Tissue-Engineered Osteochondral Constructs in the Shape of an Articular Condyle

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Background: An entire articular condyle engineered from stem cells may provide an alternative therapeutic approach to total joint replacement. This study describes our continuing effort to optimize the chondrogenic and osteogenic differentiation from mesenchymal stem cells toward engineering articular condyles in vivo.

Methods: Primary rat bone-marrow mesenchymal stem cells were induced to differentiate into chondrogenic and osteogenic lineages in vitro and were suspended in polyethylene glycol-based hydrogel. The hydrogel cell suspensions, each at a density of 20 × 10^6 cells/mL, were stratified into two separate layers that were molded into the shape and dimensions of an adult human cadaveric mandibular condyle by sequential photopolymerization. The osteochondral constructs fabricated in vitro were implanted in the dorsum of immunodeficient mice for twelve weeks.

Results: De novo formation of articular condyles in the shape and dimensions of the adult human mandibular condyle occurred after a twelve-week period of in vivo implantation. Histological evaluation demonstrated two stratified layers of cartilaginous and osseous tissues, and yet there was mutual infiltration of cartilage-like and bone-like tissues into each other's territories. The cartilaginous portion was stained intensively to safranin O and expressed immunolocalized type-II collagen. Chondrocytes adjacent to the tissue-engineered osteochondral junction were enlarged and expressed type-X collagen, typical of hypertrophic chondrocytes. The osseous portion contained bone trabeculae-like structures and expressed immunolocalized type-I collagen, osteopontin, and osteonectin.

Conclusions: A cell encapsulation density of 20 million cells/mL with in vivo incubation for twelve weeks yields further tissue maturation and phenotypic growth of both cartilage-like and bone-like tissues in the tissue-engineered articular condyle.

Clinical Relevance: Tissue engineering of an entire condyle with chondral and osseous components derived from a single population of adult stem cells, as described in the present study, may have therapeutic implications in total joint replacement.

The prevalence of synovial joint degeneration has motivated tremendous advances in the field of bone and cartilage tissue engineering in the past decade. Despite ongoing applications of cell and tissue-based therapies, such as mosaicplasty and chondrocyte transplantation for the treatment of articular cartilage defects, prosthetic replacement of the entire articular condyle continues to be the predominant practice for total joint replacement. Alternative therapeutic approaches for large osteochondral defects and condylar replacement, including autografts, allografts, and xenografts, are also used to replace small synovial joints, such as the phalanges and the temporomandibular joint. Collectively, prosthetic implants and grafting procedures share certain deficiencies, such as implant dislocation, wear, suboptimal biocompatibility, donor site limitation and morbidity, immunological challenge, and potential pathogen transmission. An entire articular condyle derived from adult stem cells should overcome most of the deficiencies associated with the current prosthetic and grafting approaches.

Adult mesenchymal stem cells are capable of differentiating into all connective tissue cell lineages, including cartilaginous and osseous phenotypes. For the purpose of bone and cartilage tissue engineering, adult mesenchymal stem cells have an advantage over embryonic stem cells or differentiated osteoblasts and chondrocytes because of the shorter differentiation journey that they may have toward their natural derivatives such as bone and cartilage. Since mesenchymal stem cells can be harvested readily by needle aspiration, donor site morbidity may be reduced compared with that associated with procedures for harvesting autologous bone grafts. Although mature os-
teoblasts can also be harvested in the same fashion, mesenchymal stem cells are more expandable ex vivo than are mature osteoblasts. The isolated mesenchymal stem cells can be reliably induced to differentiate into bone-forming and cartilage-forming cells after exposure to well-established osteogenic and chondrogenic supplements in cell culture, respectively.

Our previous work has demonstrated, with use of different outcome measurements, the feasibility of tissue engineering of an entire articular condyle with stratified cartilaginous and osseous components from a single population of adult mesenchymal stem cells. However, in our previous work, we used a cell encapsulation density of 5 million cells/mL for each of the chondrogenic and osteogenic components and up to four and eight weeks of in vivo implantation. Although this cell encapsulation density led to in vivo chondrogenesis and osteogenesis, the level of tissue formation needed to be improved. As argued in our previous work, because cell densities at given stages of natural synovial joint development are unknown, cell encapsulation densities need to be explored in tissue-engineered articular condyles. Accordingly, the present study was designed to tissue engineer articular condyles with both cartilaginous and osseous components from a single population of adult stem cells in the shape and dimensions of an adult human cadaveric mandibular condyle by increasing the cell encapsulation density to 20 million cells/mL followed by an extended twelve-week period of in vivo implantation in immunodeficient mice.

Materials and Methods
Isolation and In Vitro Cultivation of Mesenchymal Stem Cells

Bone marrow-derived mesenchymal stem cells were harvested from two to four-month-old (200 to 250-g) male Sprague-Dawley rats (Harlan, Indianapolis, Indiana). Following CO2 asphyxiation of the rats, the tibias and femora were dissected under aseptic conditions. The whole bone marrow plugs were flushed out with use of a 10-mL syringe with Dulbecco modified Eagle medium-low glucose (DMEM-LG; Sigma, St. Louis, Missouri) supplemented with 10% fetal bovine serum (Biocell, Rancho Dominguez, California) and 1% antibiotic-antimycotic (Gibco, Carlsbad, California).

Marrow samples were collected and mechanically disrupted by passage through 16, 18, and 20-gauge needles. Cells were centrifuged, resuspended in serum-supplemented medium, counted, and plated at 5 x 10⁶ cells/100-mm culture dish and incubated in 95% air and 5% CO2 at 37°C, with fresh medium change every three to four days. Upon formation of large colonies (typically after two to three weeks), primary mesenchymal stem cells were trypsinized, counted, and passaged at a density of 5 to 7 x 10⁶ cells/100-mm dish. All animal experiments received approval from the institutional animal care committee.

Treatment of Mesenchymal Stem Cells with Chondrogenic and Osteogenic Supplements

First-passage mesenchymal stem cells were cultured separately in chondrogenic or osteogenic medium for one week. Chondrogenic medium contained a supplement of 10 ng/mL TGF-β1 (transforming growth factor-β1; RDI, Flanders, New Jersey), whereas osteogenic medium contained 100 nm dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate (Sigma). Monolayer mesenchymal stem-cell cultures for control constructs were grown for the same period in a basic culture medium consisting of Dulbecco modified Eagle medium, fetal bovine serum, and antibiotic-antimycotic solution but without any chondrogenesis or osteogenesis-inducing supplements. All cultures were incubated in 95% air and 5% CO2 at 37°C with medium changes every three to four days.

Preparation of Polyethylene Glycol-Based Hydrogel-Photoinitiator Solution

Polyethylene glycol diacrylate (MW 3400; Shearwater Polymers, Huntsville, Alabama) was dissolved in sterile phosphate-buffered saline solution supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) to a final solution of 10% weight per volume. A photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Ciba, Tarrytown, New York), was added to the polyethylene glycol diacrylate solution to obtain a final photoinitiator concentration of 0.05% weight per volume.

Fabrication and In Vivo Implantation of Osteochondral Constructs

After a one-week incubation in medium with either chondrogenesis-inducing or osteogenesis-inducing supplements, mesenchymal stem cell-derived chondrogenic and osteogenic cells were trypsinized, counted, and resuspended in polyethylene glycol diacrylate polymer-photoinitiator solution at a density of 20 x 10⁶ cells/mL. A 150-µL aliquot of cell-polymer suspension with mesenchymal stem cell-derived chondrogenic cells was loaded into a hollow bivalved polysiloxane negative mold that had been previously fabricated from a positive replica of an adult human cadaveric mandibular condyle (Fig. 1, A and B). The chondrogenic layer was photopolymerized by a long-wave, 365-nm ultraviolet lamp (Glomark, Upper Saddle River, New Jersey) at an intensity of approximately 4 mW/cm² for five minutes. A cell-polymer suspension containing mesenchymal stem cell-derived osteogenic cells (approximately 600 µL) was then loaded to occupy the remainder of the mold followed by the same photopolymerization protocol. The polymerized osteochondral constructs were then removed from the mold, washed twice with phosphate-buffered saline solution, and implanted in the subcutaneous pockets in the dorsum of SCID (severe combined immunodeficient strain) mice (Harlan), which had been prepared by blunt dissection under general anesthesia with an intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg xylazine. A total of six fabricated osteochondral constructs and five control constructs were implanted in three immunodeficient mice. Among the five control constructs, three constructs encapsulated mesenchymal stem cells grown in basic medium without either chondrogenesis or osteogenesis-inducing supplements, and two control hydrogel constructs were cell-free.
Histological and Immunohistochemical Phenotyping of Tissue-Engineered Osteochondral Constructs

After a twelve-week period of in vivo implantation in the dorsum of the immunodeficient mice, harvested experimental osteochondral constructs and control hydrogel constructs were fixed in 10% formalin overnight, decalcified in 0.5-M EDTA solution, and embedded in paraffin with use of standard histological procedures. Sequential sections were stained with either hematoxylin and eosin or safranin O-fast green (Sigma). Serial consecutive sections adjacent to those used for histological examination were deparaffinized, washed in phosphate-buffered saline solution, and digested for thirty minutes at room temperature with bovine testicular hyaluronidase (1600 U/mL) in sodium acetate buffer, pH 5.5, with 150 mM of sodium chloride. Sections were treated with 5% bovine serum albumin for twenty minutes at room temperature to block nonspecific reactions. All antibodies for immunohistochemical analysis were obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa). Type-I, II, and X collagens were immunolocalized with monoclonal antibodies to type-I collagen (SP1.D8; 1:2)\(^{18}\), type-II collagen (II-I6B3; 1:2)\(^{19}\), and type-X collagen (X-AC9; 1:2)\(^{20}\), respectively. Osteopontin and osteonectin were immunolocalized with monoclonal antibodies to osteopontin (MPIIIB10; 1:2)\(^{21}\) and to osteonectin (AON-10; 1:4)\(^{22}\). After overnight incubation with the primary antibody in a humidity chamber, sections were rinsed with phosphate-buffered saline solution and incubated with immunoglobulin G anti-mouse secondary antibody (1:500; Antibodies Incorporated, Davis, California) for thirty minutes. Sections were then incubated with streptavidin-horseradish peroxidase conjugate for thirty minutes in a humidity chamber. After washing in phosphate-buffered saline solution, the double-linking procedure with the secondary antibody was repeated. Slides were developed with diaminobenzidine solution and were counterstained with Mayer hematoxylin for three to five minutes. Counterstained slides were dehydrated in graded ethanol and cleared in xylene. The same procedures were performed for negative controls except for the omission of the primary antibody. In addition, all histological and immunohistochemical characterization procedures were performed for positive-control normal rat mandibular condyles obtained from adult Sprague-Dawley rats (Harlan) that were age-matched to those from which the mesenchymal stem cells were originally harvested.

Results

Gross Examination of Tissue-Engineered Articular Condyles

Following twelve weeks of in vivo implantation in the dorsum of immunodeficient mice, osteochondral constructs in the shape and dimensions of real-sized adult human mandibular condyle formed de novo (Fig. 1). The tissue-engineered articular condyles measured 18 mm long by 9 mm wide by 11 mm high (Fig. 1, C), proportionally analogous to...
the original adult human cadaveric mandibular condyle and the acrylic positive replica of the mandibular condyle. The constructs were firm and unyielding upon physical manipulation and demonstrated multiple areas of calcification upon radiographic examination (Fig. 1, D). The chondrogenic and osteogenic layers of the tissue-engineered osteochondral constructs were inseparable with no observable seam at the interface, signifying positive material integration between the two stratified hydrogel layers.

**Histogenesis of Chondral and Osseous Tissues and Immunohistochemical Phenotyping**

The chondrogenic and osteogenic layers of the tissue-engineered articular condyles demonstrated distinctive histological characteristics (Fig. 2). Histological examination of the interface between the top polyethylene glycol-based hydrogel layer encapsulating the chondrogenic cells derived from mesenchymal stem cells and the bottom polyethylene glycol-based hydrogel layer encapsulating the osteogenic cells derived from mesenchymal stem cells revealed distinctive phenotype-specific characteristics of cartilage-like and bone-like tissues, respectively (Fig. 2, A and B). The chondrogenic and osteogenic portions of the tissue-engineered articular constructs largely remained in their respective layers (Fig. 2, A and B). However, there was mutual infiltration of the cartilaginous and osseous components into each other’s territory (Figs. 2, A and B; 3, A; and 4, A, C, and D), especially in comparison with our previous work of four-week and eight-week periods of in vivo

![Fig. 2](image)
incubation. This mutual infiltration of cartilage-like and bone-like tissues may resemble, to a rudimentary degree, the normal osteochondral interface of the mandibular condyles in an age-matched normal rat (Fig. 2, C and D).

The chondrogenic layer consisted of chondrocyte-like cells in lacunae and surrounded by an abundant intercellular matrix that showed intense reactions to cartilage-specific safranin-O staining (Fig. 2, B). Type-II collagen was immunolocalized throughout the chondrogenic portion (Fig. 3, A). Sparse areas of positive reaction to safranin O and immunolocalized type-II collagen were also observed within the osteogenic layer near the osteochondral interface (Figs. 2, B and 3, A). The deep chondrogenic layer adjacent to the tissue-engineered osteochondral junction consisted of cells with a hypertrophic appearance (Fig. 2, A and B) and expressed type-X collagen (Fig. 3, B), characteristic of the extracellular matrix of hypertrophic and degenerating chondrocytes. Type-II collagen immunolocalization within the cartilaginous portion and at the osteochondral interface in a normal mandibular condyle from an age-matched rat (Fig. 3, C) showed rudimentary morphological resemblance to type-II collagen immunolocalization observed in tissue-engineered condyle constructs (Fig. 3, A). Control constructs encapsulating mesenchymal stem cells that were not preconditioned with chondrogenesis-inducing or osteogenesis-inducing supplements lacked the osteochondral organization, and they were mostly negative to immunolocalization of chondrogenic and osteogenic markers (Fig. 3, D).

Histological examination of the control (cell-free) polyethylene glycol diacrylate hydrogel scaffolds revealed the intact borders of the hydrogel material, the absence of infiltration of the hydrogel by the host cells, and a lack of immunolocalization of chondrogenic or osteogenic markers (Fig. 4, B).

The osseous portion of the tissue-engineered articular condyle showed bone trabeculae-like structures that were occupied by cells both on the surface and in the center that were embedded within abundant extracellular matrix (Figs. 2, A and B; 3, A; and 4). Type-I collagen, osteopontin, and osteonectin were immunolocalized in this osteogenic layer of the tissue-engineered articular condyle from an age-matched rat (Fig. 3, C) showed rudimentary morphological resemblance to type-II collagen immunolocalization observed in tissue-engineered condyle constructs (Fig. 3, A). Control constructs encapsulating mesenchymal stem cells that were not preconditioned with chondrogenesis-inducing or osteogenesis-inducing supplements lacked the osteochondral organization, and they were mostly negative to immunolocalization of chondrogenic and osteogenic markers (Fig. 3, D). Histological examination of the control (cell-free) polyethylene glycol diacrylate hydrogel scaffolds revealed the intact borders of the hydrogel material, the absence of infiltration of the hydrogel by the host cells, and a lack of immunolocalization of chondrogenic or osteogenic markers (Fig. 4, B).

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engineered osteochondral constructs, whereas the chondrogenic layer lacked positive immunolocalization of these bone markers (Fig. 4, A, C, and D). The osseous portion of the tissue-engineered articular condyle showed negative reaction to safranin O (Fig. 2, B) and a lack of immunolocalization of type-II collagen (Fig. 3, A).

**Discussion**

The present study is a necessary extension of our previous work, as we increased the encapsulation density of adult stem-cell-derived tissue-forming cells in osteochondral constructs from 5 million to 20 million cells/mL, with an extended in vivo implantation period of twelve weeks. Because increasing the in vivo cultivation period alone from four weeks to eight weeks without increasing the initial cell density (5 million cells/mL) failed to enhance the maturation level of the tissue-engineered structures, we believe that the greater encapsulation density (20 million cells/mL), in addition to the longer in vivo implantation period (twelve weeks), probably accounts for the advanced tissue maturation stage of the engineered bone-like and cartilage-like tissues seen in the present study. This observation is in agreement with similar cell-based tissue-engineering investigations. For example, increased maturation of tissue-engineered cartilage is observed upon seeding greater densities of isolated chondrocytes. Moreover, clustering of marrow stromal cells, as observed in the present study of clustering of mesenchymal stem cell-derived chondrocytes and osteoblasts, enhances their differentiation toward the osteogenic lineage.

The results of the present study substantiate the findings in our previous reports on the feasibility of de novo formation of osteochondral constructs in the shape of a small human articular condyle with two stratified layers of chondrogenic and osteogenic histogenesis from a single population of bone marrow-derived chondrogenic and osteogenic cells encapsulated in a hydrogel scaffold. The exposure of mesenchymal stem cell cultures to the media containing chondrogenesis-inducing and osteogenesis-inducing supplements for one week prior to in vivo implantation is aligned with our previous work and the work done by others. This short...
ex vivo manipulation period may facilitate eventual therapeutic applications. The presence of abundant matrix biosynthesis and immunolocalization of chondrogenic and osteogenic markers in corresponding layers of the tissue-engineered articular condyles indicates continuing phenotypic differentiation of chondrogenic and osteogenic cells from a single population of mesenchymal stem cells. The morphological appearance of the tissue-engineered costochondral interface and the expression of cartilaginous and osseous markers within the corresponding chondrogenic and osteogenic layers rudimentarily resembles that of a native articular condyle.

Specifically, mutual infiltration of tissue-engineered cartilaginous and osseous tissues offers encouraging morphological evidence of interactions between mesenchymal stem cell-derived chondrogenic and osteogenic cells stratified in two hydrogel layers. The appearance of hypertrophic chondrocyte-like cells with immunolocalization of type-X collagen in the deep region of the chondrogenic layer likely indicates a transformation from chondrocyte hypertrophy to osteogenic phenotype. In support of this assumption is the continuing expression, although sparse and less intense, of chondrogenic markers such as type-II collagen and safranin O within the osteogenic portion of the tissue-engineered osteochondral construct, pointing to a phenotypic transition at the osteochondral interface. The nature of scattered tissue formation in the osteogenic layer, evident from both radiographic and histological images, appears to suggest that in the absence of directional cues that are embedded in cells during development, mesenchymal stem cell-derived osteogenic cells demonstrate that cell-to-cell contact may be necessary for continuing cell differentiation and matrix synthesis. In addition, longer in vivo cultivation and the application of mechanical stresses may accelerate tissue maturation.

Although previous approaches to the engineering of osteochondral constructs with use of isolated mature chondrocytes and osteoblasts have yielded important data, upon which the present study was based, the current work on tissue-engineering of articular condyles with both cartilaginous and osseous components from a single population of adult mesenchymal stem cells may represent another step toward the eventual goal of an alternative total joint replacement therapy. The design of osteochondral constructs in the shape and dimensions of a human articular condyle is likely a key parameter since cell survival and viability are increasingly challenging for large three-dimensional scaffolds used for bone and cartilage tissue engineering. The stratified fabrication of the osteochondral constructs from a single hydrogel system in the present study has the potential to promote physical integration between their cartilaginous and osseous components and to minimize the potential ingrowth of host fibrous tissue between the chondrogenic and osteogenic components after in vivo implantation. An empirical concern coupled with seeding cells in prefabricated three-dimensional scaffolds is the tendency for the seeded cells and the synthesized extracellular matrix to localize toward the outer surface of the scaffold, where most cells are initially seeded. The present work has adopted several approaches to encapsulate mesenchymal stem cell-derived chondrogenic and osteogenic cells in the aqueous phase of the polyethylene glycol-based hydrogel, thus maximizing the possibility of homogeneous cell distribution in the hydrogel and encouraging de novo tissue formation from within the photopolymerized constructs. The ease of fabrication and the ability to mold the photopolymerizable hydrogel systems into given proportions are advantages toward their potential applications for tissue engineering of osteochondral constructs.

Despite the advantages described above, a potential concern associated with polyethylene glycol-based hydrogel is the degradation rate. Increasing efforts are being made to investigate the enhancement of the degradation rate of the polyethylene glycol-based hydrogel system by the addition of degradable linkages to the macromere backbone such as polyester and phosphate groups. Our experience with in vivo implantation of the chondrogenic and osteogenic cells derived from rat mesenchymal stem cells encapsulated in a polyethylene glycol diacrylate hydrogel has revealed a positive relationship between the hydrogel degradation and the period of in vivo implantation. Nonetheless, the experimental divergence in our approach with ex vivo incubation of mesenchymal stem cells with chondrogenesis-inducing and osteogenesis-inducing supplements prior to the hydrogel encapsulation, the environmental differences between in vivo and in vitro incubation, and the extended implantation periods may have contributed to the differences observed in the degradation behavior of polyethylene glycol diacrylate relative to that seen in other studies. The ultimate goal of the present approach is to tissue engineer autologous articular condyles ex vivo that are structurally and functionally sound to serve as replacements for missing or degenerated articular condyles. However, a number of challenging issues need to be addressed before reaching this ambitious goal. Larger animal models are probably necessary to test the feasibility of in vivo implantation of tissue-engineered articular condyles derived from autologous mesenchymal stem cells. In addition, the tissue maturation and the mechanical strength of the tissue-engineered articular condyles should be attained ex vivo before in vivo implantation.

Zonal organization of articular cartilage is of structural and functional importance. Thus, tissue-engineered articular cartilage may need to incorporate appropriate molecular cues to recapitulate the developmental process of normal articular cartilage. The present selection of a photopolymerizable hydrogel system may facilitate this goal. A recent study has shown successful fabrication of a multilayered articular cartilage construct with zonal organization by encapsulating bovine chondrocytes from corresponding zones of the femoral articular cartilage. Encapsulating growth factors for the in vivo modulation of chondrogenesis and osteogenesis may be necessary for further differentiation and phenotypic maintenance of mesenchymal stem cell-differentiated chondrogenic and osteogenic cells in an in vivo environment. The most challenging task appears to be functional enablement of tissue-engineered articular condyles to withstand the mechanical loads that are experienced by normal articular condyles.
Increasing evidence of the potential enhancement of the extracellular matrix properties of both bone and cartilage by mechanical modulation is recognized as functional tissue engineering, a field that currently studies the responses of progenitor cells to mechanical stresses with an ultimate goal of enhancing the maturation and mechanical strength of tissue-engineered structures\textsuperscript{1-3}. Much additional work is warranted along several fronts before the present approach can be used for therapeutic applications.

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