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Tissue-engineered Neogenesis of Human-shaped Mandibular Condyle from Rat Mesenchymal Stem Cells

ABSTRACT

The temporomandibular joint is susceptible to diseases and trauma that may ultimately lead to structural degeneration. Current approaches for replacing degenerated mandibular condyles suffer from deficiencies such as donor site morbidity, immunorejection, implant wear and tear, and pathogen transmission. The hypothesis of this study was that a human-shaped mandibular condyle can be tissue-engineered from rat mesenchymal stem cells (MSCs) encapsulated in a biocompatible polymer. Rat bone marrow MSCs were isolated and induced to differentiate into chondrogenic and osteogenic cells *in vitro*, and encapsulated in poly(ethylene glycol)-based hydrogel in two stratified layers molded into the shape of a cadaver human mandibular condyle. Eight weeks following *in vivo* implantation of the bilayered osteochondral constructs in the dorsum of immunodeficient mice, mandibular condyles formed *de novo*. Microscopic evaluation of the tissue-engineered mandibular condyle demonstrated stratified layers of histogenesis of cartilaginous and osseous phenotypes. The current approach is being refined toward ultimate therapeutic applications.

KEY WORDS: TMJ, osteochondral, tissue engineering, cartilage, bone.

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INTRODUCTION

Temporomandibular disorders (TMD) affect approximately 30 million individuals in the United States alone, with more than one million new patients added each year (LeResche, 1997; Stohler, 1999). TMDs may manifest as pain, myalgia, headaches, and structural destruction known as degenerative joint disease (Okeson, 1996). The temporomandibular joint (TMJ), like other synovial joints, is also prone to rheumatoid arthritis, injuries, and congenital anomalies (LeResche, 1997; Stohler, 1999). The severe form of TMJ-associated degenerative disorders necessitates surgical replacement of involved mandibular condyle (Sarnat and Laskin, 1991). Currently available materials for surgical replacement of the mandibular condyle—such as autologous, allogenous, xenogenous grafts or artificial prosthesis—suffer from deficiencies such as donor site morbidity, limited tissue supply, immunorejection, potential transmission of pathogens, and complications of wear and tear (Henning *et al.*, 1992; Wolford and Karras, 1997; Baird and Rea, 1998; Bell *et al.*, 2002). A tissue-engineered mandibular condyle from the patient's own tissue-forming cells should overcome these deficiencies.

Previous attempts to tissue-engineer mandibular condyles have utilized several meritorious approaches (for review, see Glowacki, 2001) that inspired various components of the present work. For instance, chondrocytes or osteoblasts encapsulated in various hydrogels survive *in vitro* fabrication and synthesize cell-associated extracellular matrices (Poshusta and Anseth, 2001; Schliephake *et al.*, 2001; Springer *et al.*, 2001; Weng *et al.*, 2001). Increasingly sophisticated scaffold design influences cell differentiation patterns (Hollister *et al.*, 2002; Sherwood *et al.*, 2002). The premolded shape of the mandibular condyle is retained after marrow-derived osteoblasts are seeded in scaffolds consisting of poly-lactic-glycolic acid or natural coral (Weng *et al.*, 2001; Chen *et al.*, 2002; Abukawa *et al.*, 2003). However, an unmet challenge is to tissue-engineer a mandibular condyle from adult stem cells that differentiate into both chondrogenic and osteogenic lineages, an approach that not only mimics the developmental processes of the mandibular condyle, but also is necessary for ultimate clinical applications. Stem cells are necessary because full-thickness osteochondral defects, such as those in severe arthritis, heal poorly in the absence of corresponding tissue-forming cells (Hunziker, 2002; Lietman *et al.*, 2002). Adult mesenchymal stem cells have advantages over embryonic stem cells for tissue engineering of the mandibular condyle, because adult mesenchymal stem cells (MSCs) can be obtained from the same individual and readily induced to differentiate into both chondrogenic and osteogenic cells (Caplan, 1994).

Hydrogels are hydrophilic polymers capable of absorbing biological fluids while serving as a three-dimensional scaffold, thus providing tissue-forming cells with a mimicked environment of the extracellular matrix (Lee and Mooney, 2001). Polyethylene-glycol-based hydrogel, such as that used

in the present work, is biocompatible and has been shown to maintain the viability of encapsulated cells (Poshusta and Anseth, 2001; Burdick *et al.*, 2002). The objective of the present study was to tissue-engineer a human-shaped mandibular condyle from a single population of rat mesenchymal stem cells that had been induced to differentiate into chondrogenic and osteogenic lineages.

MATERIALS & METHODS

Harvest and Culture of MSCs

Rat bone-marrow MSCs were harvested from two- to four-month-old (200-250 g) male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA). Following CO_2 asphyxiation, the tibia and femur were dissected under aseptic conditions. Bone-marrow plugs were flushed by means of an 18-gauge needle and 10-mL syringe loaded with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biocell, Rancho Dominguez, CA, USA) and 1% antibiotic-antimycotic (Gibco). The marrow samples were mechanically disrupted by passage through 16-, 18-, and 20-gauge needles. Marrow cells were centrifuged, re-suspended in serum-supplemented medium, counted, plated at 5 x 10^7 cells/100-mm culture dish, and incubated in 95% air/5% CO_2 at 37°C for 2 wks, with fresh medium change every 3-4 days. Upon reaching 80-90% confluence, primary MSCs were trypsinized, counted, and passaged at a density 5-7 x 10^5 cells/100-mm culture plate. The animal protocol was approved by the institutional Animal Care Committee.

Treatment of MSCs with Chondrogenic and Osteogenic Differentiation Factors

The same population of first-passage MSCs was treated separately with chondrogenic or osteogenic specially formulated medium. The chondrogenic medium was supplemented with 10 ng/mL TGF-β1, whereas the osteogenic medium contained 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. Cultures were incubated for 1 wk in 95% air/5% CO_2 at 37°C, with fresh medium change every 3-4 days.

Hydrogel Preparation and Cell Photoencapsulation

Poly(ethylene glycol) diacrylate (PEGDA) (Shearwater, Huntsville, AL, USA) was dissolved in PBS supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco) to a final solution of 10% w/v. A biocompatible ultraviolet photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Ciba, Tarrytown, NY, USA) was added to the PEGDA solution to make a final concentration of 0.05% w/v. After trypsinization and counting, MSC-derived chondrogenic and osteogenic cells were re-suspended separately in the polymer/photoinitiator solution at a concentration of 5 x 10^6 cells/mL.

For *in vivo* experiments, a 200-μL aliquot of cell/polymer suspension containing MSC-derived chondrogenic cells was loaded in the human mandibular condyle-shaped polyurethane mold (Fig. 1D,1E), followed by photopolymerization with UV light at 365 nm (Glowmark, Upper Saddle River, NJ, USA) for 5 min (Elisseeff *et al.*, 2000). MSC-derived osteogenic cells suspended in polymer/photoinitiator solution were loaded to occupy the remainder of the mold (approx. 400 μL), followed by photopolymerization. For the *in vitro* assay, a 100-μL aliquot of cell/polymer suspension containing either MSC-derived chondrogenic cells or MSC-derived osteogenic cells was loaded in tissue culture inserts (diameter, 5 mm), followed by photopolymerization.

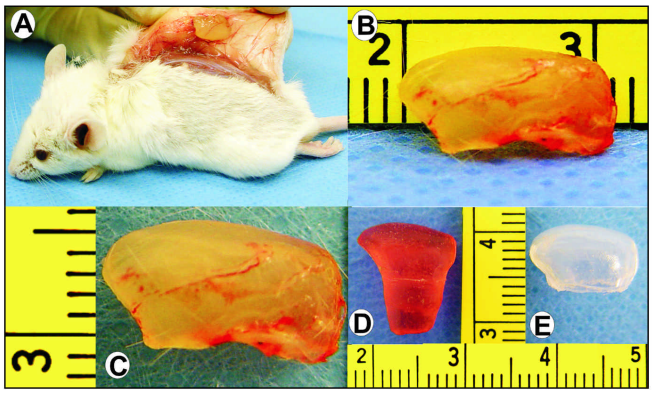

Figure 1. Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. **(A)** Recovery process of a tissue-engineered mandibular condyle after eight-week *in vivo* implantation in immunodeficient mouse. **(B,C)** Harvested osteochondral construct retained the shape and size of the cadaver human mandibular condyle mold. **(D)** Acrylic model of a cadaver human mandibular condyle. **(E)** Polyurethane mold used to load the cell/polymer suspensions.

In vivo Implantation and *in vitro* Incubation of Hydrogel Constructs

Following photopolymerization, the osteochondral construct was removed from the mold and washed with PBS supplemented with 1% antibiotics. After anesthesia of severe combined immunodeficient (SCID) mice (four- to five-week-old males) (Harlan) by I.P. injection of 100 mg/kg ketamine plus 5 mg/kg xylazine, the osteochondral constructs were implanted into dorsal subcutaneous pockets formed by blunt dissection. Four fabricated constructs were implanted into 2 SCID mice. Three experimental constructs contained MSC-derived chondrogenic and osteogenic cells encapsulated in 2 stratified layers of poly(ethylene glycol) diacrylate hydrogel, whereas the fourth construct, containing untreated MSCs, served as a control.

To demonstrate chondrogenesis and osteogenesis *in vitro*, the resulting constructs (6 samples *per* group) were removed from tissue culture inserts and then incubated in six-well tissue culture plates with either chondrogenic or osteogenic medium, respectively. Control samples consisted of 6 constructs encapsulating untreated MSCs and 6 constructs with no cells. Control constructs were incubated with DMEM/FBS without exposure to chondrogenic or osteogenic factors. MSC monolayer cultures (6 culture plates *per* group) were incubated with chondrogenic or osteogenic medium, or with DMEM/FBS as control. All hydrogel constructs and monolayer cultures for the *in vitro* assay were incubated statically at 95% air/5% CO_2 at 37°C for 4 wks, with fresh medium change every 3-4 days.

Harvest of Tissue-engineered Mandibular Condyles and Histologic Phenotyping

Eight wks following subcutaneous implantation, tissue-engineered osteochondral constructs were harvested from SCID mice. Following CO_2 asphyxiation, an incision was made aseptically in the dorsum of the SCID mice (Fig. 1A). After careful separation

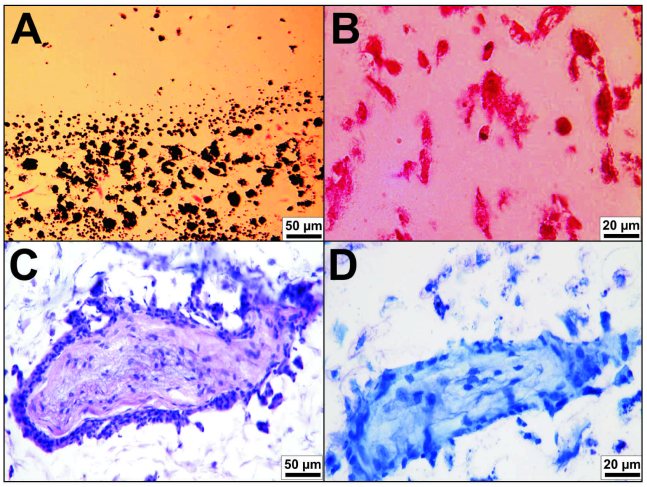

Figure 2. Photomicrographs of histologic phenotypes of a representative tissue-engineered mandibular condyle following 8 wks of *in vivo* implantation. **(A)** Von Kossa silver-stained section showing the interface between chondral and osseous layers. Multiple mineralization nodules were present in the osseous layer (lower half of the photomicrograph), but absent in the chondral layer (upper half of the photomicrograph). **(B)** Positive safranin O staining of the chondrogenic layer was represented by intense red, indicating the synthesis of negatively charged cartilage-specific glycosaminoglycans in the extracellular matrix. **(C)** H&E-stained section of the osteogenic layer showing a representative island structure consisting of MSC-differentiated osteoblast-like cells on the surface and in the center. **(D)** Positive toluidine blue staining of an island structure in the osseous layer, suggesting osteogenic phenotype.

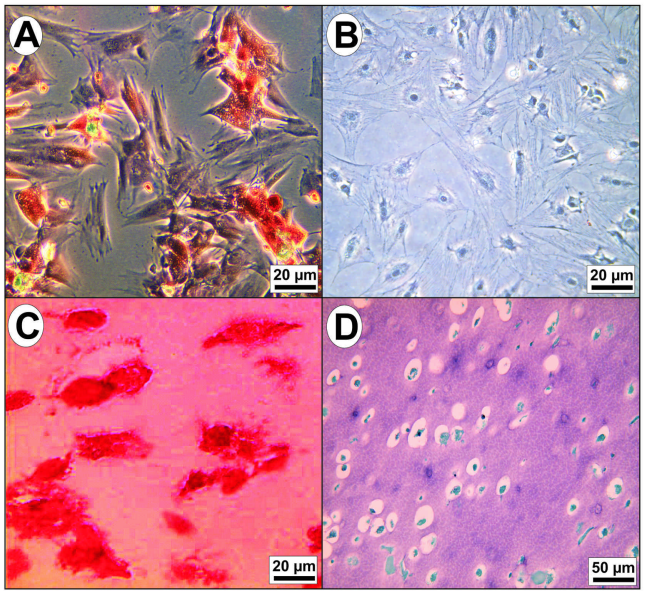

Figure 3. Chondrogenesis driven by MSC-derived chondrogenic cells in *ex vivo* samples. **(A)** Positive reaction of MSC-derived chondrogenic cells to safranin O in monolayer culture following four-week treatment with chondrogenic medium containing TGF-β1. **(B)** Monolayer culture of MSCs from the same population as in (A), cultured for 4 wks with DMEM/FBS but without TGF-β1, showed no positive reaction to safranin O. **(C)** Positive reaction of PEG hydrogel encapsulating MSC-derived chondrogenic cells to safranin O, demonstrating the presence of cartilage-specific glycosaminoglycans after four-week incubation in chondrogenic medium containing TGF-β1. **(D)** PEG hydrogel encapsulating the same population of MSCs as in (C), but without exposure to TGF-β1, showed negative reaction to safranin O.

from the surrounding fibrous capsule, the tissue-engineered mandibular condyles were removed (Figs. 1B, 1C), rinsed with PBS, fixed in 10% formalin overnight, embedded in paraffin, and sectioned in the sagittal plane and parallel to the long axis of the construct at 5-µm thickness according to standard histological procedures. Sequential sections were stained with hematoxylin and eosin, toluidine blue, von Kossa's silver stain, and safranin O/fast green so that osseous and cartilaginous phenotypes could be distinguished. The same histologic preparations were used for *in vitro* constructs. Monolayer cultures were stained with either safranin O or von Kosssa and alkaline phosphatase stain. A fresh mixture of Naphthol, DMF (N, N-Dimethylformamide), Tris-HCl, and red violet LB salt (Sigma) stained monolayer cultures for alkaline phosphatase, followed by standard von Kossa staining.

RESULTS

Tissue-engineered mandibular condyles formed *de novo* in the dorsum of immunodeficient mice (Fig. 1A) from osteochondral constructs consisting of a single population of MSC-derived chondrogenic and osteogenic cells encapsulated in two stratified layers of PEG-based hydrogel. The tissue-engineered mandibular condyle measured 11 x 4 x 7 mm (length and height measurements in Figs. 1B and 1C, respectively), virtually the same size as the polyurethane mold of the human cadaver mandibular condyle (*cf.* Fig. 1E). Gross examination and physical manipulation indicated that the tissue-engineered mandibular condyles were opaque, firm, and retained the macroscopic shape of the cadaver human mandibular condyle from which the polyurethane mold was made (Figs. 1D, 1E).

Nodules of mineral deposits in the osteogenic layer containing MSC-derived osteogenic cells encapsulated in PEG-based hydrogel were revealed with von Kossa silver staining (the lower half of Fig. 2A). In contrast, the chondrogenic layer, consisting of MSC-derived chondrogenic cells encapsulated in PEG-based hydrogel, lacked mineralization nodules (the upper half of Fig. 2A). The chondrogenic layer contained sparse chondrocyte-like cells within abundant extracellular matrix (ECM) that reacted positively to safranin O (Fig. 2B). Multiple islands of dark-stained structures with H & E were present in the osteogenic layer, consisting of MSC-derived osteogenic cells encapsulated in PEG-based hydrogel (Fig. 2C). Osteoblast-like cells resided on the surface and in the center of these islands. These island structures reacted positively to toluidine blue, indicating their osteogenic phenotype (Fig. 2D). The control construct, consisting of hydrogel encapsulating untreated-MSCs, reacted negatively to safranin O, von Kossa, and toludine blue staining (data not shown).

Marrow-derived MSCs treated with chondrogenic medium in monolayer culture exhibited positive reaction to safranin O after four-week incubation in chondrogenic medium (Fig. 3A), whereas the same population of MSCs cultured without TGF-β1 showed negative reaction to safranin O (Fig. 3B). Positive reaction to safranin O was also evident after encapsulation of MSC-derived chondrogenic cells in PEG-based hydrogel,

especially in the matrix after four-week incubation of the hydrogel-MSC construct in the chondrogenic medium (Fig. 3C). In contrast, the same population of MSCs without exposure to chondrogenic medium prior to encapsulation in the hydrogel showed negative reaction to safranin O (Fig. 3D).

Marrow-derived MSCs in monolayer culture incubated for 4 wks in osteogenic medium reacted positively to both alkaline phosphatase (white arrow in Fig. 4A) and von Kossa silver stain (green arrow in Fig. 4A), indicating their osteogenic phenotype and mineral deposition, respectively. In contrast, the same population of MSCs without exposure to osteogenic medium showed negative reaction for both alkaline phosphatase and von Kossa staining (Fig. 4B). Further, the osteogenic constructs, consisting of PEG-hydrogel encapsulating MSC-derived osteogenic cells incubated in osteogenic medium for 4 wks, showed the formation of mineral nodules and positive reaction to von Kossa silver stain (Fig. 4C), whereas the same population of MSCs without exposure to osteogenic medium showed negative reaction to von Kossa staining (Fig. 4D). The PEG hydrogel encapsulating MSC-derived chondrogenic cells showed negative reaction to osteogenic markers such as von Kossa stain, whereas PEG hydrogel encapsulating MSC-derived osteogenic cells demonstrated negative reaction to chondrogenic markers such as safranin O (data not shown). In addition, control hydrogel constructs without any cells reacted negatively to both chondrogenic and osteogenic markers (data not shown).

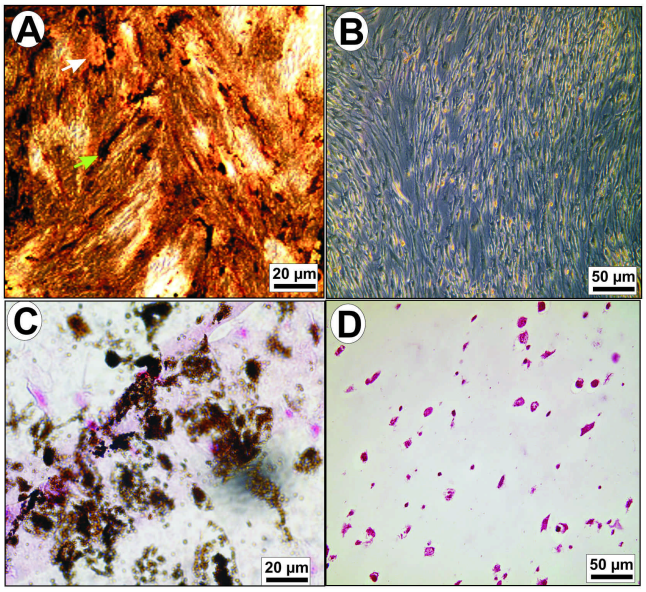

Figure 4. Osteogenesis driven by MSC-derived chondrogenic cells in *ex vivo* samples. **(A)** Positive reactions of MSC-derived osteogenic cells to alkaline phosphatase (white arrow) and von Kossa silver stain (green arrow) following four-week incubation in oseteogenic medium. **(B)** Monolayer culture of MSCs from the same population as in (A), cultured for 4 wks with DMEM/FBS but without osteoinduction factors, showed no positive reaction to either alkaline phosphatase or von Kossa silver stains. **(C)** Von Kossa silver-stained section of PEG-hydrogel encapsulating MSC-derived osteogenic cells showing mineral nodules. **(D)** The same population of MSCs encapsulated in the PEG-hydrogel construct without exposure to osteogenic medium showed no evidence of mineralization by von Kossa silver staining.

DISCUSSION

The present approach of engineered neogenesis of human-shaped mandibular condyle with stratified chondrogenic and osteogenic layers from a single population of rat bone marrow mesenchymal stem cells addresses several issues in this field. The outcome of tissue-engineered mandibular condyles from MSC-derived chondrogenic and osteogenic cells represents another step toward therapeutic applications of total joint replacement in comparison with approaches using isolated mature chondrocytes or osteoblasts (Niederauer *et al.*, 2000; Weng *et al.*, 2001). The differentiation of MSCs into chondrogenic cells and osteogenic cells *in vitro* is consistent with previous work (*e.g.*, Goldberg and Caplan, 1994; Schaefer *et al.*, 2000; Gao *et al.*, 2001), leading to active chondral and osseous matrix syntheses. Although the present encapsulation density of both MSC-derived chondrogenic and osteogenic cells at 5 x 10^6/mL has led to *in vivo* chondrogenesis and osteogenesis, the optimal densities of both MSC-derived chondrogenic and osteogenic cells should be determined.

Analysis of the present data demonstrates that MSC-derived chondrogenic and osteogenic cells continued their phenotypic differentiations both *in vitro* and *in vivo*. This is remarkable, since MSC-derived chondrogenic and osteogenic cells were encapsulated into the shape of a human mandibular condyle with a dimension of 11 x 4 x 7 mm. The *in vitro* osteogenic potential of MSC-derived osteogenic cells in the present work is evidenced by their positive reactions to alkaline phosphatase and von Kossa staining. *In vitro* chondrogenesis in the present work is evidenced by positive reaction to safranin O, a cationic dye that binds to cartilage-specific glycosaminoglycans such as chondroitin sulfate and keratan sulfate (Lammi and Tammi, 1988; Mao *et al.*, 1998; Wang and Mao, 2002). On the other hand, chondrogenesis and osteogenesis *in vivo* were demonstrated by strong safranin O labeling of the chondrocytes' extracellular matrix, and positive reaction to von Kossa staining as well as by the formation of dark HE-stained island structures occupied by osteoblast-like cells, respectively. Matrix synthesis by MSC-derived chondrogenic and osteogenic cells in 2 stratified, and yet integrated, layers of PEG hydrogel corroborates previous findings from the use of similar PEG-based hydrogel systems (Elisseeff *et al.*, 2000; Poshusta and Anseth, 2001; Burdick and Anseth, 2002; Halstenberg *et al.*, 2002; Martens *et al.*, 2003). In the present study, histological examination of the chondrogenic layer revealed abundant safranin-O-positive matrices of chondrocyte-like cells. In contrast, the present observation of osteoblast-like cells on both the surface and the center of toluidine-blue-positive island structures warrants further characterization for genetic and biochemical osteogenic markers. The selection of eight-week *in vivo* implantation was based on both our preliminary data and the anticipated clinical requirement for the shortest possible *ex vivo* incubation time (Temenoff and Mikos, 2000; Gao *et al.*, 2001; Altman *et al.*, 2002).

The use of a uniform polymer such as PEG-based hydrogel for both chondral and osseous components of osteochondral constructs has additional advantages, such as the ease of fabrication, and improved adhesion and interpenetration between the 2 layers (Lu and Anseth, 1999; Lee and Mooney,

2001; Nguyen and West, 2002). In the present study, physical manipulation of the *ex vivo* photopolymerized constructs and the harvested *in vivo* constructs failed to separate the 2 layers. PEGDA is biocompatible, biodegradable, and FDA-approved for several medical applications (Fu *et al.*, 2002). Despite a somewhat slow degradation rate, degradation of PEGDA in the present study is evident from both cell-associated matrix synthesis and formation of distinctive microscopic structures in both the chondrogenic and osteogenic layers. A common tendency associated with seeding cells in prefabricated three-dimensional scaffolds is their localization in the scaffold's surface (*e.g.*, Abukawa *et al.*, 2003). In the present study, loading MSC-derived chondrogenic and osteogenic cells in PEG hydrogel solution before photopolymerization likely has allowed for relatively homogenous cell distribution. On the other hand, copolymer may be necessary to promote differential needs of chondrogenesis and osteogenesis (Schaefer *et al.*, 2000; Gao *et al.*, 2001; Sherwood *et al.*, 2002).

Much additional work is needed before tissue-engineered mandibular condyles are ready for therapeutic use in patients suffering from osteoarthritis, rheumatoid arthritis, injuries, and congenital anomalies. A meritorious approach is *in vivo* growth factor delivery to maintain phenotypic differentiations of chondrogenic and osteogenic cells (Blunk *et al.*, 2002; Burdick *et al.*, 2002; Pei *et al.*, 2002a). The mechanical strength of tissue-engineered mandibular condyles must be enhanced so that they are capable of withstanding the mechanical stresses that normal condyles experience. Mechanical stresses with tailored peak magnitudes and frequencies are capable of modulating bone and cartilage growth at different levels of organization in both the appendicular and craniofacial skeletal lineages (Carter *et al.*, 1998; Goldstein, 2002; Mao, 2002; Wang and Mao, 2002; Kopher and Mao, 2003). Recently, both hydrodynamic stresses and bioreactors have been shown to enhance the biophysical properties of tissue-engineered cartilage constructs (Buschmann *et al.*, 1995; Vunjak-Novakovic *et al.*, 1999; Mauck *et al.*, 2000; Altman *et al.*, 2002; Davisson *et al.*, 2002; Pei *et al.*, 2002b). The enhancement of mechanical properties of tissue-engineered mandibular condyles likely will be a critical step toward clinical applications. Nonetheless, the present findings represent a proof of concept for further development of tissue-engineered mandibular condyles.

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