STEM CELLS AND DEVELOPMENT 13:436–448 (2004)

Mesenchymal Stem Cells: Isolation and Therapeutics

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ABSTRACT

Mesenchymal stem cells (MSCs) are progenitors of all connective tissue cells. In adults of multiple vertebrate species, MSCs have been isolated from bone marrow (BM) and other tissues, expanded in culture, and differentiated into several tissue-forming cells such as bone, cartilage, fat, muscle, tendon, liver, kidney, heart, and even brain cells. Recent advances in the practical end of application of MSCs toward regeneration of a human-shaped articular condyle of the synovial joint is one example of their functionality and versatility. The present review not only outlines several approaches relevant to the isolation and therapeutic use of MSCs, but also presents several examples of phenotypic and functional characterization of isolated MSCs and their progeny.

INTRODUCTION

MESENCHYMAL STEM CELLS (MSCs), also known as marrow stromal cells (1) or mesenchymal progenitor cells (2), are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages (3). To date, MSCs of multiple adult vertebrate species have been demonstrated to differentiate into lineage-specific cells that form bone, cartilage, fat, tendon, and muscle tissue (4–6). In addition to differentiation into their natural derivatives, MSCs have the potential to differentiate into other types of tissue-forming cells such as hepatic (7), renal (8), cardiac (9), and neural cells (10,11). Hence, the descriptive terms "pluripotent" or "multipotent" are reciprocally used to describe the capacity of MSCs to differentiate into a wide arrange of mammalian tissues (12).

The first successful isolation of fibroblast-like colonies from bone marrow, i.e., MSCs, was described about 4 decades ago by Friedenstein *et al.* (13). The isolation method was based on the adherence of marrow-derived, fibroblast-like cells to the plastic substrate of the cell culture plate, and a concomitant lack of adherence of marrow-derived hematopoietic cells. To date, Friedenstein's procedure is considered a standard protocol to isolate bone marrow (BM) MSCs (4,14,15). Isolation and phenotypic characterization of MSCs have been demonstrated in several vertebrate species, including human (6,12,16), murine (4,5,15), lapine (17), canine (18), ovine (19), avian (20), porcine (21), equine (22), and bovine (23). However, MSC colonies isolated by adherence to the plastic culture plate are heterogeneous, likely containing osteoblasts and/or osteoprogenitor cells, fat cells, fibroblasts, reticular cells, macrophages, endothelial cells, and a fraction of blood cells and hematopoietic stem cells (24,25). The fraction of hematopoietic cells is higher in initial cultures of murine marrow cells than in human marrow cell cultures (1,26). Ultimately, it can only be ascertained that a subset of the isolated BM cells adherent to the plastic culture plate are indeed MSCs if the isolated cells are demonstrated to differentiate into multiple cell lineages (4,5).

Following the demonstrated multipotentiality of the isolated BM cells by Friedenstein's protocol (13,27), several approaches have been investigated to prepare primary cultures of BM-derived MSCs with more homogeneous cell population using rather sophisticated isolation protocols (6,12,24,28–33). Because most MSC populations lack specific cell-surface markers, many isolation protocols are based on the process of negative selection—

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cells lacking the expression of endothelial and hematopoietic cell markers are sorted out and maintained as primary cultures.

The objective of the present review is to compare multiple methodological approaches to isolate MSCs with elaboration on new technical advances and their implications toward potential therapeutic applications. Specific examples of isolation and culturing protocols of BM-derived rat and human MSCs will be provided with some phenotypic and functional characterization assays performed on isolated MSCs and their progeny.

EMERGING TECHNOLOGIES FOR ISOLATION AND CHARACTERIZATION OF MSCs

In contrast to other cell types that express specific cell-surface markers such as hematopoietic cells (CD14, CD34, CD45) (6,34) and endothelial cells (CD31) (35), the phenotypic identify of MSCs is not unique, sharing features of multiple cell lineages, including mesenchymal, hematopoietic, endothelial, epithelial, and muscle cells (25,36,37). In addition, efforts to characterize phenotypic features of MSCs have been confounded by the fact that MSCs display a variety of morphological characteristics and express various cell lineage-specific antigens that can vary between different preparations and as a function of time in culture (34,36).

Initial approaches to enrich primary MSC cultures isolated from BM have utilized simple spatial separation of the hematopoietic cells from marrow stromal elements within liquid suspension culture systems (38,39). Cells adhered to culture plates are considered more likely to be MSCs, whereas other cells from the BM stroma such as adipocytes, macrophages, and plasma cells do not typically adhere to culture plate (38). Other isolation procedures have utilized the property of distinctive cell membrane sensitivity of MSCs and hematopoietic cells to certain extracellular treatments such as to adenosine triphosphate (ATP) ions (29,40). Whereas macrophages and hematopoietic cells are sensitive to applied extracellular ATP ions and form membrane lesions subject to the entrance of lethal substances, MSCs are insensitive to ATP (29). Also, a subset of quiescent population of MSCs has been identified by inducing metabolic death of proliferative cells as a result of culture-treatment with 5-fluorouracil (41).

There is continuous effort to characterize the morphological and cytochemical properties of BM-derived MSCs (42,43). When BM cells are cultured in vitro, the adherent population tends to form colonies of spindle-shaped cells, similar to fibroblasts in two-dimensional culture. Hence the term colony-forming unit-fibroblast (CFU-F) is frequently used when studying the proliferation of MSCs in culture (43,44). The cultured fibroblast-like cells have been initially described as alkaline phosphatase-positive, Sudan Black-positive, collagen IV-positive, fibronectin-positive, and esterase-negative (42,43). In addition, the extracellular matrix surrounding cultured MSCs contains type I collagen and laminin of the basement membrane (6,45). A group of colonies may also synthesize factor VIII-associated antigen, probably indicating their endothelial progeny (1).

Initial cultures of the adherent MSC population have been labeled by a panel of antibodies targeting a wide range of cell-surface antigens and peptides such as SH2 (CD105), SH3, SH4 (CD73), SB-10, and a group of other adhesion molecules and growth factor/cytokine receptors including CD166, CD54, CD102, CD121a,b, CD123, CD124, CD49, and so forth (6,46–50). Also, initial cultures of MSCs are known to co-express a heterogeneous group of genes characteristic of hematopoietic (36,49) and multiple mesenchymal lineages such as the osteogenic lineage (cbfa1, alkaline phosphatase, osteocalcin, and osteopontin) and the adipocytic lineage (lipoprotein lipase), suggesting a lack of commitment of MSCs (41,51). On the other hand, MSCs are negative to cell markers of endothelial cells (CD31), monocytes/macrophages (CD14), lymphocytes (CD11a/LFA-1), leukocytes (CD45), red blood cells (glycophorin A), and other hematopoietic cells (e.g., CD3, CD14, CD19, CD34, CD38, and CD66b) (12,34,46). Also, cultured MSCs synthesize a wide range of cytokines and growth factors, including stem cell factor (c-kit ligand), interleukin-7 (IL-7), IL-8, IL-11, transforming growth factor-β (TGF-β), cofilin, galectin-1, laminin-receptor 1, cyclophilin A, and matrix metalloproteinase-2 (MMP-2) (52,53). The reader is referred to several valuable reviews of the biological and phenotypic characteristics of MSCs (1–3,6,12, 51–55).

In spite of extensive molecular and cytochemical characterization of BM-derived MSCs isolated by negative selection, the first antibody (Stro-1) capable of providing positive identification of BM-derived MSCs was available years after their first successful isolation (24). In addition, monoclonal antibodies such as (anti-Sca-1) (30) and HOP-26 (56) were shown to enrich osteoprogenitor cells in BM cultures. Other protocols have attempted to generate more homogenous population of MSCs by supplementing the initial BM cultures with single or a combination of growth factors such as TGF-β1, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (57–59). For example, while bFGF has been demonstrated to support the maintenance of multilineage differentiation of MSCs (60), a combination of cytokines such as kit-ligand, thrombopoietin (TPO), IL-3, and IL-11 fails to improve the supportive role of MSCs toward hematopoietic cells in long-term bone marrow cultures (61). Nonetheless, de-

spite previous meritorious effort, most of the isolation protocols appear to be species-specific and result in heterogeneous cell populations in regards to morphology, surface-marker profile, and phenotype characteristics.

Previous approaches have investigated the isolation of increasingly homogenous population of MSCs based on differential cellular morphological and dimensional features within the bone marrow stroma using flow cytometry (61,62). Recently, a relatively simple culture protocol based on size-dependent sieving of a cell population from human BM aspirates through a porous membrane resulted in a relatively homogeneous population that had the capacity of self-renewal and the multilineage differentiation potential, as indicated by morphology and a wide range of cell-surface markers (63). In addition, more sophisticated approaches such as positive selection of MSCs with microbeads combined with fluorescence-activated cell sorting (FACS) (64) or magnetic-activated cell sorting (65) are promising techniques, not only for more defined isolation and precise characterization of MSCs, but also for potential disclosure of their role under physiologic and/or pathologic condition and ultimately toward infinitive therapeutic applications (66–68). Recent examples include the potential tracking and fate determination of implanted nanoparticle-labeled stem cells by magnetic resonance imaging (MRI) (69,70), targeted delivery of MSCs to the therapeutic site using magnetic resonance fluoroscopy (71), and unbounded potential for biological and functional studies of magnetically labeled MSCs at a single-cell resolution level (72).

EXAMPLES OF BM-DERIVED MSC ISOLATION AND CULTURE PROCEDURES

Isolation and culture of rat BM-derived MSCs

Rat BM-derived MSCs were harvested from 2- to 4-month-old (200–250 g) male Sprague-Dawley rats (Harlan, Indianapolis, IN). After removing epiphyses and gaining access to the marrow cavities, whole BM plugs were flushed out from tibial and femoral bones using a 10-ml syringe with Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Biocell, Rancho Dominguez, CA) and 1% antibiotic-antimycotic (Gibco, Carlsbad, CA). The lot of the FBS was specifically selected for its support of proliferation and differentiation of rat MSCs (73). Marrow samples were collected and mechanically disrupted by sequential aspiration through 16-, 18-, and 20-gauge needles attached to the same 10-ml syringe. The resulting cell suspension was centrifuged for 5 min at 1000 rpm with cells collected and resuspended in serum-supplemented medium.

To perform a cell count, a small volume of the resulting suspension was mixed with 4% acetic acid to lyse red blood cells. Nucleated cells were counted using a hemocytometer. After counting, cells were plated at 5×10^7 cells/100-mm culture dish and incubated in 5% CO_2 at 37°C, with fresh medium changes every 3–4 days. Typically, cells were maintained for 12–14 days as primary culture or upon formation of large colonies.

When large cell colonies developed (~80–90% confluence), cultures were washed twice with phosphate-buffered saline (PBS) and cells were trypsinized with 0.25% trypsin in 1 mM EDTA (Sigma, St. Louis, MO) for 5 min at 37°C. After centrifugation, cells were resuspended with serum-supplemented medium, counted, and plated at a density of $5–7 \times 10^5$ cells/100-mm dish. The resulting cultures were referred to as first-passage cultures. All animal experiments received approval from the institutional animal care committee.

This isolation and culturing protocol for rat BM-derived MSCs follows in general the original isolation procedure described by Friedenstein (13) and is in widespread practice including our laboratory (4,5). Despite ongoing intensive efforts toward isolation of increasingly homogenous population of MSCs within the adherent colonies of murine BM cultures (15,59,74–76), currently available in vitro and in vivo evidence supports the multipotential nature of the plastic-adherent subpopulation of cells in murine BM cultures (3–5).

Isolation and culture of human BM-derived MSCs

BM samples were obtained from the posterior iliac crest of a healthy 22-year-old adult male donor after informed consent (AllCells LLC, Berkeley, CA). To prepare a more homogenous population of MSCs, the whole BM sample was thoroughly mixed with mesenchymal cell enrichment cocktail (RosetteSep™; StemCell Technologies, Inc., Vancouver, Canada), at 50 μl of RosetteSep for each 1 ml of BM sample, and the mixture was allowed to incubate for 20 min at room temperature. RosetteSep is a PBS solution of a combination of mouse and rat monoclonal antibodies that target specific cell-surface antigens on human hematopoietic cells such as CD3, CD14, CD19, CD38, and CD66b, and glycophorin A on red blood cells. After incubation with RosetteSep, the marrow sample was diluted with twice the volume of PBS containing 2% FBS and 1 mM EDTA, followed by gentle mixing. Diluted sample was then layered on top of a density gradient solution (Ficoll-Paque®; StemCell Technologies, Inc., Vancouver, Canada) and centrifuged for 25 min at $300 \times g$.

Following centrifugation, enriched cells were removed from the Ficoll-Paque/plasma interface, washed with PBS containing 2% FBS and 1 mM EDTA, and resuspended in complete culture medium: basal medium for human MSCs (Mesencult™; StemCell Technologies, Inc., Van-

couver, Canada), 10% human MSC stimulatory supplement (StemCell Technologies, Inc., Vancouver, Canada), and 1% antibiotic-antimycotic (Gibco, Carlsbad, CA).

Following standard nucleated cell counting, cells were plated at $\sim 2 \times 10^7$ cells/100-mm culture plate and incubated in 5% CO_2 at 37°C, with fresh medium change every 3–4 days. Primary cultures were maintained for 12–14 days. When nearly confluent, the cultured cells were harvested using 0.25% trypsin in 1 mM EDTA (Sigma, St. Louis, MO) for 5 min at 37°C and centrifuged. After centrifugation, cells were resuspended with complete culture medium, replated at a ratio of 1:4 (each primary culture plate yields four new plates), and referred to as first-passage cultures.

The negative selection approach for the isolation and culture of enriched population of MSCs from bone marrow, such as the one described above, are more widely practiced for human-derived BM samples (6,12,77) than for murine BM (76). We have utilized this negative selection protocol to enrich initial cultures of human BM-derived MSCs with a multipotent cell population that is capable of differentiating into multiple lineages including bone, cartilage, and adipose tissue.

EXAMPLES OF FUNCTIONAL AND PHENOTYPIC ASSESSMENT OF MSCs AND PROGENY

We have performed histological, immunohistochemical, biochemical, as well as mechanical assays to characterize the phenotypic differentiation of rat- and human-derived MSCs into chondral and osseous tissue phenotypes (4,5), as well as adipose tissue (unpublished data).

Histochemical and biochemical in vitro assays

The chondrogenic and osteogenic differentiation potential of MSCs has been demonstrated in monolayer cultures by incubating first-passage rat-derived MSC cultures separately in chondrogenic or osteogenic medium, respectively, for 4 weeks. Chondrogenic medium contained a supplement of 10 ng/ml of TGF-β1 (R&D Systems Inc., Minneapolis, MN), whereas osteogenic medium contained 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich, St. Lois, MO). Cultures were incubated in 5% CO_2 at 37°C with medium change every 3–4 days.

Following 4-week incubation period with chondrogenic-inducing culture medium, monolayer cultures of rat MSC-derived chondrogenic cells showed positive reaction to safranin O staining relative to control cultures without exposure to the chondrogenic supplement (Fig. 1, A and B, respectively). Safranin O is a cationic stain that binds to cartilage glycosaminoglycans (GAG) such as chondroitin sulfate and keratan sulfate (78,79). In parallel, monolayer cultures of rat MSCs cultured for 4 weeks with medium containing osteoinductive supplement showed a positive reaction to alkaline phosphatase and von Kossa silver staining (Fig. 2A), relative to control monolayer MSC cultures that were incubated with regular culture medium without the osteoinductive supplement (Fig. 2B).

To perform histological, biochemical, and functional characterization of the MSC-derived chondral and osseous phenotypes in a cell-based three-dimensional construct model, MSC-derived chondrogenic and osteogenic cells were photoencapsulated in Poly(ethylene glycol) diacrylate (PEGDA) Hydrogel polymer (MW 3400; Shearwater Polymers, Huntsville, AL), as previously described (4,5). Briefly, PEGDA polymer was dissolved in sterile PBS supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, Carlsbad, CA) to a final solution of 10% wt/vol and a biocompatible photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Ciba, Tarrytown, NY), was added to the PEGDA solution to obtain a final initiator concentration of 0.05% wt/vol. Following 1-week exposure of rat-derived MSC cultures to chondroinductive or osteoinductive culture medium, MSC-derived chondrogenic and osteogenic cells were trypsinized, counted, and resuspended in PEGDA polymer/photoinitiator solution at a concentration of 5×10^6 cells/ml. The cell/polymer suspension was then loaded into small plastic molds (8 mm diameter and 150 μl volume each) and photopolymerized using long-wave, 365-nm ultraviolet lamp (Glowmark, Upper Saddle River, NJ) at an intensity of ~ 4 mW/cm^2 for 5 min. The polymerized chondrogenic and osteogenic constructs were then removed from the molds, washed twice with sterile PBS, and incubated with corresponding chondroinductive or osteoinductive medium for 8 weeks. Parallel control PEGDA constructs contained rat-derived MSCs from the same population but were not exposed to chondroinductive or osteoinductive media (cultured with serum-supplemented basic culture medium). All constructs were maintained in six-well culture plates (one construct per well) and incubated in 5% CO_2 at 37°C, with fresh medium change every 3–4 days.

Upon histological examination, Hydrogel constructs encapsulating MSC-derived chondrogenic cells and incubated with chondroinductive culture medium for 4 weeks demonstrate a positive Safranin O reaction (Fig. 1C), indicating synthesis of GAGs. In contrast, control Hydrogel constructs encapsulating MSCs that were not preconditioned with the chondroinductive medium and incubated with basic culture medium (without chondroinductive supplement) showed negative reaction to Safranin O staining (Fig. 1D). In addition, the GAG content in the chondrogenic constructs demonstrated a steady increase as a function of the incubation time in the chon-

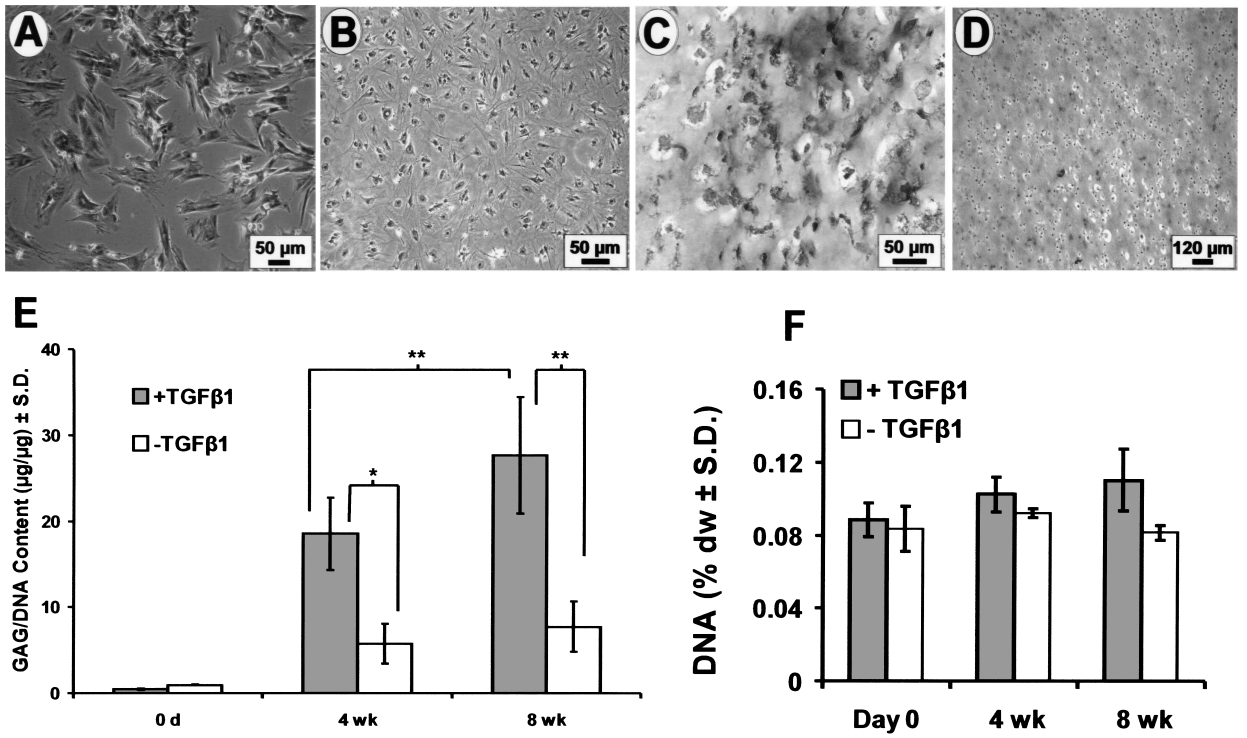

FIG. 1. MSC-driven chondrogenesis in monolayer cultures and PEGDA hydrogel constructs. (**A**) Positive Safranin O staining of monolayer culture of rat BM-derived MSCs exposed to chondrogenic medium containing TGF-β1 for 4 weeks. (**B**) Control monolayer culture of MSCs grown in basic medium (−TGF-β1) for 4 weeks showed no reaction to Safranin O staining. (**C**) Positive Safranin O staining of PEGDA hydrogel constructs encapsulating MSC-derived chondrogenic cells and incubated in chondrogenic medium (+TGF-β1) for 4 weeks. (**D**) Control PEGDA constructs encapsulating MSCs and incubated with basic medium (−TGF-β1) for 4 weeks showed negative reaction to Safranin O. (**E**) GAG content of experimental and control PEGDA Hydrogel constructs encapsulating MSCs following 0, 4, and 8 weeks of incubation with chondrogenic (+TGF-β1) or basic (−TGF-β1) medium, respectively ($^{*}p < 0.05$; $^{**}p < 0.02$). (**F**) DNA fraction analysis of experimental and control PEGDA hydrogel constructs encapsulating MSCs following 0, 4, and 8 weeks of incubation with chondrogenic (+TGF-β1) or basic (−TGF-β1) medium, respectively, indicating cell survival.

droinductive medium ($p < 0.05$) (Fig. 1E). At the same time, GAG content was significantly higher in Hydrogel constructs encapsulating MSC-derived chondrogenic cells following 4 and 8 weeks of incubation in the chondroinductive medium than control constructs encapsulating MSCs without exposure to the chondroinductive medium (incubated in basic medium lacking the chondroinductive supplement) ($p < 0.02$) (Fig. 1E). The fraction analysis of DNA content of both experimental and control constructs revealed generally consistent cell survival over the 8-week incubation period (Fig. 1F).

Osteogenic PEGDA Hydrogel constructs encapsulating MSC-derived osteogenic cells demonstrated a positive reaction to von Kossa silver staining (Fig. 2C), indicative of mineral deposition, after 4-week incubation in the osteoinductive medium. By contrast, control Hydrogel constructs encapsulating MSCs that were not preconditioned with osteoinductive medium and incubated in serum-supplemented basic culture medium showed a negative reaction to von Kossa staining (Fig. 2D). A quantitative calcium content assay revealed a positive relationship between calcium deposition in osteogenic constructs and incubation period with the osteoinductive medium over the 8-week cultivation time ($p < 0.05$) (Fig. 2E). Also, calcium content was significantly higher in osteogenic Hydrogel constructs relative to control Hydrogel constructs encapsulating MSCs unexposed to the osteoinductive supplement and incubated in basic medium lacking the osteoinductive agents for 4 and 8 weeks ($p < 0.05$ and $p < 0.02$, respectively) (Fig. 2E). DNA content analysis indicated a lack of substantial cell loss in either experimental or control constructs over the 8-week culture time (Fig. 2F).

Physical characterization of the cell membrane of human-derived MSCs and derivatives

First-passage human MSCs were cultured in basic medium (Mesencult™ + 10% human MSC stimulatory supplement), chondroinductive medium (supplemented

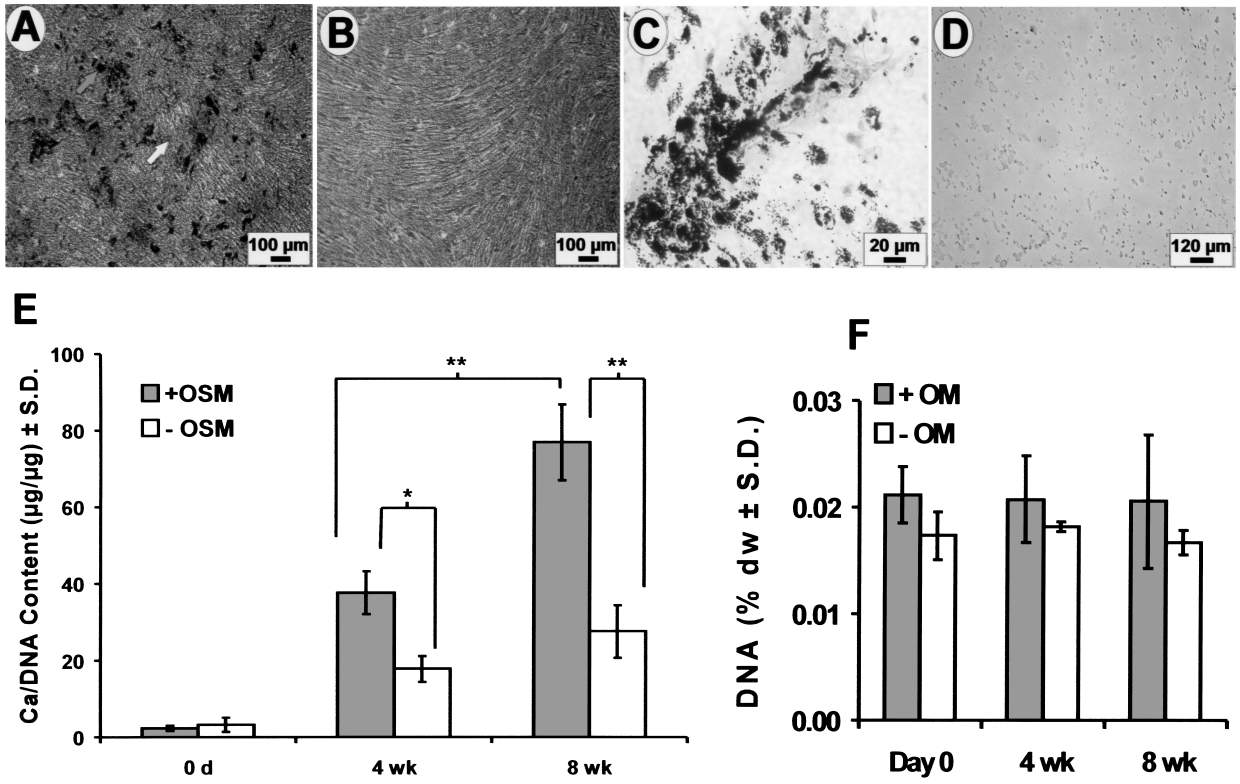

FIG. 2. MSC-driven osteogenesis in monolayer cultures and PEGDA Hydrogel constructs. (**A**) Positive von Kossa staining (green arrow) and alkaline phosphatase staining (white arrow) in monolayer culture of rat BM-derived MSCs exposed to osteogenic medium for 4 weeks. (**B**) Control monolayer culture of MSCs grown with basic medium (no osteogenic supplement) for 4 weeks showed negative reaction to von Kossa and alkaline phosphatase staining. (**C**) Positive von Kossa staining of PEGDA Hydrogel constructs encapsulating MSC-derived osteogenic cells and incubated in osteogenic medium for 4 weeks. (**D**) Control PEGDA constructs encapsulating MSCs and incubated with basic medium (no osteogenic supplement) for 4 weeks showed negative reaction to von Kossa staining. (**E**) Calcium content of experimental and control PEGDA Hydrogel constructs encapsulating MSCs following 0, 4, and 8 weeks of incubation with osteogenic or basic (no osteogenic supplement) medium, respectively ($^{*}p < 0.05$; $^{**}p < 0.02$). (**F**) DNA fraction analysis of experimental OM^{+} and control OM^{-} PEGDA Hydrogel constructs encapsulating MSCs following 0, 4, and 8 weeks of incubation with osteogenic or basic (no osteogenic supplement) medium, respectively, indicating cell survival.

with 10 ng/ml TGF-β1), or osteoinductive medium (supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate) for 4 weeks. All cultures were incubated in 5% CO_2 at 37°C, with fresh medium change every 3–4 days.

Following 4 weeks of culture, first-passage human MSCs and MSC-derived chondrogenic and osteogenic cells were trypsinized, counted, and resuspended separately with serum-supplemented culture medium to a cell density of 6×10^5 cells/ml. Aliquots of 100 μl from each cell suspension were plated on 12-mm round poly-D-lysine-treated glass coverslips (Becton Dickinson, Franklin Lakes, NJ) and cells were allowed to attach to the glass substrate for several hours before adding the corresponding culture medium to each tissue culture plate. On day 4 after plating, MSCs were nearly confluent and were used for nanoindentation and imaging with atomic force microscope (AFM).

To prepare the cells for imaging with AFM, the glass coverslips with the MSCs cultured on surface were removed from the cell culture dishes and attached by a two-sided adhesive tape to a 15-mm stainless steel disc. Subsequently, the coverslip/disc assembly was magnetically mounted onto the piezoscanner of the AFM. A standard AFM fluid cell without the O-ring seal was used for imaging, and fresh serum-free culture medium was frequently injected to keep the cells in a fluid environment during the mounting and imaging procedure. Both topographic and force spectroscopy images were obtained in contact mode using a Nanoscope IIIa atomic force microscope (Digital Instruments Inc., Santa Barbara, CA). Cantilevers with a nominal force constant of $k = 0.03$ N/m and oxide-sharpened Si_3N_4 tips were used to apply nanoindentation against cell membrane surface. Scan rates were set at 1 Hz for topographic imaging and 14 Hz for force spectroscopy, and scan size was 10×10

μm. The radius of curvature of the scanning tips used was 20 nm.

For each MSC, the average Young's modulus, which is defined as the slope of the stress versus strain curve and represents the elastic mechanical properties of the material under study, was derived from individual calculations of three randomly selected points on the membrane surface within the 10-μm^2 scanning field using the Hertz model (80,81):

$$E = \frac{3F(1 - \nu)}{4\sqrt{R}\delta^{3/2}}$$

where E is the Young's modulus, F is the applied nanomechanical load, ν is the Poisson ratio for a given region, R is the radius of the curvature of the AFM tip, and δ is the amount of indentation.

The average calculated Young's modulus of the studied human mesenchymal stem cells maintained in basic culture medium was 37.0 ± 9.62 Kilopascal (kPa ± SD) (Fig. 3A), whereas the average Young's moduli for human MSCs exposed to the chondroinductive and osteoinductive supplements were 44.85 ± 11.96 kPa and 50.68 ± 3.27 kPa, respectively (Fig. 3, B and C, respectively). The average Young's modulus for human MSC-derived osteogenic cells (50.68 ± 3.27 kPa) was significantly higher than that for human MSCs grown with basic medium (no chondroinductive or osteoinductive supplements) (37.0 ± 9.62) ($p < 0.05$). This finding of a higher average Young's modulus for human MSC-derived chondrogenic and osteogenic cells than for MSCs grown with basic medium (no chondroinductive or osteoinductive supplements) suggests molecular changes in the membrane surface of MSCs that reflect upon the membrane mechanical properties as they progress through their differentiation pathway toward chondrogenic and osteogenic lineages.

Nanomechanical characterization of cell-based Hydrogel constructs

Following 4-week in vitro incubation of PEGDA Hydrogel constructs encapsulating chondrogenic and osteogenic MSC-derived cells in corresponding chondroinductive or osteoinductive medium, functional characterization of the synthesized matrix within these constructs was performed using physical nanoindentation with AFM. Parallel control constructs consisted of plain PEGDA Hydrogel constructs encapsulating no cellular elements. Chondrogenic and osteogenic constructs demonstrated significantly different Young's moduli (5). The average Young's modulus of osteogenic constructs was 582 ± 59 kPa, significantly higher than chondral constructs (329 ± 54 kPa) ($p < 0.01$), which in turn was significantly higher than PEGDA Hydrogel alone (166 ± 23 kPa) ($p < 0.01$) (5). These nanomechanical data suggest that MSC-derived osteogenic cells encapsulated in PEGDA Hydrogel have produced stiffer matrices than matrices synthesized by MSC-derived chondrogenic cells, both of which are significantly stiffer than the PEGDA Hydrogel material itself.

Tissue engineering of articular condyles using rat-derived MSCs

We have demonstrated the feasibility of tissue engineering articular condyles with both cartilagenous and osseous components from a single population of rat BM-derived MSCs (4,5). Here, we present data from our ongoing work to optimize the initial cell encapsulation density and the in vivo cultivation period of tissue-engineered osteochondral constructs.

First-passage rat-derived MSCs were exposed separately to either chondroinductive or osteoinductive medium for 1 week (as described above). Monolayer cultures of MSCs to be encapsulated in control constructs were grown for the same period with basic culture medium (DMEM/FBS and without chondrogenic or osteogenic inducing supplements). All cultures were incubated in 5% CO_2 at 37°C with medium change every 3–4 days.

After 1 week of culturing MSCs in chondroinductive, osteoinductive, or basic medium, cells were trypsinized, counted, and resuspended in PEGDA polymer/photoinitiator solution at a density of 20×10^6 cells/ml. A 150-μl aliquot of the cell/polymer suspension with MSC-derived chondrogenic cells was loaded into a hollow, bivalved, polysiloxane negative mold that had been previously fabricated from a positive replica of a cadaver adult human mandibular condyle (4,5). Following photopolymerization of the chondrogenic portion under UV light for 5 min, the cell/polymer suspension containing MSC-derived osteogenic cells (~600 μl) was then loaded to occupy the remainder of the mold followed by the same photopolymerization protocol. For control constructs, the same fabrication protocol was followed, except that the polymer suspension contained MSCs that were grown with basic medium and were not exposed to the chondroinductive or osteoinductive supplements. The polymerized osteochondral constructs were then removed from the mold, washed twice with sterile PBS, and implanted in subcutaneous pockets prepared by blunt dissection in the dorsum of severe combined immunodeficient (SCID) mice (Harlan, Indianapolis, IN) under general anesthesia with intraperitoneal (i.p.) injection of 100 mg/kg ketamine plus 5 mg/kg xylazine.

Upon 12-week in vivo cultivation in the dorsum of SCID mice, tissue-engineered articular condyles formed de novo and retained the shape and dimensions of a real-sized adult human mandibular condyle. The chondro-

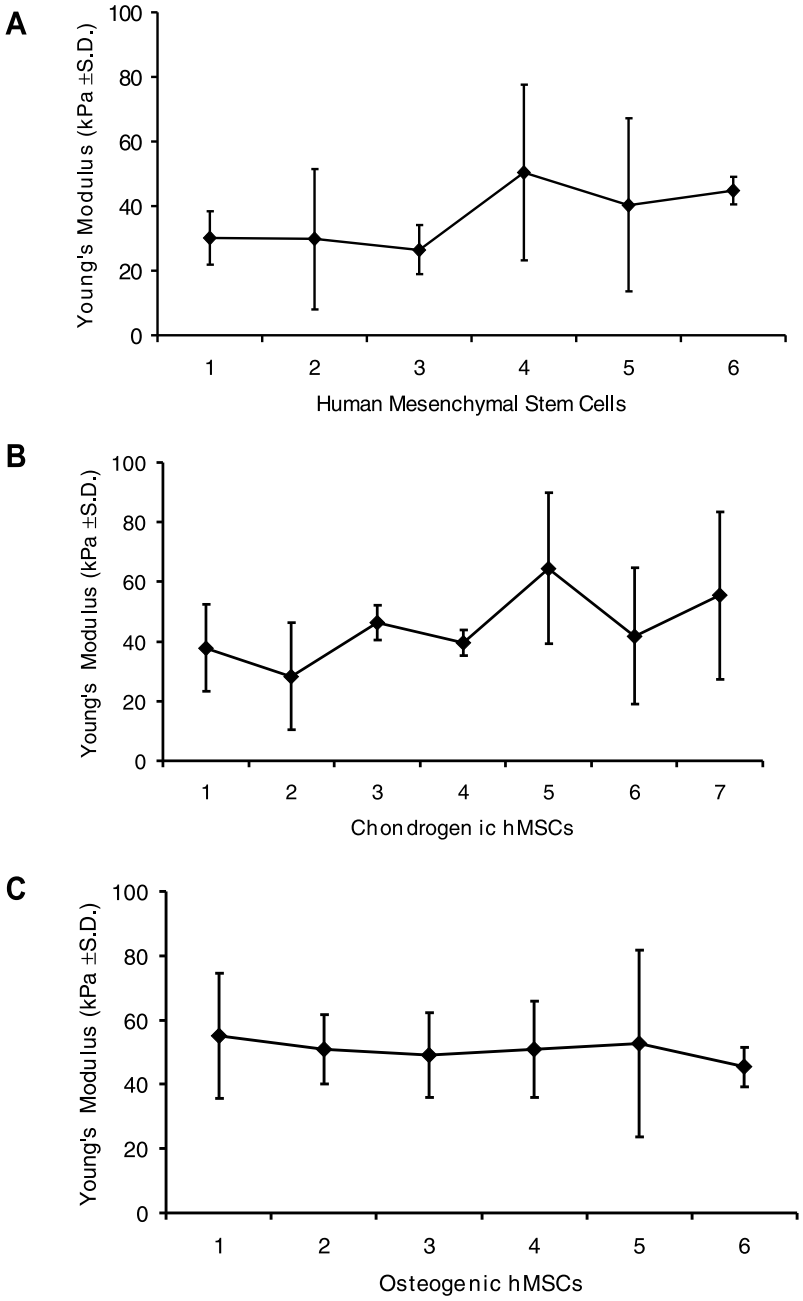

FIG. 3. Average Young's moduli of human MSCs and human MSC-derived chondrogenic and osteogenic cells upon nanoindentation with AFM. (**A**) Average Young's modulus of first-passage human MSCs ($n = 6$) grown for 4 weeks with basic medium (without chondroinductive or osteoinductive supplements). (**B**) Average Young's modulus of human MSC-derived chondrogenic cells ($n = 7$) grown for 4 weeks with chondroinductive medium. (**C**) Average Young's modulus of human MSC-derived osteogenic cells ($n = 6$) grown for 4 weeks with osteoinductive medium.

genic and osteogenic layers of the tissue-engineered articular condyles expressed chondral and osseous phenotypic characteristics, respectively, and demonstrated distinctive histological features (Fig. 4). The chondral component of the tissue-engineered constructs contained chondrocyte-like cells embedded in lacuna-like structures and surrounded by abundant intercellular matrix that reacted positively to Safranin O staining (Fig. 4A). In addition, type II collagen was immunolocalized throughout the chondrogenic portion (Fig. 4B). The deep portion of the chondrogenic layer, near the tissue-engineered osteochondral interface, contained chondrocyte-like cells with hypertrophic appearance and characterized by the expression of type X collagen (data not shown), specific

marker for normal hypertrophic and degenerating chondrocytes.

In contrast, the osseous component of the tissue-engineered articular condyle demonstrated multiple islands of bone trabecula-like structures containing abundant extracellular matrix and surrounded and occupied by osteoblast-like cells (Fig. 4A–C). Immunolocalization of bone markers such as osteopontin (Fig. 4C) and osteonectin (data not shown) within the osteogenic layer of the tissue-engineered constructs further demonstrates the osseous phenotypic nature of this layer. Generally, the chondrogenic layer lacked positive immunolocalization of bone markers and the osseous layer lacked positive immunolocalization of cartilagenous markers (Fig. 4A–C), except at the osteochondral interface where mutual infiltration and reciprocal expression of markers has occurred. Control constructs encapsulating MSCs that were not preconditioned with chondroinductive or osteoinductive supplements appeared to lack osteochondral distinctive organization and failed to express chondral and osseous immunohistochemical markers (data not shown). In addition, control acellular Hydrogel constructs (no cells encapsulated) showed intact border surrounded by fibrous tissue capsule with no host cellular invasion to the construct (Fig. 4D). The absence of host cellular invasion to the implanted Hydrogel constructs further sub-

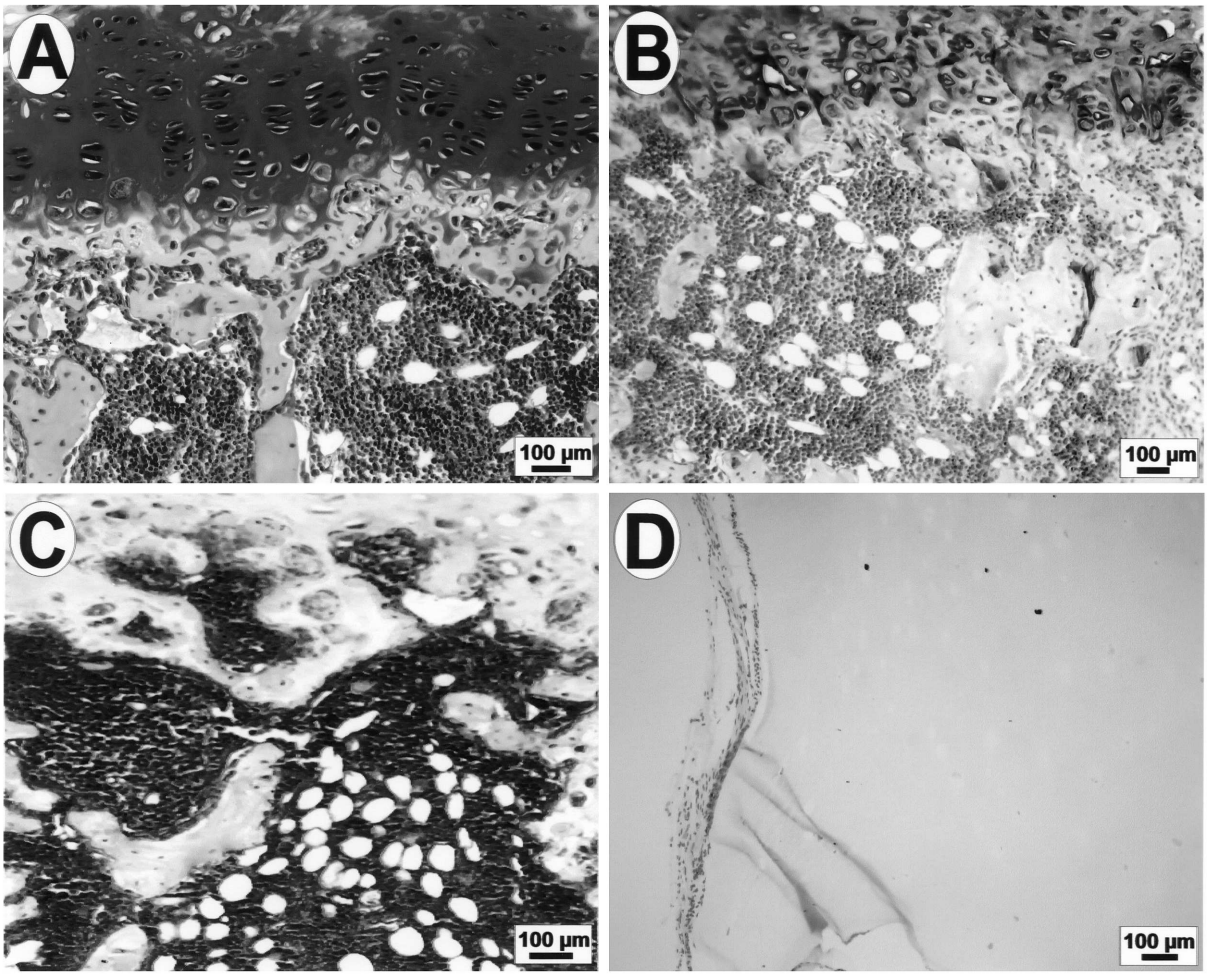

FIG. 4. Histologic and immunohistochemical characterization of tissue-engineered articular condyle. (**A**) Representative photomicrograph showing positive Safranin O red staining of the chondral portion of the tissue-engineered condyle indicating the presence of cartilage-specific glycosaminoglycans in the extracellular matrix. In contrast, the osseous portion of the tissue-engineered articular condyle showed negative reaction to Safranin O staining. (**B**) Positive immunohistochemical localization of type II collagen, a cartilage-specific marker, in the (upper) chondral portion of the tissue-engineered condyle. The osseous (lower) portion of the tissue-engineered condyle was negative to type II collagen immunolocalization. (**C**) Positive immunolocalization of ostepontin, a bone marker, within the osseous (lower) portion of the tissue-engineered condyle. By contrast, the chondral (upper) portion of the tissue-engineered condyle lacked the expression of osteopontin. (**D**) Representative micrograph of Hydrogel control construct with no cells encapsulated showing intact border of the Hydrogel and host fibrous-tissue capsule surrounding the construct. There is no host cell invasion into the Hydrogel construct, indicating that the tissue-engineered condyle was formed solely from stem cell–derived chondrogenic and osteogenic cells.

stantiates the conclusion that the synthesized matrix observed within the tissue-engineered osteochondral constructs is a result of the encapsulated chondrogenic- and osteogenic-differentiated MSCs rather than invading host cells.

The presented data substantiate our previous reports (4,5) and further demonstrate the feasibility of de novo formation of human-shaped small articular condyles with two stratified layers of chondral and osseous histogenesis from a single population of rat bone marrow-derived MSCs encapsulated in a uniform Hydrogel scaffold. The presence of abundant matrix synthesis and immunolocalization of chondral and osseous phenotypic markers in corresponding layers of the tissue-engineered articular condyles indicate survival, matrix synthesis, and continuing phenotypic differentiation of encapsulated MSC-derived chondrogenic and osteogenic cells. Many previous meritorious approaches to tissue engineer anatomically shaped osteochondral constructs have inspired various aspects of the present work (82–88). Although encouraging, much additional work is warranted along several fronts, including mechanical enablement and optimization of different structural and design parameters of the tissue-engineered condyles, before the present approach can be utilized for therapeutic applications.

CONCLUSION

Tremendous advances have been made in our understanding of the versatility of mesenchymal stem cells and their intrinsic capacity to differentiate into multiple cell lineages. Demands of the field of tissue engineering have intensified the effort to understand the potential therapeutic value of mesenchymal stem cells. The ability to understand the fundamental cell and molecular biology of mesenchymal stem cells will help attempts to manipulate these cells toward specific therapeutic goals. Our recent effort to utilize combined biological and biophysical approaches toward tissue regeneration has provided several opportunities to understand the cellular and molecular nature of mesenchymal stem cells.

ACKNOWLEDGMENTS

We thank Aurora Lopez for general technical assistance. The nanomechanical data of human MSCs have been obtained in a continuing research project that has been reported in an abstract [Patel R, Alhadlaq A, and Mao JJ. Cell membrane nanomechanical properties of human mesenchymal stem cells. J Dent Res (in press)]. The research work described in the present article has been supported by a Biomedical Engineering Research Grant from the Whitaker Foundation RG-01-0075, IRIB Grant on Biotechnology jointly from the UIC (University of Illinois at Chicago) and UIUC (University of Illinois at Urbana-Champaign), and by USPHS Research Grants DE13964, DE15391 from the National Institute of Dental and Craniofacial Research, and EB02332 from the National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health.

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Received April 9, 2004; accepted May 26, 2004.