with the contribution to lubrication attributed to increased synovial surface resulting from their anatomical location [47]. Participation of the IFP in shock absorption has also been proposed [48]. Interestingly, the weight of the synovial fat pads are unrelated to the state of nutrition, unless in extreme emaciation [49]. Even under starvation conditions, with the elimination of the subcutaneous adipose tissue, the IFP may be preserved [50]—this biological drive underscores its importance in the knee joint. As discussed later in this review, we believe this biological drive results from IFP stem cells' role in tissue maintenance and repair.

Besides its anatomical position, the embryonic origin of the IFP also highlights its potential. Synovial joints develop through the formation of an interzonal layer of flattened cells within a mesenchymal condensation, which is responsible for the cavitation and formation of the joint tissues [51]. Specifically, during human knee formation, at the 9th week of development—when the chondrification of the patella, femur, and tibia has already begun but prior to the menisci maturation and ossification—the chondral anlagen—a triangular space occupied by a mesenchymal tissue—appears below the patella. It is thought that this is the site of formation of the future IFP [52]. The cells from the interzone will further contribute to the development of the epiphyseal articular chondrocytes, ligaments, menisci, synovial lining, and fat pad [53–57] (Figure 1).

3. The IFP as an Adipose Tissue

Although the exact roles of the IFP and its development are not yet fully understood, it is important to highlight its nature as an adipose tissue and, more specifically, as an elastic adipose tissue, due to its orcein-stained elastic-fiber content [49]. Traditionally, the adipose tissue has been identified as a metabolic tissue responsible for storing energy in the form of fat. However, more recently, due to the description of adipokines and their regulation of appetite and participation in inflammation and vascular diseases, the adipose tissue is now also regarded as an endocrine tissue [58, 59]. Indeed, the adipose tissue can be divided into brown (BAT) and white (WAT) types, which are further divided into subcutaneous and visceral adipose tissue. WAT is commonly associated with energy storage, while BAT with energy dissipation in the form of heat [60]. In a similar way, subcutaneous adipose tissue is more predisposed to storing free fatty acids and triglycerides, while visceral adipose tissue is more cellularized, vascularized, innervated, and therefore more metabolically active and predisposed to insulin resistance [61]. Different sites of the adipose tissue therefore present different physiological properties [62].

Although all adipose tissues possess a mesodermal origin, different stem cell populations give rise to visceral and subcutaneous adipose tissue [63, 64], for example mesothelial cells originated mainly from the lateral plate mesoderm strongly contribute to the formation of visceral adipocytes, while paraxial mesoderm and neural crest contribute to the formation of mesenchymal/mesodermal stem cells that originate subcutaneous adipocytes [65, 66]. Based on this delineation, it is important to note that the IFP should be evaluated as an adipose tissue with singular characteristics. It does not correlate with visceral adipose tissue since its origin is not related in any way to the formation of visceral structures and has never presented a mesothelium cover. On the other hand, it is not functionally similar to subcutaneous adipose tissue either; for instance, IFP from obese patients secretes different levels of inflammatory molecules and adipokines (e.g., higher levels of IL-6, soluble IL-6 receptor and adiponectin, and lower levels of leptin), and expresses lower levels of lipid metabolism-related genes compared to subcutaneous adipose tissue [67].

4. Adipose-Derived Stem Cells

Mesenchymal stem-like cells with multilineage differentiation capacities were first isolated from the human subcutaneous adipose tissue obtained after the enzymatic digestion of lipoaspirate samples in 2001 [68]. This was in accordance with later findings that MSC with tissue/organ-specific characteristics could be found in virtually all organs, occupying a perivascular niche [69, 70]. Further investigation confirmed that although MSC derived from both subcutaneous adipose tissue and the bone marrow are multipotent, the bone marrow-derived MSC are more committed to osteogenic and chondrogenic lineages, while adipose-derived stem cells are more committed to the adipogenic lineage [71]. Moreover, while CD34 is not expressed by the bone marrow-derived MSC, it is only the CD34+ cells in the adipose tissue which are capable of multilineage differentiation and of forming fibroblastic colony-forming units (CFU-F) [72]. More recently, it has been proposed that four different nonhematopoietic (CD45−) progenitor populations exist in adipose tissue: endothelial progenitors (CD146+/CD31+/CD34+); pericytes (CD146+/CD31−/CD34−), which are more naïve; a transit amplifying progenitor population (CD146+/CD31−/CD34+); and a more adipogenic-committed supra-adventitial adipose stromal cell population (CD146−/CD31−/CD34+) [73].

In 2013, the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) published a joint statement on some definitions regarding adipose-derived stem cells. For instance, marker expression profiles of cells from the stromal vascular fraction (SVF) and the adipose tissue-derived stromal cells (ASC) have been defined. The SVF comprises the cell populations obtained after enzymatic digestion of the adipose tissue, separated from the mature adipocytes through centrifugation, such as endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes/macrophages, pericytes, and stem/progenitor populations; while the ASC comprises the adherent cells populations obtained from the SVF [74].

5. The IFP Stem Cells

In 1996—before the first isolation of adipose-derived stem cells (2001)—Maekawa et al. described a population of fibroblasts that are a "kind-of a stem cell" in the synovial tissue near the IFP. The cells described reside in a perivascular niche, expressing fibronectin and laminin, and are associated with small vessels. They participate in anterior cruciate ligament (ACL) repair after injury, by secreting extracellular matrix (ECM) components such as collagen type I and III. Moreover, these cells can also differentiate into surface-lining phagocytic fibroblasts [75]. To our knowledge, this was the first report of a stem cell-like population related to the IFP.

More recently, multipotent stem cells from IFP have been isolated and characterized as CD9+, CD10+, CD13+, CD29+, CD44+, CD49e+, CD59+, CD105+, CD106+, and CD166+ with the ability to differentiate into chondrogenic, osteogenic, and adipogenic lineages under the appropriate stimulation *in vitro*. Under chondrogenic stimulation, the cells did not produce collagen type X, a marker of hypertrophy [76]. Since then, many studies have confirmed IFP stem cells' chondrogenic capacity in different *in vitro* and *in vivo* models [28–30, 32, 51, 77–81].

Continuing the characterization studies, Hindle et al. distinguished two populations within the IFP stem cells: the pericytes (CD31−/CD34−/CD45−/CD146+) and adventitial cells (CD31−/CD34+/CD45−/CD146−), corresponding to 3.8% and 21.2% of the isolated cells at the stromal vascular fraction (SVF). The two mixed populations were termed "perivascular stem cells" (PSC). The total adherent population was termed "MSC." Interestingly, both PSC and MSC derived from IFP showed superior chondrogenic capacity

compared to the bone marrow-derived MSC. Additionally, comparing the two populations from IFP, it was found that PSC were superior to MSC [82]. Additionally, 3G5 (a pericyte marker) has been detected in IFP cells in situ. Situated in the adventitia of small blood vessels, 1–20% of cells retained 3G5 expression both in mixed and clonal population after expansion in culture [51].

When compared with other cell types, IFP stem cells were found to retain their chondrogenic potential for a longer period than chondrocytes obtained from OA articular cartilage [83]. Compared with subcutaneous adipose tissue-derived stem cells, IFP stem cells presented with a similar ability to form CFUs; however, they expressed higher levels of chondrogenic and osteogenic genes [84]. Compared with the bone-marrow MSC, IFP stem cells generated more cartilaginous ECM in pellet cultures and expressed higher levels of chondrogenic genes [82]. In one study, IFP stem cells were compared to the bone marrow-derived MSC and synovium-derived stem cells (SDSC) [85]. Importantly, SDSC had been reported to present enhanced chondrogenic potential compared to the bone marrow, adipose, and muscle MSC both *in vitro* [86] and *in vivo* [23]. Although SDSC generated the most functional and mechanically stable cartilaginous tissue *in vitro*, none of the cell types generated stable cartilage after subcutaneous implantation *in vivo*. Nevertheless, the authors stated that it is not possible to fully conclude that SDSC possess higher chondrogenic capacity compared to the other cell types, since different culture conditions may interfere with each cell's chondrogenic potential [85]. For instance, the application of physiological levels of hydrostatic pressure (HP) further enhances IFP stem cells chondrogenic capacity, as well as maintain their potential after removal of TGF-β3 stimuli [87]. Moreover, when exposed to dynamic compression and a gradient oxygen tension [88] or cultured in decellularized cartilage explants [89], IFP stem cells produced cartilaginous ECM with zonal architecture that resembled native articular cartilage.

It is possible that specific subpopulations in the heterogeneous IFP stem cell population may possess an even greater chondrogenic potential. A subpopulation of freshly isolated, that is not expanded *in vitro*, CD44+ cells (approximately 10% of the entire population) showed an impressive capability to synthesize sGAG and collagen type II *in vivo* when seeded on a cartilage ECM-derived scaffold and subcutaneously implanted [28]. The idea of using an enriched population with increased chondrogenic capacity, without the need of culture expansion, is particularly promising for clinical trials. A study comparing donor-matched articular chondrocytes, bone marrow, IFP, and subcutaneous adipose tissue stem cells also suggested that CD49c and CD39 expression positively correlate to an enhanced in vitro chondrogenic potential, besides suggesting IFP stem cells as the best stem cell alternative to chondrocytes, followed by the bone marrow and subcutaneous adipose tissue [90].

Recently, IFP stem cells were also compared to synovial fluid stem cells (SFSC). The chondrogenic capacity of both was considered similar *in vitro*, although the adipogenic and osteogenic potential of IFP stem cells was greater. Moreover, the expression of CD34 was detected in $30.1\% \pm 18.6\%$ of passage 3-4 IFP stem cells. As mentioned previously, this marker is also present in adipose-derived stem cells and is related to their multilineage potential [72]. Finally, both populations presented CD14 positivity, a marker of macrophage lineage, although there was some variation among donors ($30.5\% \pm 30.3\%$ for IFP stem cells and $7.4\% \pm 7.2\%$ for SFSC) [91].

A possible strategy in the field of cartilage repair is to use cocultures of different cell types, usually with articular chondrocytes, instead of choosing only one [92]. In this regard, several promising attempts have been performed with bone-marrow [93–96], synovium [97], and adipose-derived [96, 98] stem cells. IFP stem cells also present an enhanced chondrogenic potential when cocultured with articular chondrocytes [99] and most especially when cocultured in a structured manner (i.e., on top of the articular chondrocytes), instead of homogeneously mixing them, in an attempt to recapitulate the zonal characteristics of a progenitor population on top of the native articular cartilage [100].

One potential clinical concern would be the therapeutic potential of IFP stem cells obtained from a diseased OA joint. Encouragingly, studies have shown comparable chondrogenic capacities of OA-derived IFP stem cells with "healthy" cells (i.e., obtained from patients undergoing ligament reconstruction). Moreover, when cultured onto PLLA fiber membranes, OA-derived IFP stem cells generated hyaline cartilage-like grafts of approximately 2 cm diameter [32]. This property is of crucial importance for the future clinical translation of IFP stem cells, since it is necessary that even IFP cells from diseased joints are capable of enhancing cartilage repair.

Indeed, a rabbit model study showed improvement in the degree of cartilage degeneration, osteophyte formation, and subchondral sclerosis after allogeneic IFP stem cells injection 20 weeks after anterior cruciate ligament transection (ACLT) surgery to induce OA [101]. And in 2012, a therapeutic case control study level III was published using IFP cells to treat patients with OA in the knee. An average of 1.89 million cells was injected with platelet-rich plasma (PRP) as the carrier after arthroscopic procedure. PRP was further administered in a second and third round of treatment. The control group was treated with PRP injections without cells. There were significant clinical improvements evidenced by Lysholm score, Tegner activity scale, and visual analog scale (VAS) after 3 months and a last follow-up ranging from 12 to 18 months, on the same way that no major adverse events were reported. Interestingly, there was evidence that the therapy was more effective in younger patients and with early stage OA. Furthermore, although the degree of improvement was superior with cells, there was no statistical significance in the last follow-up comparing the treatment with and without cells [36].

A second study with the same group of patients, with level IV of evidence, was published one year afterwards with a final follow-up ranging from 24 to 26 months. At this point, clinical outcome was assessed with the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), the Lysholm score, and VAS. The results were similar, although

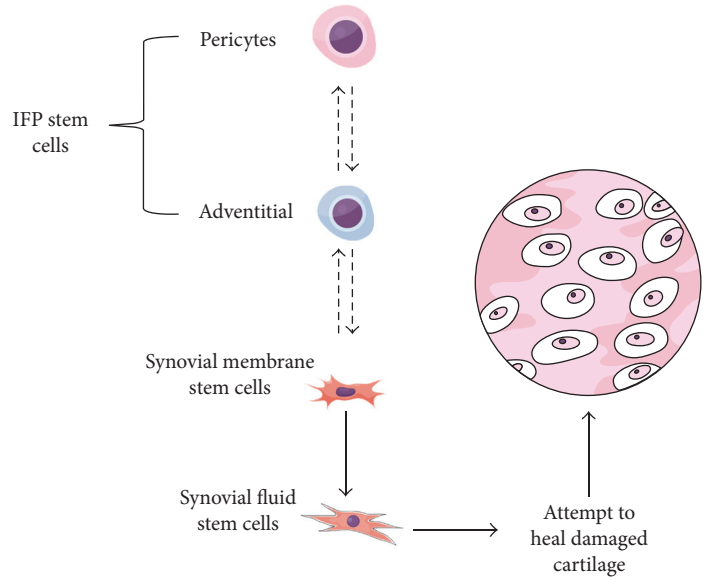

Figure 2: Hypothesis for a differentiation cascade between joint stem cells. The infrapatellar stem cells (IFP) would be divided into a perivascular (pericytes) and an adventitial population, with the pericytes being the most naive ones. Those would differentiate into synovial membrane stem cells. These can migrate into the synovial fluid, giving rise to synovial fluid stem cells, which would attempt to heal damaged cartilage. Dashed arrows represent more hypothetical relationships here proposed, while full arrows represent more proven ideas in the literature. This image was made using https://MindTheGraph.com.

the authors noticed that improvement in WOMAC score positively correlated with the amount of injected cells [37]. It is important to highlight, though, that in this study the authors used freshly isolated cells, which did not undergo *in vitro* expansion [102].

6. Ontogeny Relationships between Stem Cells within the Cartilage Joint

Along with the IFP stem cells, other stem cell populations with chondrogenic capacity reside in the articular joint, such as SDSC and SFSC. Moreover, the surface of the articular cartilage itself contains a stem cell-like population that contributes to tissue appositional growth [103, 104]. These cells have been characterized in healthy articular cartilage and have been shown to possess MSC characteristics [15, 17]. Similar populations have also been identified in OA cartilage [16]. Indeed, both healthy and OA cartilage progenitor cells, which are CD105+ and CD166+, had an adipogenic and osteogenic potential similar to the bone marrow-derived MSC. Interestingly, the percentage of these cells increases with OA, comprising 4% of cells in healthy cartilage and 8% in OA cartilage [105]. Although their origin and function are still not fully elucidated, it is believed that these cartilage-derived stem/progenitor cells, expressing NOTCH-1, reside in the surface of articular cartilage, where upon injury they may migrate to the defect site in an attempt to promote tissue repair. With lesion progression, for instance in advanced stages of OA, these cells may potentially migrate throughout the cartilaginous tissue [106].

Interestingly, there is growing evidence that cartilage stem/progenitor cells are related to other stem/progenitor cells isolated from the joint. A gene expression profile study showed that cartilage progenitor cells were more closely related to synoviocytes and synovial fluid cells than to chondrocytes [107]. Moreover, *Tgfbr2*-expressing cells in the interzone have been proposed to represent a population of joint stem cells in a murine developmental model. These cells are also found in adult mice in areas such as the synovial lining of the IFP and in part of the articular cartilage [108]. Indeed, a mouse model where *Tgfbr2* is knocked-out in the limbs, fails to develop the interzone resulting in a lack of interphalangeal joints [109]. Other molecules have also been used to illustrate the ontogenetic relationship between articular cartilage and noncartilaginous tissues in the joint. For instance, articular chondrocytes and cells from the cruciate ligament and the synovium do not express *matrilin-1*; by contrast, nonarticular chondrocytes from the developing anlagen start to express *matrilin-1* when the interzone is formed [110]. Roelofs et al. recently described a stem cell population in the synovial lining and in the vascularized sublining of connective tissue derived from interzone *Gdf5*-expressing cells. Following articular cartilage injury, this population proliferates, migrates, and differentiates into chondrocytes in the cartilage-defect sites. Interestingly, cells derived from interzone *Gdf5*-expressing cells were also detected in the adult's articular cartilage, menisci, ligaments, and fat pad [57]. These data contribute to the suggestion that, as articular cartilage is ontogenetically closely related to non-cartilaginous tissues in the joint, IFP stem cells could be used in future articular cartilage cell therapy trials.

This data also lead us to hypothesize that the IFP pericyte stem cell population described by Hindle et al. could be a tissue-resident stem cell population, as it is proposed for the subcutaneous adipose tissue [73], bone marrow [111–114], and muscle [115, 116]. Upon injury, these pericytes could

be recruited, migrating to the synovium lining and into the synovial fluid in an attempt to reach and regenerate the damaged articular cartilage (Figure 2). Indeed, the IFP is highly vascularized [117], with several perivascular niches for such stem cells. Remarkably, the first observations of Maekawa et al. resemble that hypothesis, although for ACL repair [75]. Liu et al. also hypothesized that IFP stem cells participate in patella tendon repair [118]. It is important to highlight that although this hypothesis is consistent with the interzonal origin of articular cartilage and IFP, the increased number of stem cells in the synovial fluid during OA and the pattern of surface marker expression described by Hindle et al. leaves much yet to be proved. For example, by contrast, Roelofs et al. observed that synovial stem cells were not from a perivascular niche and actually gave rise to perivascular cells upon cartilage injury [57]. Furthermore, there exact relationship between IFP stem cells and SDSC must yet be elucidated.

7. Conclusions and Future Perspectives

It has become clear that the IFP contains progenitor cells with MSC-like characteristics, such as multilineage differentiation potential and specific surface marker expressions. Interestingly, it seems that the characteristics of these cells and their subpopulations are comparable to ADSCs, particularly in regard to the fact that IFP stem cells possess a much greater chondrogenic potential. From a translational perspective, the IFP could be easily harvested arthroscopically for cell isolation.

Cartilage cell therapy has traditionally been investigated using articular the chondrocytes, bone marrow, and subcutaneous ADSCs. The recent promising clinical trial using SDSC further encourages the therapeutic use of joint-derived stem cells, and IFP stem cells are an exciting source. These observations, along with our hypothesis on the *in vivo* role of IFP stem cells in cartilage repair, strongly support the future applications of IFP stem cells for cartilage repair clinical trials in the near future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Quality Evaluation of Human Bone Marrow Mesenchymal Stem Cells for Cartilage Repair

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Quality evaluation of mesenchymal stem cells (MSCs) based on efficacy would be helpful for their clinical application. In this study, we aimed to find the factors of human bone marrow MSCs relating to cartilage repair. The expression profiles of humoral factors, messenger RNAs (mRNAs), and microRNAs (miRNAs) were analyzed in human bone marrow MSCs from five different donors. We investigated the correlations of these expression profiles with the capacity of the MSCs for proliferation, chondrogenic differentiation, and cartilage repair in vivo. The mRNA expression of *MYBL1* was positively correlated with proliferation and cartilage differentiation. By contrast, the mRNA expression of *RCAN2* and the protein expression of TIMP-1 and VEGF were negatively correlated with proliferation and cartilage differentiation. However, MSCs from all five donors had the capacity to promote cartilage repair in vivo regardless of their capacity for proliferation and cartilage differentiation. The mRNA expression of *HLA-DRB1* was positively correlated with cartilage repair in vivo. Meanwhile, the mRNA expression of *TMEM155* and expression of miR-486-3p, miR-148b, miR-93, and miR-320B were negatively correlated with cartilage repair. The expression analysis of these factors might help to predict the ability of bone marrow MSCs to promote cartilage repair.

1. Introduction

Mesenchymal stem cells (MSCs) have the capacity for self-renewal [1] and differentiation into several mesoderm-type lineages, including osteoblasts, chondrocytes, adipocytes, myocytes, and vascular cells [2] and are considered to be nonimmunogenic [3, 4]. Therefore, MSCs are one of the most promising cellular sources of stem cells that can be studied without using any immunosuppressive drugs, for both research and clinical purposes. Clinical studies using MSCs are widely available. For example, MSCs have been used in the therapy of diseases such as extended cartilage [5, 6] and osseous defects [7], intervertebral disc [8], acute myocardial infarction [9], leukemia [10], and diabetes [11]. We have started two clinical trials of intra-articular injection of autologous bone marrow MSCs for articular cartilage repair based on our previous animal experiments [5, 12, 13]. However, the functional quality of MSCs for cartilage regeneration might be diversified depending on the donor due to the heterogeneity of MSCs. There have been reports that differentiation and proliferation capacity decrease with age [14, 15] and, consequently, the use of autologous MSCs for tissue repair, which in some indications concerns elderly patients, has certain limits [16]. Quality evaluation confirming the properties of MSCs has been established and is based on cell surface markers (negative for CD14 or CD11b, CD19, CD34, CD45, CD79α, and HLA-DR and positive for CD73, CD90, CD105, CD166, and CD44 [17–19]) and

differentiation capacity [20–22]. However, quality evaluation based on the efficacy of MSCs has been not established. The evaluation criteria for quality of MSCs based on the efficacy would be required for the practical application of MSC transplantation. In this study, we aimed to devise a method for functional quality assessment of MSCs for cartilage regeneration by examining the relationships among the following data in human bone marrow MSCs (hMSCs): profiles of cartilage anabolic and catabolic factors, messenger RNAs (mRNAs), and microRNAs (miRNAs), and the capacity for cell proliferation, chondrogenic differentiation *in vivo*, and cartilage regeneration *in vivo*.

2. Materials and Methods

All procedures were approved and performed by the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

In this study, hMSCs were purchased from Lonza Walkersville Inc. (PT-2501, Walkersville, MD, USA). All these hMSCs passed the quality inspection conducted by Lonza company using cell viability (more than 75%), adipogenic and osteogenic differentiation (Oil Red O staining and calcium deposition staining), and FACS analysis of cell surface markers (more than 90% were positive for CD29, CD44, CD105, and CD166 and negative for CD14, CD34, and CD45). Assays of cell growth rate (GR) and colony formation (CF), pellet cultures *in vitro*, and transplantation of hMSCs into cartilage defect models *in vivo* were performed using hMSCs from five different donors. The age, race, and sex of the five different donors were as follows: 22-year-old black man, 20-year-old white man, 39-year-old black man, 29-year-old white woman, and 41-year-old white woman.

2.1. Cultures of hMSCs and Human Skin Fibroblasts. The hMSCs at passage two were centrifuged at 500g for 5 min. The cells were resuspended in culture medium composed of Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA, USA), 15% fetal bovine serum (FBS; SAFC Biosciences Inc., Irvine, CA, USA), 20 mmol/ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Life Technologies), 50 μg/ml gentamycin (Gentacin®, MSD, Tokyo, Japan), and 0.25 μg/ml amphotericin (Fungizon®, Bristol-Myers Squibb, New York, NY, USA). The suspension was then plated into culture dishes. The dishes were incubated in an atmosphere of 95% relative humidity and 5% CO_2 at 37°C until 70–80% confluency, and the cells were then harvested with Trypsin (TrypLE™ Express; Life Technologies, Carlsbad, CA, USA). After adding high-glucose DMEM® with 10% FBS and 1% antibiotics, the cells were neutralized and harvested by centrifugation at 200g for 5 min and the resulting supernatant frozen at −80°C with Cellbanker® 1 (LSI Medience, Tokyo, Japan) until further testing. The cells were defined as passage three (P3). The hMSCs at P3 were reseeded under high-glucose DMEM with 10% FBS and 1% antibiotics to grow the hMSCs. These adherent cells have been referred to as hMSCs at passage four (P4). The hMSCs at P4 were used in the current study. Adult normal human dermal fibroblasts (hFBs; Lonza Japan, Tokyo, Japan) at P2 were cultured with Fibroblast Cell Growth Medium-2 BulletKit (FGM™-2 BulletKit™; Lonza Japan, Tokyo, Japan) until P4 by a similar method and the cells at P4 were used.

2.2. Assay of Growth Rate for hMSCs. The hMSCs at P4 were seeded onto culture dishes at 5.0×10^3 cells/cm^2 in Mesenchymal Stem Cell Basal Medium (MSCBM™; Lonza Japan, Tokyo, Japan) and incubated in an atmosphere of 95% relative humidity and 5% CO_2 at 37°C. Cell division rate was assessed after 5 days.

2.3. Colony-Forming Assay. In a pilot study, we confirmed a positive correlation ($P < 0.001$, $R = 0.992$) between hMSC seeding density and capacity for CF. Because the CF measurement did not reflect the seeding density, the capacity for CF did not have an effect of the secretor factor and could measure quality of cells by CF measurement. The hMSCs at P4 were plated at 1.5×10^3 cells/T75 flask in MSCBM (Lonza Japan) for 14 days. The medium was changed at three- to four-day intervals. After embedding the plate in paraffin, the cells were stained by 1.0% crystal violet solution (Wako, Osaka, Japan) for 10 min. Aggregates of cells differentiated than 50 cells were counted as one colony, and we calculated the ratio of these colonies among all seeded cells.

2.4. Analysis of Protein Derived from Culture Supernatant. MSCs at 70–80% confluency were refed with culture media. After 48 h incubation, the media were collected. To remove debris, the media were centrifuged at 600g for 5 min and the supernatants were collected as MSC-conditioned media (MSC-CMs). They were stored at −80°C until they were used for the following assays.

The MSC-CMs were analyzed by enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA kits purchased from R&D Systems (Minneapolis, MN, USA) were used for bone morphogenetic protein- (BMP-) 2, BMP-7, fibroblast growth factor- (FGF-) 2, insulin-like growth factor- (IGF-) 1, transforming growth factor- (TGF-) α, TGF-β1, TGF-β2, tissue inhibitor of metalloproteinase- (TIMP-) 1, TIMP-2, platelet-derived growth factor- (PDGF-) AA, interleukin- (IL-) 2, IL-17, monocyte chemotactic protein- (MCP-) 1, matrix metalloproteinase- (MMP-) 1, MMP-3, MMP-9, MMP-13, RANTES, stromal cell-derived factor- (SDF-) 1α, macrophage inflammatory protein- (MIP-) 1α, MIP-1β, and MIP-3α. Kits from Life Technologies (Carlsbad, CA, USA) were used for hepatocyte growth factor (HGF), IL-1β, IL-4, IL-8, IL-10, TNF-α, interferon- (IFN-) γ, and vascular endothelial growth factor (VEGF). Assays were performed according to the manufacturer's instructions in duplicate. As a control, culture media were also analyzed. The hFBs at P4 were cultured at the same time and were compared with hMSCs as a control.

2.5. The Assessment of Chondrogenic Differentiation Using Pellet Culture for hMSCs. The capacity for chondrogenic differentiation of the hMSCs from each donor was evaluated using pellet culture.

A pellet culture system for chondrogenesis was used [23–26]. About 2.5×10^5 hMSCs at P4 were placed in a 15 ml polyethyleneterephthalate tube (Sumitomo Bakelite) and centrifuged at 450g for 10 min. The pellet was cultured at 37°C with 5% CO_2 in 500 μl of chondrogenic medium containing 500 ng/ml BMP-6 (27) (R&D Systems, Minneapolis, MN, USA) in addition to high-glucose DMEM supplemented with 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA), 10^{-7} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate (Sigma-Aldrich, St. Louis, MO, USA), and 50 mg/ml ITS+ Premix (Becton Dickinson; 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml BSA, and 5.35 mg/ml linoleic acid). The medium was replaced every 3 to 4 days for 21 days. For microscopy, the pellets were embedded in paraffin, cut into 5 μm sections, and stained with 0.05% toluidine blue solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The production of extracellular matrix was evaluated by measuring the percentage of metachromasy in pellets (PMP) derived from hMSCs stained with toluidine blue by image processing software (WinROOF®, Mitani, Tokyo, Japan). We calculated the PMP for all areas in pellets.

2.6. Real-Time Quantitative Polymerase Chain Reaction (qPCR) of Pellets. Total RNA was isolated from pellets using a Qiagen RNeasy Micro Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from RNA using Super Script VILO Master Mix (Life Technologies). As a control, total RNA was obtained from normal knee cartilage dissected from skeletally matured cadaveric donors (Articular Engineering, Northbrook, IL, USA). qPCR was performed using Power SYBR Green Master Mix (Life Technologies). cDNA samples were analyzed for both collagen type II (*COL II*) and the reference gene (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)). Assays were performed according to the manufacturer's instructions. The primer sequences used for the experiment are shown in Table 1. The amount of *COL II* mRNA expressed was normalized to *GAPDH* expression. In addition, the amount of mRNA expressed in hMSCs from donors (hMSC-1 to hMSC-5) was normalized to expression in hMSC-1 for the purpose of comparing the mRNA expressions among five donors.

2.7. The Assessment of Capacity for Cartilage Regeneration Using Cartilage Repair. In this study, male nude rats aged 9 to 10 weeks were used and were anesthetized with an intraperitoneal administration of 1.0 ml/kg sodium pentobarbital before surgery. The medial parapatellar approach was used to expose the knee joint. We created full thickness articular cartilage defects of 2 mm in diameter and 1 mm in depth at the patellar groove of the distal femur using a power drill, and the joint capsule and skin incision were closed with 6-0 nylon sutures. Rats were divided into two groups. In the control group, phosphate-buffered saline (PBS, 25 μl) was injected into the five operated joints ($n = 5$). This group indicated the natural course of healing of the osteochondral defect. In the hMSC group, 3×10^5 hMSCs from the five donors were injected into each operated joint ($n = 5$/donor).

Table 1: Primer sequences used for qPCR.

Gene	Primer sequence (5′→3′)
Type II collagen	GGCAATAGCAGGTTCACGTACA
	CGATAACAGTCTTGCCCCACTT
Type X collagen	CAAGGCACCATCTCCAGGAA
	AAAGGGTATTTGTGGCAGCATATT
Aggrecan	TACGAAGACGGCTTCCACCA
	CTCATCCTTGTCTCCATAGC
SOX9	GTACCCGCACTTGCACAAC
	GTAATCCGGGTGGTCCTTCT
CD44	AAGACACATTCCACCCCAGT
	GGTTGTGTTTGCTCCACCTT
GAPDH	ATGGGGAAGGTGAAGGTCG
	TAAAAGCAGCCCTGGTGACC

After transplantation, all nude rats were allowed to move freely in their cages.

2.8. Histological Evaluation. All nude rats were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital sodium at 12 weeks after the injection. The patellar groove was resected and fixed in 4% paraformaldehyde for 24 h. The specimens were then decalcified with 0.5 M ethylenediaminetetraacetic acid. After that, the specimens embedded in paraffin block were cut into 5 μm sections serially along the sagittal plane that included the center of the defect. For histological assessment, these sections were stained with Safranin-O/Fast green. The specimens were graded semiquantitatively. The grading scale was based on a histological grading scale for cartilage regeneration as previously described [27].

2.9. 3D-Gene® Human Oligo Chip 25k for mRNA and TaqMan® Low-Density Array for miRNA Expression Profiling. The hMSCs and hFBs at P4 were homogenized on plate using TRIzol Reagent (Life Technologies), and total RNAs were isolated according to the manufacturer's instructions. For mRNA microarray analysis, 3D-Gene Human Oligo chip 25k (3D-Gene; Toray Industries, Tokyo, Japan) was used (24,460 distinct genes). The gene expression of hFBs at P4 was used as a control for normalization. Experimental procedures for TaqMan low-density array analysis (TLDA®; Life Technologies) were performed using TaqMan Array Human microRNA Cards® (card A v2.0 and card B v3.0) according to the manufacturer's instructions to identify differentially expressed miRNAs in hMSCs of each donor.

2.10. Statistical Analysis. All assays were performed in triplicate. The results are shown as mean values and standard deviations. Pearson's correlation coefficient calculated using software of the statistic program for Windows by Statcel4® of Excel Statistics (Statcel4: Nebula Company, Bunkyo-ku, Tokyo, Japan) was used to evaluate associations among capacity for cell proliferation, chondrogenic differentiation, and cartilage regeneration, and expression pattern of proteins, mRNAs, and miRNAs in hMSCs. Multiple comparison was performed for the evaluation of histological scores of

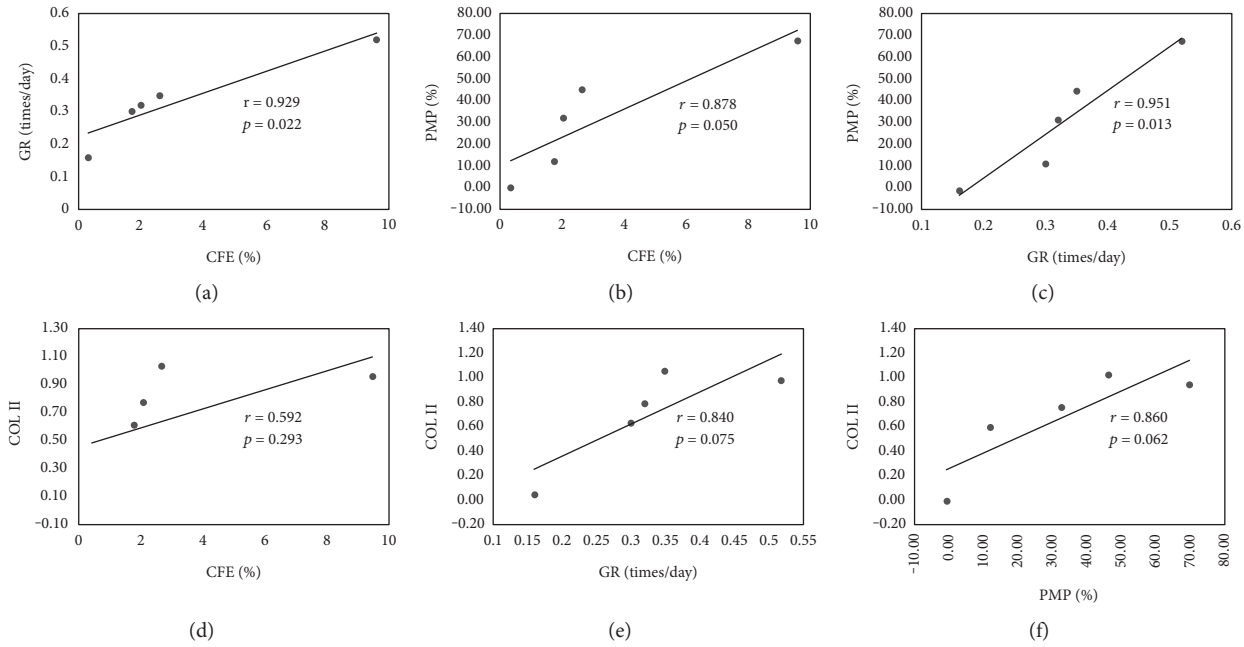

Figure 1: Correlations between (a) CFE and GR, (b) CFE and PMP, (c) GR and PMP, (d) CFE and COLII, (e) GR and COLII, and (f) PMP and COLII were $r = 0.929$ and $P = 0.022$, $r = 0.878$ and $P = 0.050$, $r = 0.951$ and $P = 0.013$, $r = 0.592$ and $P = 0.293$, $r = 0.840$ and $P = 0.075$, and $r = 0.860$ and $P = 0.062$, respectively.

specimens from the knee of nude rats between each group using Bartlett's test and one-way analysis of variance. When a significant p value was found, the Tukey-Kramer method was used to identify significant differences among the groups. The significance level was defined at $P < 0.05$ for all tests.

3. Results

3.1. Growth Rate and Colony Forming. The hMSCs from five different donors were ranked from hMSC-1 to hMSC-5 in order of GR, and the hMSC-1 to hMSC-5 were from a 22-year-old black man, 20-year-old white man, 39-year-old black man, 29-year-old white woman, and 41-year-old white women, respectively. GR and colony-forming efficiency (CFE) for hMSC-1 to hMSC-5 and hFBs were 0.52 times/day and 9.6%, 0.35 times/day and 2.6%, 0.32 times/day and 2.0%, 0.30 times/day and 1.7%, 0.16 times/day and 0.3%, and 0.73 times/day and 0.0%, respectively. CFE showed a positive correlation with GR ($r = 0.929$, $P = 0.022$) (Figure 1(a)).

3.2. Proteins Derived from Culture Supernatant. To evaluate the protein secretion from MSCs relating to cartilage repair, antianabolic and catabolic factors for cartilage in culture supernatant were chosen for assessment. In the assessment of protein expression using ELISA, the anabolic factors TIMP-1, TIMP-2, TGF-β1, TGF-β2, PDGF-AA, HGF, and IGF-1 and the catabolic factors IL6, IL8, SDF-1a, MMP13, VEGF, MCP-1, MMP1, and MMP-3 were detected in the culture supernatant for each donor (Table 2). On the other hand, we were not able to validate protein expression for BMP-2, BMP-7, IL-4, IL-10, FGF-2, IFNγ, IL1β, IL2, IL17, MMP9, RANTES, TNFα, MIP1α, MIP1β, MIP3α, and TGFα. The 15 factors out of 31 could be detected using ELISA.

3.3. Percentage of Metachromasy in Pellets. PMP of hMSC-1 to hMSC-5 were 69.3%, 46.16%, 32.8%, 12.4%, and 0.0%, respectively (Figure 2). The capacity for the production of extracellular matrix, demonstrating chondrogenic differentiation, was positively correlated with GR and CFE (GR: $r = 0.951$, $P = 0.013$; CFE: $r = 0.878$, $P = 0.050$) (Figures 1(b) and 1(c)).

3.4. Real-Time PCR Assays of Pellets. COLII gene expressions in hMSC-1 to hMSC-5 were 1.00, 1.08, 0.80, 0.63, and 0.00, respectively. Gene expression of *COL II* showed a correlative trend with CFE ($r = 0.592$, $P = 0.293$) and a correlation with GR ($r = 0.840$, $P = 0.075$) and PMP ($r = 0.860$, $P = 0.062$) (Figures 1(d), 1(e), and 1(f)). These findings indicate that chondrogenic capacity of hMSCs is positively correlated with proliferation capacity.

3.5. Histological Evaluation of Cartilage Repair. In the control group, the chondral defect area was not stained with Safranin-O. The mean histological score of the control samples was 12.40 ± 1.52 (SD) points. In hMSC-1, hMSC-2, and hMSC-5, the chondral defect area was partially stained with Safranin-O. However, in hMSC-3 and hMSC-4, the margins of the defect were irregular and the repair cartilage was composed of fibrous tissue. In hMSC-1 to hMSC-5 groups, the mean histological score was 4.4 ± 4.51 (SD), 4.6 ± 3.13 (SD), 6.0 ± 1.22 (SD), 6.2 ± 3.90 (SD), and 3.2 ± 2.28 (SD), respectively (Figure 3).

3.6. Gene Expression of hMSCs Assessed by 3D-Gene Human Oligo Chip 25k and TaqMan Low-Density Array. Of 24,460 mRNAs analyzed by 3D-Gene, we detected mRNA expressions of *MYBL1* (MYB proto-oncogene like 1) and *RCAN2*

Table 2: Secretional capacity of proteins per 10,000 hMSCs (pg/10,000 hMSCs).

	TIMP-1	TIMP-2	TGF-β1	TGF-β2	PDGF-AA	HGF	IGF-1	
hMSC-1	5841.6	2790.4	192.3	19.8	1.1	2.5	17.7	
hMSC-2	12129.6	3714.5	283.6	31.8	2.0	4.3	44.3	
hMSC-3	14791.6	4227.6	333.2	40.5	2.1	1.4	64.8	
hMSC-4	23319.9	4394.7	367.0	38.5	1.7	85.8	44.4	
hMSC-5	41621.9	7640.3	479.5	54.6	4.2	60.3	80.8	
hFB	5879.2	3176.6	128.9	11.6	0.5	0.0	27.4	
	IL6	IL-8	SDF-1a	MMP13	VEGF	MCP-1	MMP-1	MMP-3
hMSC-1	123.9	0.4	150.1	1.7	449.8	30.0	55.6	10.4
hMSC-2	355.8	1.4	301.8	2.4	513.7	54.8	7.5	8.7
hMSC-3	606.8	1.1	278.8	3.1	742.0	59.0	32.1	22.8
hMSC-4	314.7	5.0	378.6	16.1	789.3	133.0	42.8	12.6
hMSC-5	966.5	8.4	284.5	4.1	1253.7	322.0	99.3	75.5
hFB	58.4	1.6	380.5	0.5	49.5	81.5	1096.2	1279.9

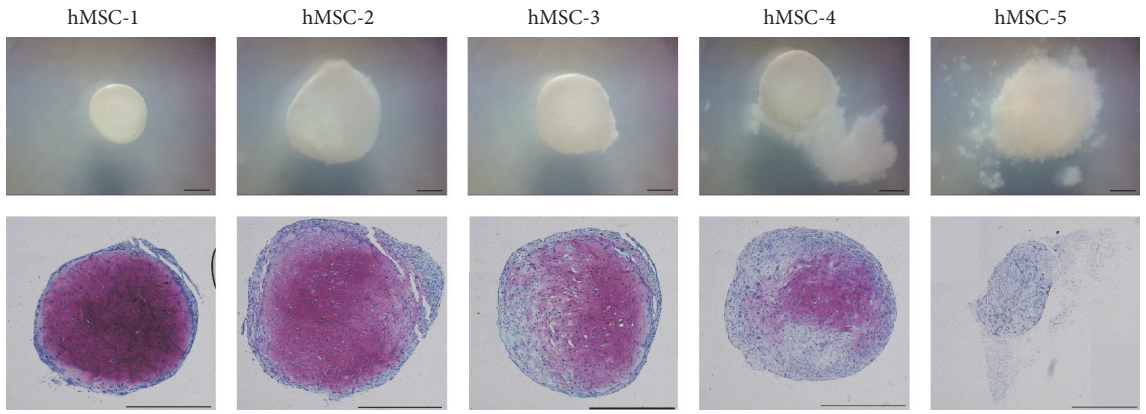

Figure 2: Percentages of metachromasy in pellets of hMSC-1 to hMSC-5 were 69.3%, 46.16%, 32.8%, 12.4%, and 0.0%, respectively. Scale bars, 500 μm.

(regulator of calcineurin 2) related to proliferation capacity *in vitro* and *HLA-DRB1* (major histocompatibility complex, class II, DR beta 1) and *TMEM155* (transmembrane protein 155) related to cartilage repair *in vivo* (Table 3). Of 768 miRNAs analyzed by TLDA, we detected miR-486-3p, miR-148b, miR-93, and miR-320B in cartilage repair *in vivo* (Table 4). The gene expression in hMSCs from each donor was evaluated for fold changes compared with the gene expression in hFBs as a control for normalization. In the assessment of genes that were not detected in hFBs, the lowest gene expression in hMSCs was used as a control for normalization.

3.7. Relation In Vitro. The hMSCs from the five donors were divided into two groups of hMSC-1 to hMSC-3 and hMSC-4 to hMSC-5 according to their results in chondrogenic differentiation. In the toluidine blue staining and RT-PCR assays of pellets from hMSCs, hMSC-1 to hMSC-3 showed rich, whereas hMSC-4 and hMSC-5 showed poor, production of extracellular matrix. In addition, hMSC-1 to hMSC-3 showed good capacity, whereas those of hMSC-4 and hMSC-5 showed poor capacity, for cell proliferation. The mRNAs and miRNAs that could be divided into two groups of hMSC-1 to hMSC-3 and hMSC-4 to hMSC-5 according to their results in chondrogenic differentiation were selected and assessed for their correlation with chondrogenic differentiation.

In the assessment of mRNAs and miRNAs, the expression of *MYBL1* was higher and that of *RCAN2* lower in hMSCs from the three donors with good cell proliferation and production of extracellular matrix than in hMSCs from the other two donors. In addition, protein expression levels of both TIMP-1 and VEGF were negatively correlated with cell proliferation and production of extracellular matrix (Table 5).

3.8. Relation In Vivo. The hMSCs from the five donors were divided into two groups of hMSC-1, hMSC-2, and hMSC-5 and hMSC-3 to hMSC-4 according to their results in evaluation of cartilage regeneration using Wakitani's scales. The mRNAs and miRNAs that could be divided into two groups of hMSC-1, hMSC-2, and hMSC-5 and hMSC-3 to hMSC-4 according to their results in cartilage regeneration were selected and assessed for their correlation with cartilage regeneration.

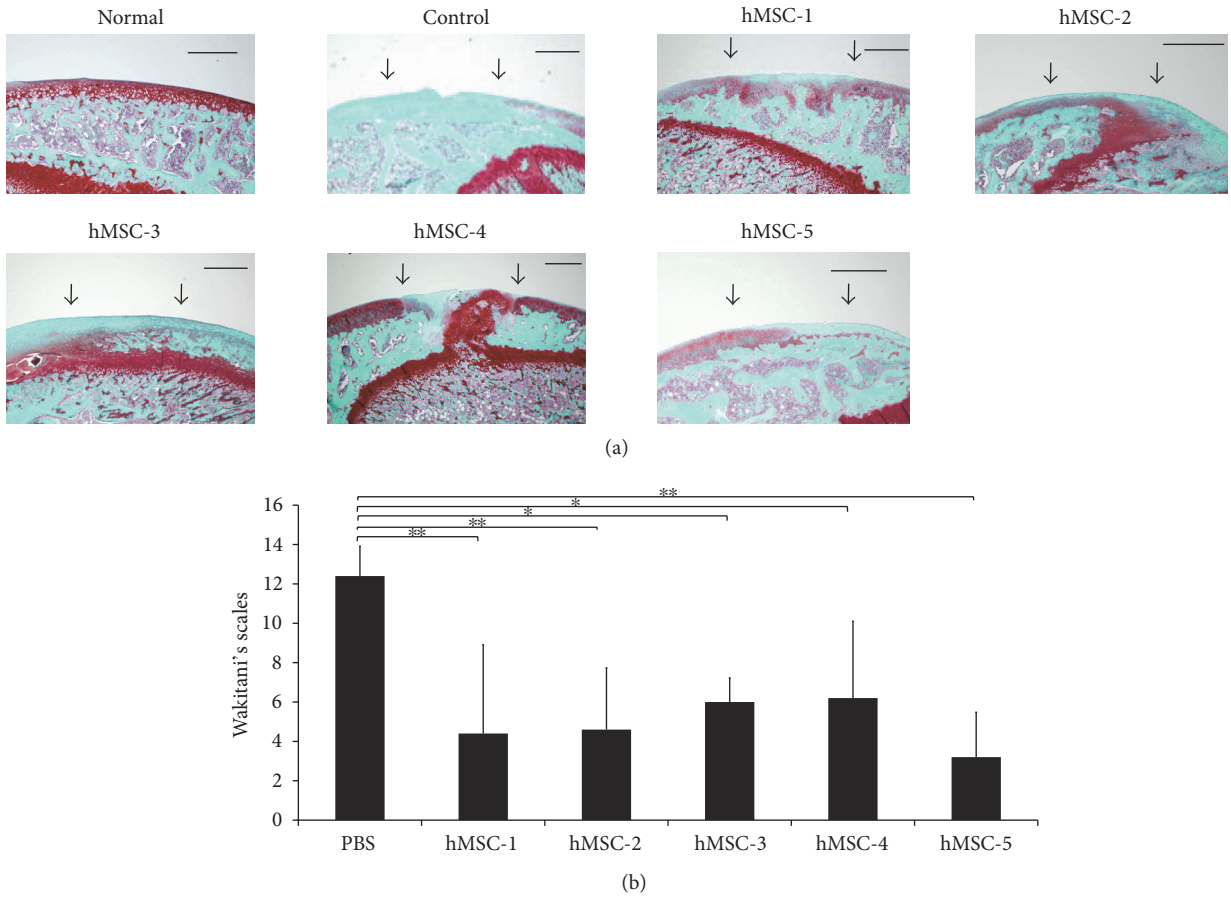

Figure 3: (a) Histological findings with Safranin-O/Fast green staining at 12 weeks after injection of hMSCs into the cartilage defect models. Scale bars, 500 μm. (b) Assessment of the five different donors using Wakitani's scales ($^{**}P < 0.01$, $^{*}P < 0.05$).

Table 3: Changes in mRNA expression in hMSCs.

	Gene	hMSC-1	hMSC-2	hMSC-3	hMSC-4	hMSC-5
In vitro	MYBL1	5.44	4.38	4.76	3.33	2.97
	RCAN2	0.07	0.07	0.08	0.14	0.15
In vivo	HLA-DRB1	0.07	0.15	0.28	0.45	0.03
	TMEM155	4.25	4.16	3.37	2.03	4.86

Fold changes.

Table 4: Changes in miRNA expression in hMSCs.

	Gene	hMSC-1	hMSC-2	hMSC-3	hMSC-4	hMSC-5
In vivo	miR-486-3p	2.12	1.55	0.88	1.00	3.11
	miR-148b	2.37	3.85	1.00	1.98	6.57
	miR-93	4.16	4.27	3.60	2.76	4.55
	miR-320B	2.34	3.46	1.99	0.89	4.53

Fold changes.

The expressions of *MYBL1*, *RCAN2*, TIMP-1, and VEGF showing relevance with cell proliferation and chondrogenic differentiation had no correlation with cartilage repair *in vivo*. On the other hand, the expression of *HLA-DRB1* was higher and that of *TMEM155*, miR-486-3p, miR-148b, miR-93, and miR-320b lower in hMSCs from the three donors that showed good cartilage repair *in vivo* than in hMSCs from the other two donors (Table 6). However, protein expression levels of anabolic and catabolic factors for cartilage were not correlated with cartilage repair *in vivo*.

Table 5: Correlation analysis of specific factors *in vitro*.

mRNA		CFE	GR	PMP
MYBL1	*P*	0.098	0.042*	0.014*
	r	0.808	0.891	0.947
RCAN2	*P*	0.271	0.122	0.032*
	r	−0.614	−0.777	−0.909
Protein				
TIMP1	*P*	0.164	0.026*	0.025*
	r	−0.727	−0.923	−0.923
VEGF	*P*	0.179	0.030*	0.034*
	r	−0.711	−0.914	−0.906

P = probability, *r* = correlation coefficient, $^*P < 0.05$.

Table 6: Correlation analysis of specific factors *in vivo*.

mRNA and miRNA		CFE	GR	PMP	Wakitani
HLA-DRB1	*P*	0.605	0.918	0.666	0.024*
	r	−0.315	−0.065	−0.265	0.726
TMEM155	*P*	0.779	0.937	0.763	0.031*
	r	0.175	−0.050	0.187	−0.911
miR-486-3p	*P*	0.932	0.645	0.766	0.008*
	r	0.054	−0.282	−0.185	−0.966
miR-148b	*P*	0.567	0.301	0.444	0.045*
	r	−0.347	−0.584	0.453	−0.887
miR-93	*P*	0.799	0.950	0.735	0.037*
	r	0.158	−0.039	0.209	−0.900
miR-320B	*P*	0.725	0.490	0.784	0.032*
	r	−0.218	−0.413	−0.171	−0.910

P = probability, *r* = correlation coefficient, $^*P < 0.05$.

Finally, there was no correlation between the cell proliferation and production of extracellular matrix *in vitro* and the cartilage repair *in vivo*.

4. Discussion

This study demonstrated that the mRNA expression of *MYBL1* was positively correlated with proliferation and cartilage differentiation of hMSCs and that the mRNA expression of *RCAN2* and the protein expressions of TIMP-1 and VEGF were negatively correlated with proliferation and cartilage differentiation of MSCs. However, we also showed that MSCs from all five donors had the capacity to promote cartilage repair *in vivo* regardless of their capacity for proliferation and cartilage differentiation. The mRNA expression of *HLA-DRB1* was positively correlated with cartilage repair *in vivo*, and the mRNA expression of *TMEM155* and expressions of miR-486-3p, miR-148b, miR-93, and miR-320b were negatively correlated with cartilage repair.

In vitro, the capacity for chondrogenic differentiation, which is an index of extracellular matrix production, was high in cells with high proliferation, as indicated by CFE and the GR. However, we found that the cell proliferation and the chondrogenic differentiation cannot be used for the quality assessment of the MSCs based on the efficacy of cartilage repair *in vivo*. Thus, CFE, the gene expressions of *MYBL1* and *RCAN2*, and the protein expressions of TIMP-1 and VEGF can be used for the quality assessment of the MSCs based on the capacity of proliferation and chondrogenic regeneration, but not for the quality assessment of the MSCs based on the efficacy of cartilage repair. Previous studies reported that the tissue regeneration promoted by MSC transplantation might be mediated predominantly through the indirect paracrine mechanisms rather than the direct regeneration from transplanted MSCs [28–31]. Our previous study of intra-articular injection of green fluorescent protein (GFP) expressing rat MSCs into a rat cartilage defect model also showed that GFP-positive cell could be observed at the injured site at four weeks after the treatment but could not be detected in the posttreatment specimens at eight and 12 weeks [5]. This might be the reason for discrepancy between the *in vitro* chondrogenic capacity of MSCs and the cartilage repair *in vivo*. On the other hand, the expression levels of *HLA-DRB1*, *TMEM155*, miR-486-3p, miR-148b, miR-93, and miR-320b might be used for the quality assessment of MSCs based on the efficacy of cartilage repair. *HLA-DRB1* is part of a family of genes called the human leukocyte antigen (HLA) complex that has a critical role in the immune system. *HLA-DRB1* was reported to have participated in the pathopoiesis of rheumatoid arthritis [32, 33]. However, the function of *HLA-DRB1* in MSCs has not been reported. The function of TMEM in MSCs is also unknown. MiR-93 has been implicated in multiple cell processes, including proliferation, apoptosis, invasion, and extracellular matrix degradation [34–37]. Jing and Jiang reported that miR-93 is lower in human degenerative nucleus pulposus tissues and that its level is associated with disc degeneration grade. In addition, overexpression of miR-93 increases expression of type II collagen by targeting MMP3 and might thereby promote cartilage repair [38]. On the other hand, the functions of miR-486-3p, miR-148b, and miR-320b relating to MSCs or cartilage have not been previously studied. We found expression of specific mRNAs and miRNAs in hMSCs to be related to the capacity for cartilage regeneration. These genes might be used for the quality assessment of hMSCs before their use in treatment for cartilage repair.

In this study, the cartilage repair *in vivo* after MSC transplantation was incomplete. The xenograft of human MSCs to nude rats might be the reason of insufficient repair of articular cartilage. Another possible reason for insufficient repair of articular cartilage is the use of purchased hMSCs. The commercialized hMSCs were extremely expanded and frozen. This might have an undesirable influence on the quality of hMSCs for cartilage repair. In the next step, the qualities of hMSCs from the patients who take part in clinical trials should be assessed in the same way.

5. Conclusions

The cell proliferation and chondrogenic differentiation of hMSCs *in vitro* have no correlation with cartilage

regeneration *in vivo*. On the other hand, we found expression of *HLA-DRB1*, *TMEM155*, miR-486-3p, miR-148b, miR-93, and miR-320B in hMSCs to be related to the capacity for cartilage regeneration. These factors might be useful for the quality assessment of hMSCs before their use in treatment for cartilage repair.

Disclosure

An earlier version of this work was presented as a poster at ORS 2017 Annual Meeting, March 19–22, 2017.

Conflicts of Interest

The authors declare no competing interests.

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Research Article

IGF-1 Gene Transfer to Human Synovial MSCs Promotes Their Chondrogenic Differentiation Potential without Induction of the Hypertrophic Phenotype

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Mesenchymal stem cell- (MSC-) based therapy is a promising treatment for cartilage. However, repair tissue in general fails to regenerate an original hyaline-like tissue. In this study, we focused on increasing the expression levels for insulin-like growth factor-1 (IGF-1) to improve repair tissue quality. The IGF-1 gene was introduced into human synovial MSCs with a lentiviral vector and examined the levels of gene expression and morphological status of MSCs under chondrogenic differentiation condition using pellet cultures. The size of the pellets derived from IGF-1-MSCs were significantly larger than those of the control group. The abundance of glycosaminoglycan (GAG) was also significantly higher in the IGF-1-MSC group. The histology of the IGF-1-induced pellets demonstrated similarities to hyaline cartilage without exhibiting features of a hypertrophic chondrocyte phenotype. Expression levels for the Col2A1 gene and protein were significantly higher in the IGF-1 pellets than in the control pellets, but expression levels for Col10, MMP-13, ALP, and Osterix were not higher. Thus, IGF-1 gene transfer to human synovial MSCs led to an improved chondrogenic differentiation capacity without the detectable induction of a hypertrophic or osteogenic phenotype.

1. Introduction

Articular cartilage (AC) has limited self-repair capabilities due in part to poor vascularity, no lymphatic system, and no innervation. In addition, AC is rich in a hyaline-like extracellular matrix abundant in type II collagen, glycosaminoglycan, and water, a composition which can interfere with rapid cellular migration and repopulation and thus negatively affect tissue repair processes. In order to compensate for such disadvantages, a variety of approaches have been investigated to improve cartilage healing [1–7]. Among them, stem cell therapy could be a promising option to facilitate regenerative repair. Mesenchymal stem cells (MSCs) have the capability to differentiate into a variety of connective tissue cells including bone, cartilage, tendon, muscle, and adipose tissue [8]. These MSCs may be readily isolated from many sources such as the bone marrow, skeletal muscle, synovial membranes, adipose tissue, and umbilical cord blood [9–16]. Specifically, MSCs isolated from synovial membrane may be well suited for cell-based therapies for cartilage repair because of the relative ease of their harvest and their strong capability for chondrogenic differentiation

[14, 17, 18]. Recent implantation studies of synovial MSC (Syn-MSC) have shown successful repair of cartilage defects [18–21]. However, detailed observation revealed that in most cases, the repair tissues differed from normal hyaline cartilaginous tissue with contamination of some fibrocartilaginous or fibrous tissues [22]. Such findings suggest a need to further improve the quality of the repair tissue towards more complete regeneration.

One of the options to address the need for improvements could be biological manipulation of the differentiation capacity of MSC. Insulin-like growth factor (IGF-1) is known as one of the important growth factors that can regulate the chondrogenic potential of cells and chondrocyte status. Previous reports suggested that IGF-1 could play important roles in cartilage repair [23]. IGF-1 promotes the proliferation of MSCs [24] and also leads to increased production of aggrecan and type 2 collagen and thus contributes to maintaining the phenotype of chondrocytes [23, 25]. A recent study revealed that transducing the IGF-1 gene into chondrocytes contributed to improved cartilage repair with more intense staining for safranin-O and the maintenance of a three-layer structure as compared with the nontransduced control chondrocytes [26]. Another study demonstrated that IGF-1 promoted the chondrogenic differentiation capacity of bone marrow-derived MSCs, but in that study, IGF-1 also stimulated expression of a hypertrophic phenotype along with elevated osteogenic markers, suggesting the promotion of an osteogenic phenotype in this bone marrow-derived MSC population as well [27].

Since Syn-MSCs exhibit an inferior osteogenic differentiation capacity compared to bone marrow-derived MSCs [28, 29], the above discussed results could be source specific. To address this issue, it is of interest to assess the impact of enhanced IGF-1 expression on the biological phenotype of Syn-MSCs. In the present study, the IGF-1 gene was transferred into human Syn-MSCs and the influence of elevated expression of IGF-1 on their chondrogenic differentiation capacity was assessed.

2. Materials and Methods

2.1. Cell Culture. Human synovial-derived mesenchymal stem cells (hSyn-MSCs) were isolated and expanded as previously reported [30]. Briefly, synovium was obtained from 5 human donors (age = 17–35 years old; 2 females and 3 males) at the time of arthroscopic surgery of the knee (ex., ACL reconstruction, meniscus repair, or synovectomy). And they were free from any diagnosed diseases (Table 1). Before surgery, the patients received an explanation of the study, and consent was provided in accordance with a protocol approved by the Institutional Ethics Committee of Osaka University School of Medicine and affiliated hospital.

The synovial specimens were minced and digested with 0.2% collagenase (Collagenase-AOF Type A; Worthington Biochemical, Lakewood, NJ) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Rockville, MD) for 2 hours in a shaking water bath at 37.0°C. The cells were collected by centrifugation and then cultured in a DMEM growth medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin (Sigma-Aldrich), with the medium replaced twice per week. These cells were defined as synovium-derived MSCs. When the cells had reached confluence, they were then washed with sterile phosphate-buffered saline (PBS), detached with trypsin-EDTA (0.25% trypsin + 1 mM EDTA; Gibco BRL, Life Technologies), and replated at a density of $3.0 \times 10E3$ cells/cm^2. Cell passages were continued in the same manner when cells reached confluence. Cells at passages 3–5 were used in the present studies.

Table 1: Donor information of synovial mesenchymal stem cells.

Patient no.	Age	Gender	Surgery	Comorbid diagnosis
1	17	Male	Synovectomy	None
2	22	Male	ACLR	None
3	29	Female	ACLR	None
4	33	Male	ACLR + MR	None
5	35	Female	ACLR	None

ACRL: anterior cruciate ligament reconstruction; MR: meniscal repair.

2.2. Construction of Lentiviral Vector. The plasmids used previously by Madry et al. [31] were initially provided for the current studies. The IGF-1 cDNA fragment was released from the plasmid and recombined with the SIN type lentiviral vector plasmid pLVSIN-IRES-ZsGreen1 (TAKARA, Shiga, Japan). The IGF-1-expressing lentiviral plasmid pLVSIN-IRES-IGF-1 ZsGreen and control plasmid pLVSIN-IRES-ZsGreeen1 stocks were generated by transfection of Lenti-X 293T cells (TAKARA). The Lenti-X HTX Packaging System (Clontech Laboratories, CA) was used. Lenti-X 293T cells were plated in DMEM with 10% dialyzed FBC (TAKARA) at a concentration of $1 \times 10E6$ cells per 100 mm diameter dish and then transfected the following day with 7 g of the plasmid. Supernatants of these transfected cells were collected on days 2 and 3 posttransfection and titrated using a Lenti-X-PCR Titration Kit (Clontech).

2.3. Lentiviral Vector. Syn-MSCs were cultured on retronectin (recombinant human fibronectin fragment (TAKARA BIO INC., Kusatsu, Shiga, Japan)) and coated on 6-well plates. The plates of cells and aliquots of the lentiviral stock were centrifuged at 1000 ×g for 2 hours at 32°C following retronectin-binding infection methods (MOI = 50). At 12 hours postviral infection, the medium was changed to the growth medium. After induction for 72 hours, the Syn-MSCs were subjected to triple lineage cell differentiation capability assays. The lentiviral-mediated gene induction in the cells was assessed by expression of mRNA transcript levels (RT-qPCR) and protein levels (Western blotting) for IGF-1.

2.4. Chondrogenic Differentiation. Chondrogenic differentiation of the MSCs was induced using a pellet-culture method. Briefly, the cells were cultured in polypropylene tubes at a density of $1 \times 10E5$ cells/tube in chondrogenic medium

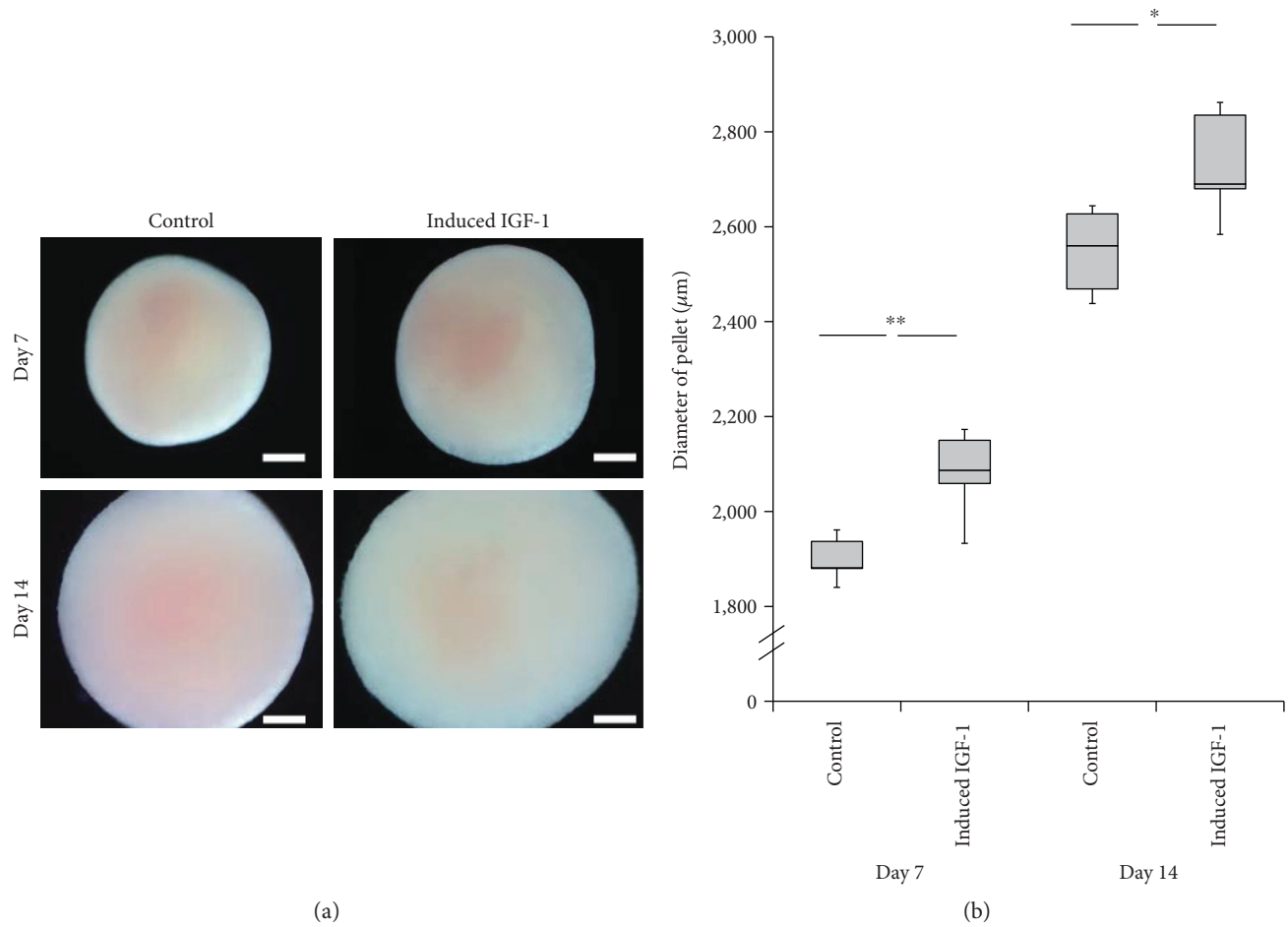

Figure 1: Diameters of the pellet cultures after 7 and 14 days of chondrogenic differentiation. (a) Microscopic findings: control (left) versus IGF-1 gene transferred cell-derived pellets (right). Showing results after 7 days (upper) and 14 days (lower). Scale bars = 500 μm. (b) Graph showing the diameter of the pellet cultures after 7 and 14 days. Vertical axis: diameters (μm); horizontal axis: the control (left) versus induced IGF-1 (right). Statistical analysis using independent samples t-test, $^{**}p < 0.01$, $^{*}p < 0.05$.

containing 1% insulin-transferrin-selenium supplement (ITS + Premix; Corning Inc., NY), 0.2 mM Asc-2P (Sigma-Aldrich), 50 ng/mL recombinant human BMP-2 (Osteopharma Inc., Osaka, Japan), and 2.5 ng/mL recombinant human TGF-beta3 (Peprotech, Rocky Hill, NJ). The chondrogenic medium was changed three times a week. To evaluate chondrogenic differentiation, the size of pellets was assessed, glycosaminoglycan (GAG) content determined, gene expression levels assessed, and histochemical assays performed. The size of pellets was measured using the transverse and longitudinal diameters of pellets under microscopic image processing software (Olympus Microscope, Nikon Image Plus, Japan) on days 7 and 14 after induction of differentiation. Glycosaminoglycan content was quantified using a Blyscan sulfated GAG assay kit (Biocolor Ltd., Carrick Fergus, UK). For histological and immunohistochemical assay, the pellets were fixed with 4% paraformaldehyde and embedding in paraffin. Approximately 5 μm sections of the pellets were stained with safranin-O fast green or toluidine blue dye to detect sulfated GAGs. Proteinase K (DAKO, CA)-treated sections were probed with an anti-human collagen II (F-57, Kyowa Pharmachemical Co., Takaoka, Japan) or a collagen II antibody (eBioscience, San Diego, CA).

To examine gene expression levels in the pellets, total RNA was extracted with TRIzol (Life Technologies, MD), and complementary DNAs (cDNA) were synthesized with SuperScript VILO (Life Technologies). The mRNA expression levels for type II collagen alpha1 (Col2a1; Hs 00264051_m1) and Sox9 (Hs1001343_g1) genes were assessed by TaqMan real-time polymerase chain reaction (qRT-PCR, Applied Biosystems, Carlsbad, CA) using a StepOnePlus Real-Time PCR instrument (Applied Biosystems). The expression levels for type X collagen (Col10; Hs00166657_m1) and matrix metalloproteinase-13 (m-13; Hs00233992_m1) as hypertrophic chondrocyte markers and alkaline phosphatase (ALP; Hs03046558_s1) and Osterix (Hs01822874_m1) as osteogenic markers were evaluated by RT-qPCR. The values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs02758891_g1) mRNA levels as a house keeping gene and an internal control.

2.5. Osteogenic and Adipogenic Differentiation. Osteogenic differentiation was induced when the cells were at

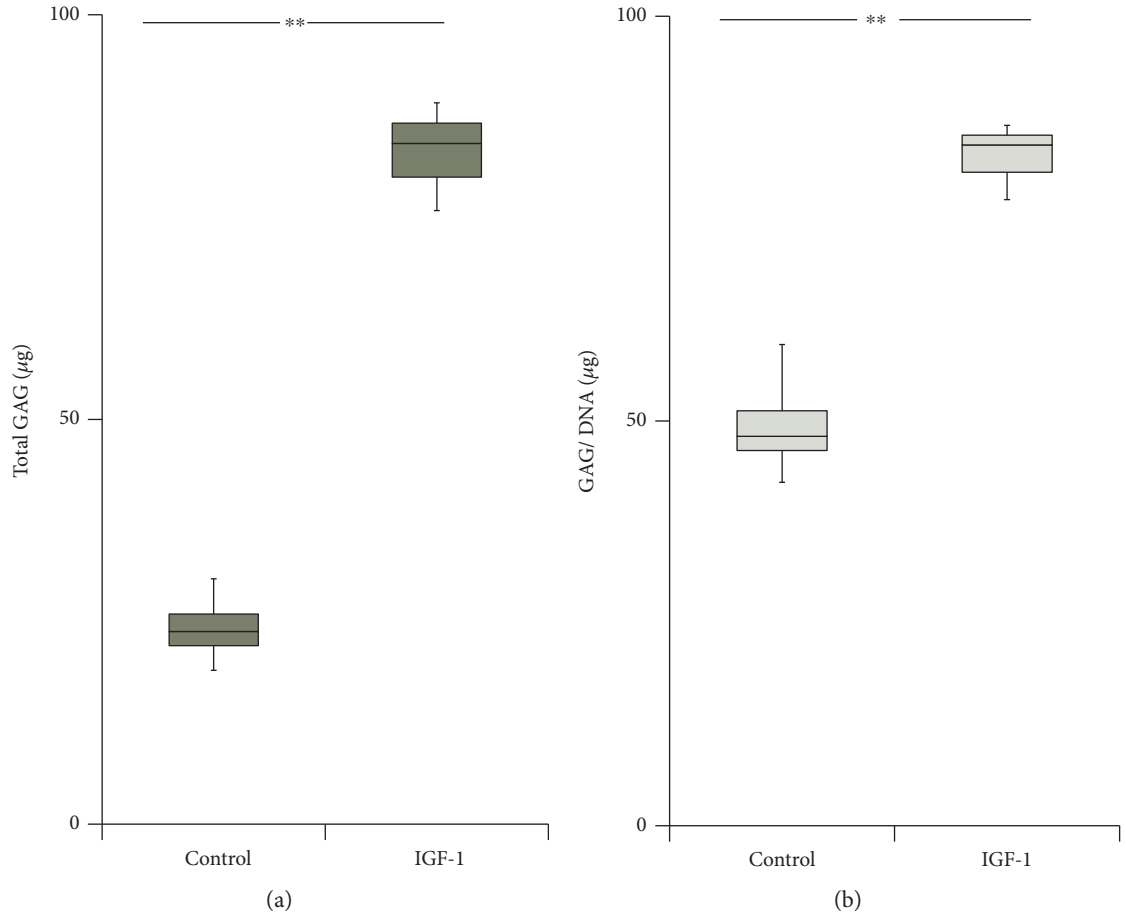

Figure 2: Gross and DNA-corrected glycosaminoglycan (GAG) content of pellet cultures (in μg). The pellets were analyzed for GAG after 14 days in the differentiation medium using a Blyscan sulfated GAG assay kit. Control values (left) versus IGF-1 gene transfer values (right). (a) Box plot of gross GAG content of the pellet cultures. (b) Box plot of DNA-corrected GAG content. $^{**}p < 0.01$.

approximately 80% confluency using a commercially osteogenic medium (StemPro Osteogenesis Differentiation Kit, Life Technologies GmbH, Darmstadt, Germany). The medium was changed three times a week. After one week of differentiation culture, the cells were evaluated for osteogenic differentiation by detection of ALP expression using an ALP staining method (BCIP/NBT Color Development Substrate, Promega). After three weeks, the cells were again evaluated for osteogenic differentiation using alizarin red staining (Muto Pure Chemicals Co., Tokyo, Japan). Quantitation of alizarin red staining was performed using the absorbance at 415 nm of the solution eluted from stained cells. Adipogenic differentiation induction of the cells was initiated when they had proliferated and were overconfluent using a commercially available adipogenic differentiation medium (StemPro Adipogenic Differentiation Kit, Life Technologies GmbH). After three weeks of differentiation, the cells were stained with oil red solution (0.5% Oil-Red, Sigma-Aldrich) to evaluate the lipid content of the cells as an indicator of adipogenic capability.

2.6. Statistical Analysis. Each in vitro experiment was repeated at least three times independently using different donor cell sources. The data were subjected to an independent samples *t*-test. The results are presented as mean ± standard deviation (SD) for triplicate determinations. For assessment of statistical difference, p values less than 0.05 were considered significant. All data analysis was performed using the statistical software "EZR" (Easy R) which is based on the R and R commander [32].

3. Results

3.1. Effect of IGF-1 Gene Transfer on Chondrogenic Differentiation. Previous research has demonstrated that the size of the pellet cultures is a good assessment parameter to evaluate achievement of chondrogenesis using pellet-culture differentiation methods [33]. Accordingly, we measured the diameters of the pellet developed from the pLVSIN-IGF-1-transfected MSCs. The mean diameters were 2080 ± 94.4 mm on day 7 and 2730 ± 116.0 mm on day 14. These values were significantly larger than those of the control group at 7 (1899 ± 47.8 mm ($p < 0.01$)) and 14 days (2548 ± 91.5 mm ($p < 0.05$)) (Figures 1(a) and 1(b)).

Likewise, glycosaminoglycan (GAG) content was significantly higher by 3.6-fold ($p < 0.01$) in the IGF-1-induced cell pellets than in the control pellets at day 14 (Figure 2(a)). After normalizing to the DNA content, the GAG

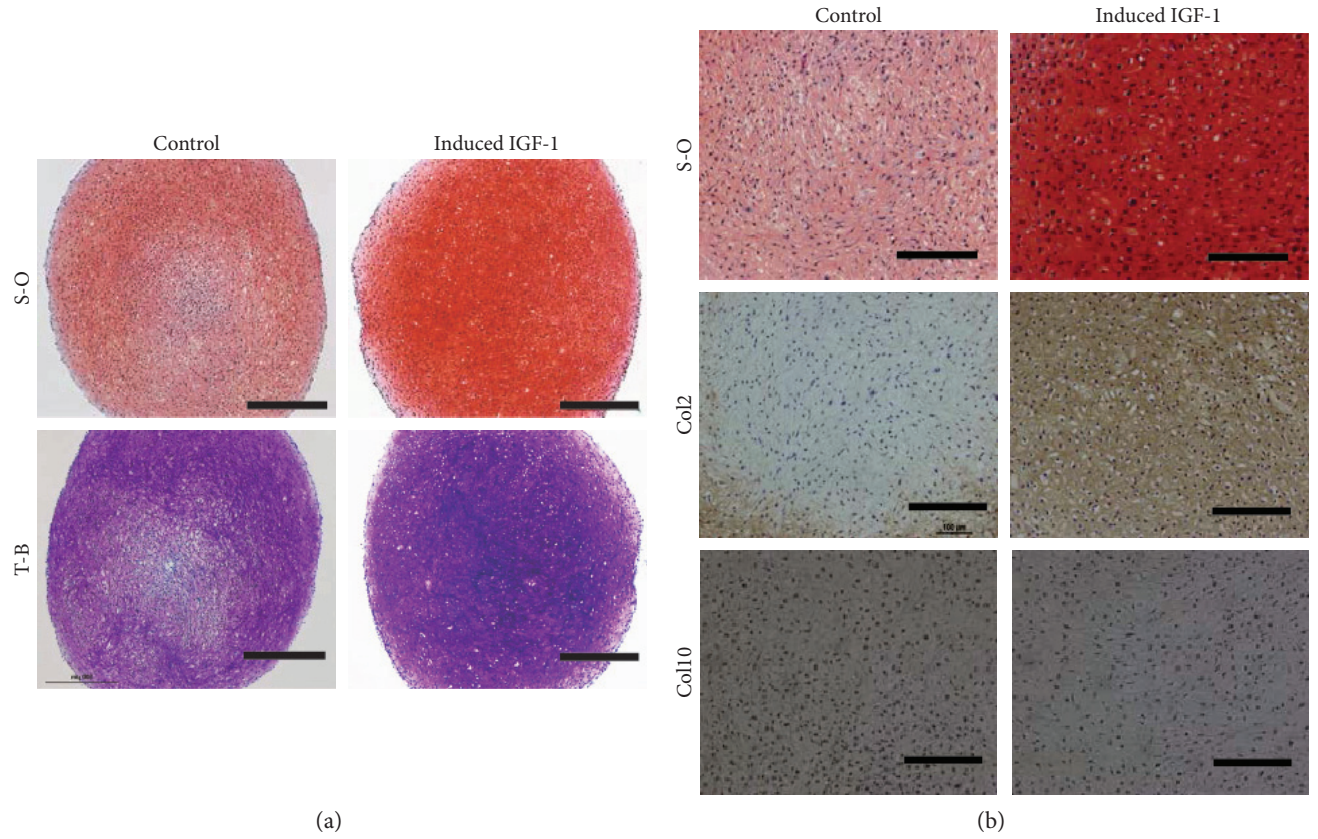

Figure 3: The results of histology and immunostaining assessments. (a) S-O; safranin-O (upper lane), T-B; toluidine blue (lower lane). Control (left) versus IGF-1 gene transferred cell-derived pellets (right). Scale bars = 500 μm. (b) S-O; safranin-O (upper lane), Col2; type II collagen (middle), and Col10; type X collagen (lower) immunostaining. Control (left) versus IGF-1 gene transfer (right). Scale bars = 200 μm.

content in the IGF-1-transduced group was still significantly (1.7-fold) higher ($p < 0.01$) compared with values for the control group (Figure 2(b)).

Although all the pellets showed positive staining for safranin-O and toluidine blue regardless of IGF-1 introduction, the staining intensity was higher in samples from the IGF-1-transduced group than in those of the control group (Figure 3(a)). Thus, histology of the IGF-1-induced pellets exhibited more similarities to those of normal, hyaline cartilage. Notably, there were no apparent increases in the presence of hypertrophic chondrocytes with large and round shapes in the IGF-1 transduced pellets as compared with the control pellets (Figure 3(b) upper lane). In other donor samples, similar results were obtained (Supplementary Figure 2A, 2B available online at https://doi.org/10.1155/2017/5804147).

The results of immunostaining revealed (Figure 3(b)) more intense staining for type II collagen within the IGF-1-transduced pellets than in the control pellets. Conversely, staining for type X collagen, a marker for the hypertrophic phenotype was negligible in both groups, suggesting the presence of hypertrophic chondrocytes with in the pellets of both groups was minimal (Figure 3(b)).

We also assessed RNA from IGF-1-transduced cells and control cells for mRNA transcript levels for the chondrogenic marker gene type II collagen alpha1 (Col2A1), as well as the hypertrophic marker type X collagen (Col10), matrix metalloproteinase 13 (MMP-13), and the osteogenic marker genes ALP and Osterix. Expression levels for the chondrogenic marker gene Col2A1 were significantly higher (3.1-fold) in the IGF-1-transduced group compared with those in the control group ($p < 0.05$). In contrast, mRNA expression levels for the hypertrophic chondrocyte marker genes (ColX and MMP-13), and the osteogenic marker genes (ALP and Osterix) were not significantly higher in the pellets from the IGF-1-transduced cells than those from the control pellets (Figure 4).

3.2. Osteogenic and Adipogenic Differentiation of IGF-1-Transduced Syn-MSCs. The effect of IGF-1 introduction into Syn-MSCs on osteogenic and adipogenic lineage differentiation capacity was also investigated. There were no detectable differences in ALP staining at 7 days between the IGF-1 group samples and those for the control group (Figure 5(a)). After 21 days of osteogenic differentiation, both groups exhibited similar staining with alizarin red (Figure 5(a)), and quantitated values for both groups were not significantly different (Figure 5(b)).

After 21 days of exposure to adipogenic differentiation culture conditions, detection of lipid droplets associated with adipogenic differentiation was observed to be similar in both groups (Figure 5(c)).

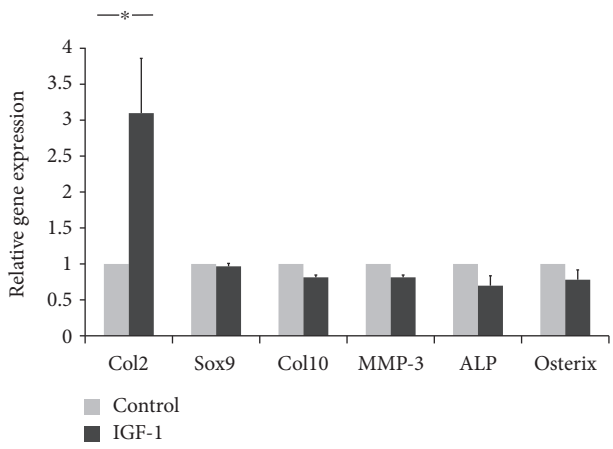

Figure 4: Specific gene expression levels in MSCs under chondrogenic conditions. Gene expression analyses of human synovial MSCs overexpressing IGF-1 after induction of chondrogenic differentiation were performed using real-time RT-PCR. The MSCs were transduced with a pLVSIN-IRES-ZsGreen1 (control; left) or the pLVSIN-IRES-IGF-1 ZsGreen vector (IGF-1; right) and cultured in a chondrogenic differentiation medium for 14 days. The values for each gene were normalized to the GAPDH house keeping gene levels used as the internal control. Ct values were generated for each target gene. *Statistically significant compared with control values ($p < 0.05$). GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Col2 (type II collagen alpha1) and Sox9 were assessed as chondrogenic markers. Col10 (type X collagen) and MMP-13 (matrix metalloproteinase 13) were assessed as hypertrophic chondrocyte markers. ALP (alkaline phosphatase) and Osterix were assessed as osteogenic markers.

The above described results are a representative of similar findings obtained with three of the donors of Syn-MSCs. Other donors were used to confirm only parts of the complete characterization. The control group, which was transduced with the pLVSIN-IRES-ZsGreen1 plasmid lacking the IGF-1 insert was used for cell proliferation assays and found to not differ from the properties of nontransduced cells (Supplementary Figure 3). Therefore, the nontransduced cells were defined as the control group for all subsequent assessments.

4. Discussion

The present study has demonstrated that lentiviral vector-mediated IGF-1 gene transfer specifically enhances the chondrogenic differentiation capacity of human Syn-MSCs and does so without any detectable impact on osteogenesis and adipogenesis differentiation of these cells.

The therapeutic use of MSCs for cartilage repair has been advocated to overcome the limitations of autologous chondrocyte implantation approaches, such as issues related to the dedifferentiation of chondrocytes during expansion culturing and the impact of removal of undamaged cartilage from the same joint. In their review, Filardo et al. [9] indicated that one-half of the 60 clinical trials which were using MSCs for cartilage repair were reported in the last 3 years. However, it has also been revealed that MSC-based therapies have some limitations regarding the quality of the repair tissue due to the contamination with fibrocartilaginous tissue [22]. Such incompletely regenerated tissue was shown to have inferior biomechanical properties as compared with normal cartilage [22]. Therefore, it is necessary to improve the repair quality of MSC-based therapies in order to move towards tissue regeneration. A number of approaches have been investigated for this purpose. Along with the alternative use of pluripotent stem cells such as embryonic stem cells or induced pluripotent stem (iPS) cells as the cell source [34–39], manipulation of the biological phenotype of MSC could be a one potential direction out of several that include inclusion exertion of low oxygen tension during cell culture [40], application of exogenous growth factors or cytokines [41–43], and gene transfer of specific target molecules [44, 45].

We hypothesized that IGF-1 gene transfer could improve the chondrogenic capacity of human Syn-MSCs, analogous to the study by Madry et al. [26], where the IGF-1 gene was introduced into bovine chondrocytes. To enhance the efficiency of IGF-1 gene introduction, we utilized an established lentiviral vector system. Recently, the SIN type lentiviral vector has become one of the most popular tools for gene transfer to mammalian cell based on its efficiency, safety, and convenience [46]. Transfer of the IGF-1 gene transfer into MSCs was confirmed at the levels of mRNA transcripts and protein expression (Supplementary Figure 1A, 1B), and it occurred without detectably altering the cell surface marker expression profile (Supplementary Figure 1C) or the differentiation capacity of the cells towards the osteogenic and adipogenic lineages. In contrast, introduction of the IGF-1 gene promoted chondrogenic differentiation of Syn-MSCs to a tissue phenotype which was morphologically more similar to hyaline cartilage. It should also be pointed out that in the present study, IGF-1 gene transfer to Syn-MSCs promoted chondrogenesis without detectable enhancement of the hypertrophic or osteogenic phenotype. Frisch et al. reported that expression of osteogenic and hypertrophic marker genes was increased by overexpression of IGF-1 in bone marrow-derived MSCs, possibly related to the propensity of bone marrow-derived MSCs to differentiate towards the osteogenic lineage along with some chondrogenesis [27, 45]. Although the differences observed between the two studies may have been affected by differing conditions for gene transfer and cell culture, it is also likely that the action of IGF-1 on the differentiation characteristics of Syn-MSCs versus bone marrow-derived MSCs may be also be quite different. In support of that conclusion, Goodrich et al. reported ectopic bone formation within repair tissue following the implantation of bone marrow-derived MSCs into chondral defects [47]. Such findings suggest that potential osteogenic differentiation of MSCs can occur in vivo during cartilage repair. Along with the previous report that premature induction of hypertrophy during in vitro chondrogenesis of MSCs correlates with calcification after ectopic implantation [48], stimulation of the osteogenic differentiation capacity of MSCs should be avoided when being used for cartilage repair. In this regard, exposure to IGF-1 during bone marrow-derived MSC-based cartilage

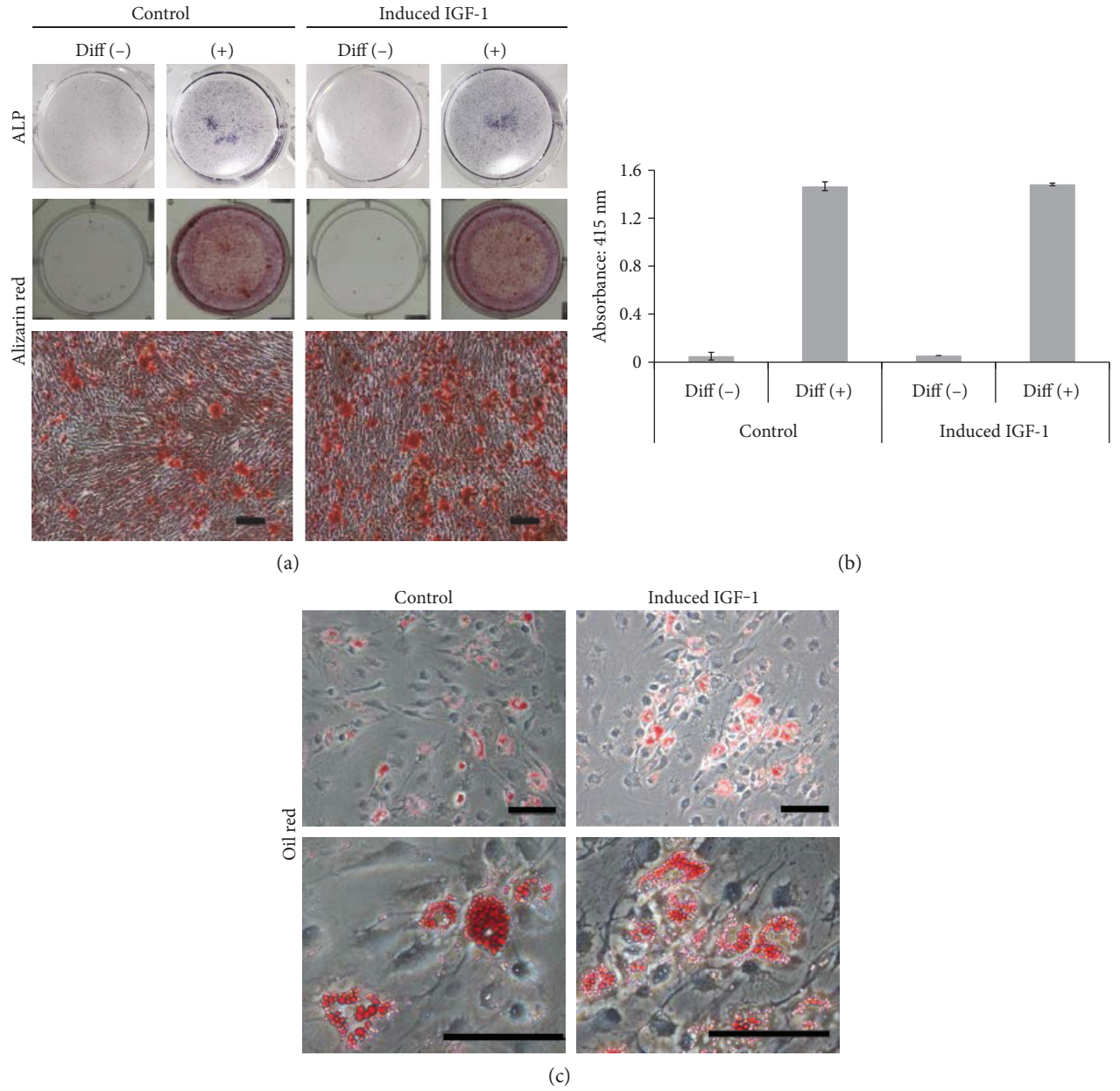

Figure 5: Effect of IGF-1 transduction on osteogenic and adipogenic differentiation of human Syn-MSC. (a) Osteogenic differentiation assay using ALP staining after 1 week of differentiation (upper). Alizarin red staining after 3 weeks of differentiation (lower). Scale bars = 500 μm. (b) Quantitated alizarin red staining values after osteogenic differentiation in the control versus induced IGF-1 groups. (c) Adipogenic differentiation after 3 weeks in a differentiation medium using oil red staining. Scale bars = 100 μm.

repair could increase the risk of unfavorable bone formation and thus is not recommended. Conversely, IGF-1-transduced Syn-MSCs appear to exhibit lineage-specific enhanced chondrogenic capacity, and the clinical implication of these observations is that such cells could be a promising approach for manipulating cartilage repair towards more complete tissue regeneration.

One of limitations of this study is that although we identified a unique phenotypic change towards an enhanced chondrogenic differentiation capacity of human Syn-MSCs via IGF-1 gene transfer, we could not assess whether the differences are dependent on donor ages and sex. We used cell sources that were available, but these were young donors (17–35 years old) and the numbers were limited. However, we have previously investigated the detailed chondrogenic capacities of a number of human Syn-MSCs of differing ages, and we did not detect any obvious differences in the phenotype of individual Syn-MSCs assessed [49]. It does however remain controversial whether human Syn-MSCs populations exhibit different chondrogenic capacities dependent on age or sex. In addition, we did not assess the biomechanical characterization of the cartilage tissues or the prototypical stability of the cartilaginous tissues in vivo after implantation. Further studies in these areas are needed

for detailed clarification of the potential of the IGF-1-transduced MSC to foster cartilage regeneration.

5. Conclusion

Lentivirus-mediated IGF-1 gene transfer to human Syn-MSC promotes the chondrogenic differentiation capacity of the cells without stimulating either the hypertrophic or osteogenic phenotype, and thus, may have enhanced potential for cartilage repair.

Abbreviations

GAG: Glycosaminoglycan
M.O.I.: Multiplicity of infection
MSCs: Mesenchymal stem cells.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

Authors' Contributions

Yasutoshi Ikeda, Morito Sakaue, Ryota Chijimatsu, Hidenori Otsubo, David A. Hart, Henning Madry, Kazunori Shimomura, Tomoyuki Suzuki, and Norimasa Nakamura are responsible for the project conception and experimental design. Yasutoshi Ikeda, Morito Sakaue, and Ryota Chijimatsu collected and assembled the data. Yasutoshi Ikeda, Morito Sakaue, Ryota Chijimatsu, and Norimasa Nakamura analyzed and interpreted the data. Yasutoshi Ikeda, Morito Sakaue, David A. Hart, and Norimasa Nakamura wrote the manuscript. Hideki Yoshikawa, Toshihiko Yamashita, and Norimasa Nakamura are responsible for the administrative support and final approval of the manuscript. Dr. Norimasa Nakamura was responsible for the project conception and experimental design, data analysis and interpretation, manuscript writing, administrative support, and final approval of the manuscript.

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