Phenotypic changes in proliferation, differentiation, and migration of chondrocytes: 3D *in vitro* models for joint wound healing

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Abstract: We aim to establish a 3D model of cartilage wound healing, and explore the involvement of chondrocytes in its repair. To characterize chondrocyte involvement in wound healing, an *in vitro* 3D model composed of chondrocyte mixing with either type II/I collagen or type I collagen matrix was established. The "defects" measuring 5 mm in diameter were made on each collagen matrix-chondrocyte construct to mimic *in vivo* cartilage defects. The effects of basic fibroblast growth factor (bFGF) on chondrocytes migration and differentiation were studied. The migration and Glucosaminoglycan (GAG) synthesis of chondrocytes in the defect areas were observed by microscopy after Alcian-blue staining. In the presence of bFGF, GAG expression increased significantly when chondrocytes were cultured in type II/I collagen matrix compared to type I collagen matrix. However, mild GAG accumulation was also found when cells were cultured in either type I or type II/I collagens without bFGF. In a 3D model of cartilage wound healing, bFGF promote chondrocyte proliferation, migration and differentiation in the presence of type II/I collagen matrix, and showed potential to regulate wound healing. These wound healing models may provide feasible methods to explore various drugs prior to human trials. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 1115–1122, 2010

Key words: chondrocyte; collagen; basic fibroblast growth factor; cartilage; wound healing

INTRODUCTION

Articular cartilage has a limited capacity to heal itself. If injury persists, osteoarthritis (OA) may result.[1,2] The mechanism underlying OA features chondrocyte degeneration and insufficient repair of cartilaginous extracellular matrices (ECMs).[3]

Chondrocytes are reported to gradually lose their capacity to synthesize cartilage-specific type II collagen in the arthritic joint and increase the synthesis of type I collagen.[4,5] The change in collagen typing is the major feature of fibrosis during wound healing.[6] Once the cartilage is damaged, the three-dimensional (3D) structure of the ECM is destroyed. Biocompatible 3D scaffolds such as collagen or polymeric matrices incorporated with growth factors have been used in attempts to decrease fibrosis and enhance wound healing of cartilage and skin defects.[7–9] Therefore, collagen 3D scaffolds are designed to act as carriers for the implantation of cells into cartilage defects. The structure of scaffolds should be able to maintain

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the chondrocyte phenotype. Furthermore, the porosity of a well-seeded matrix is crucial to facilitating chondrocyte migration and proliferation within the scaffold and to decrease fibrosis/scar formation.[10]

After serial passages in Petri dishes, chondrocytes appear flattened and produce type I collagen, a phenomena called "dedifferentiation." However, if chondrocytes are grown in 3D ECM structures, they maintain a differentiated phenotype and function. Collagen 3D scaffolds can also recruit chondrocytes to reside and proliferate[11] within them.

Several *in vitro* and *in vivo* models have been developed to study the mechanisms of joint wound healing, and evaluate arthritic changes. Animal models include the rat,[12] rabbit,[13] ovine,[14] canine,[15,16] and equine.[17] Most *in vivo* animal models involve creation of a defect and repair of the defect with various scaffolds in the presence or absence of various cells. The *in vivo* models have limitations such as an inability to control temperature and unpredictable biological conditions that can be overcome by *in vitro* models. Attempts also have been made in bovine[18] and rabbit[19] explant culture to investigate cartilage degeneration or repair, although tissue function and utilization are still limited. An *in vitro* model that mimics the *in vivo* environment would afford greater versatility in experimental design. In addition, once the preliminary projects are conducted in an *in vitro* environment, *in vivo* animal and human experiments can proceed.

Basic fibroblast growth factor (bFGF) has been shown to play a crucial role in maintaining chondrogenic capacity of expanded chondrocytes and cartilage development.[20–23] In the present study, an *in vitro* 3D model is used to assess the respective effects of type I collagen, type II collagen, and bFGF on chondrocytes.[24] Numerous studies have now shown the importance of chondroitin sulfate and *heparan* sulfate GAGs on cell and ECM proteoglycans in the binding of a variety of cell signaling molecules. Specifically, the sulfated saccharide within the GAG chains allows the binding and regulation of signaling molecules, thereby regulating the intracellular signaling pathways that affect differentiation and matrix synthesis. In particular, chondroitin sulfate GAG acts as a biomarker for cartilage progenitors.[25] We therefore utilize Glucosaminoglycan (GAG) expression as a biomarker for the chondrocyte phenotype in our study.[26]

METHODS

Isolation and cultivation of primary chondrocytes

Primary chondrocytes isolated from newborn New Zealand white rabbit hyaline cartilage were previously described in detail.[27,28] The subepithelium was obtained from rat skin. Cartilage and skin were separately sliced and incubated in Hank's balanced salt solution (HBSS) (Gibco) containing 1 mg/mL collagenase (Sigma), and 1 mg/mL hyaluronidase (Sigma) overnight. The chondrocytes and fibroblasts (8×10^5 cell per dish) obtained by this method were seeded on 10-cm Petri dishes in DMEM/F12 medium (Hyclone) with 10% fetal bovine serum (FBS) (Gibco/Invitrogen), 50 μg/mL gentamycin sulfate (Gibco), 100 units/mL penicillin G sodium (Gibco), 100 μg streptomycin sulfate (Gibco) and 0.25 μg/mL fungizone (Gibco). Cells were cultured in a 5% CO_2 incubator at 37°C.

Analysis of collagen typing of rabbit chondrocytes

β-aminopropionitrile (β-APN) at 100 μg/mL and 50 μg/mL ascorbic acid were added to the chondrocytes after they reached confluence at the following two time points: passage 1 (day 13) and passage 2 (day 18), and cultured in a 5% CO_2 incubator at 37°C for 48 h. Cells were collected with the medium. The cell culture was supplemented with ammonium sulfate (25%, weight/volume) and stood at 4°C overnight. After centrifuging, the supernatant was discarded, and the pellet was re-dissolved in 10 mL 0.5*M* acetic acid at 4°C for 24 h. The precipitate was discarded after centrifugation. Crystal sodium chloride was added to the supernatant to a final concentration of 0.9*M*. It was then stirred vigorously and stood at 4°C for 2 h. After centrifugation, the pellet was dissolved in 5 m*M* acetic acid. The collagen typing was analyzed using 8% SDS-PAGE.

Manufacture of engineered cartilage-3D model

Engineered neocartilage was manufactured (1 mm in thickness and 35 mm in diameter; 80% type II collagen mixed with 20% type I collagen) as described in a previous report[28] (Fig. 1). The first-passage chondrocytes were recovered and suspended to a concentration of 2×10^6 cells/mL in 2× concentrated DMEM/F12 medium with 20% fetal bovine serum. Cells were then mixed with an equal volume of a 2 mg/mL collagen solution consisting of 80% type II collagen and 20% type I collagen in 5 m*M* acetic acid. The collagen-cell mixture was incubated at 37°C for gel polymerization. After formation of the collagen matrix-chondrocyte construct, 2 mL DMEM/F12 medium with 10% fetal bovine serum and 50 μg/mL L-ascorbic acid were added. The medium was changed once every 2 days for 4 weeks.

Cartilage defect and wound healing model

Seven "defects" measuring 5 mm in diameter were made on each collagen matrix-chondrocyte construct to mimic *in vivo* cartilage defects. Type I collagen only or the combined collagens made of 80% type II collagen and 20% collagen solution (Type II/I matrix)) (final, 1 mg/mL) was applied into the "defect" and allowed to form a cell-free matrix for the subsequent tests. The defect created-collagen matrix- chondrocyte constructs were transferred to 6-well plates containing DMEM/F12, 10% FBS, 50 mg/mL L-

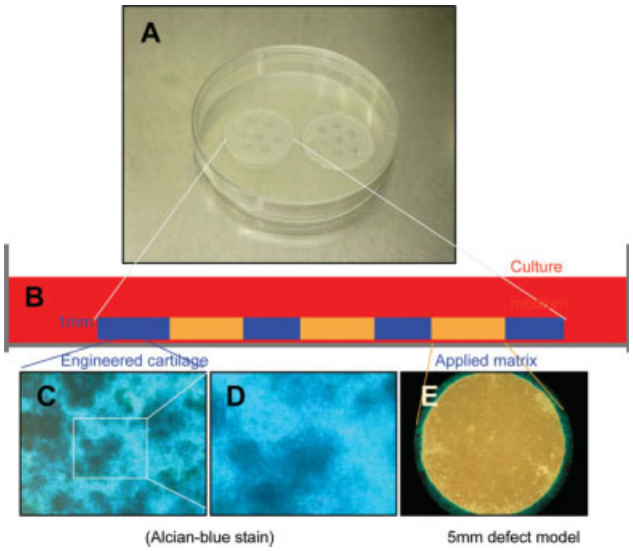

Figure 1. A 3D model for cartilage wound healing. As shown in (A) and (B), the engineered 1 mm thick cartilage disc contains seven defects measuring 5 mm in diameter. The defects were repaired with the cell-free matrices made of type II/I or I collagen alone (A–E). Note that mature chondrocytes and extracellular matrix were stained with Alcian blue (C, D), chondrocyte migration can be observed (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ascorbic acid (Merck), and 1× antibiotics (penicillin/streptomycin). Three collagen-cell constructs were made for each condition, and each construct contained seven collagen matrices. Total 21 samples were used for each condition. Migration distance was measured from circumference toward the center of the defect. The migration area of the defect was divided into quarters, and the three maximal distances measured. The mean of 12 maximal migration distances was considered to be the migration distance. After the cell-free collagen matrices had formed, the media were supplemented with or without 10 ng/mL bFGF (R&D, Minneapolis, MN). The medium was changed once every 2 days for 4 weeks [Fig. 1(A–E)].

The migration and GAG synthesis of chondrocytes in the defect areas were observed by microscopy after Alcian-blue staining respectively on the10th, 14th, 18th, and 26th days [Fig. 3(A)]. Spectrum absorption was used at 600nm after the GAG-retained Alcian blue was eluted with extraction buffer, 4*M* gluanidine-HCl with 33% n-propyl alcohol[26] to estimate the relative expression of GAG. Effects of bFGF on the cartilage defects were further determined.

Histological evaluation

Collagen matrix-chondrocyte constructs were removed on days 18 and 26. The constructs were grossly examined and fixed in 10% formalin, embedded in paraffin, and serially sectioned (Sacura Sledge microtome) at 5-10 μm. Tissue sections were respectively stained with Alcian-blue, and Hematoxylin. The results were examined under light microscopy and recorded by photography.

Statistical analysis

The data of migration, and expressions of GAG from chondrocyte cultures incorporated with collagens and with or without bFGF, were analyzed using Student's *t*-test. Data are reported as the mean ± SD. $p < 0.05$ was considered statistically significant. All analyses were performed in SAS for Windows version 9.0.1 (SAS Institute, Chicago, IL).

RESULTS

Analysis of collagen typing of rabbit chondrocytes in cell culture

During the entire course of the study, chondrocytes in cell culture contained primarily type II collagen on day 1 (passage 1) with a small quantity of type I collagen. On day 18 (passage 2), chondrocytes showed a decrease in the synthesis of type II collagen and an increase in type I collagen (Fig. 2).

The effects of collagen and bFGF on the migration and GAG synthesis of chondrocytes in 3D cartilage defect model

In the presence of bFGF (from days 10 to 26), the migration pattern in the type I matrix appeared slightly faster compared with the type II/I matrix; however, the differences were not statistically significant [Fig. 3(A,B)]. Chondrocytes migrated approximately 2.25 mm on day 26 in constructs of either collagen with or without bFGF [Fig. 3(A,C)].

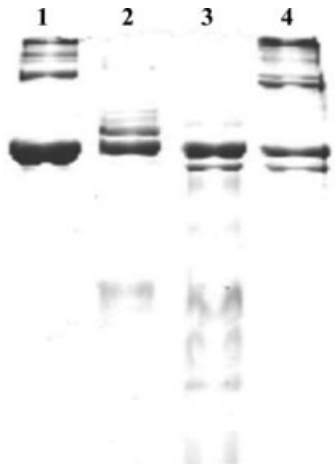

Figure 2. PAGE analysis of collagens extracted from chondrocytes at various days of culture. Lanes 1 and 4 are standard type II and I collagen, respectively. Lane 2 shows a major band of type II collagen on day 1. Note that lane 3 exhibits a decrease in type II collagen and an increase in type I collagen synthesis on day 18.

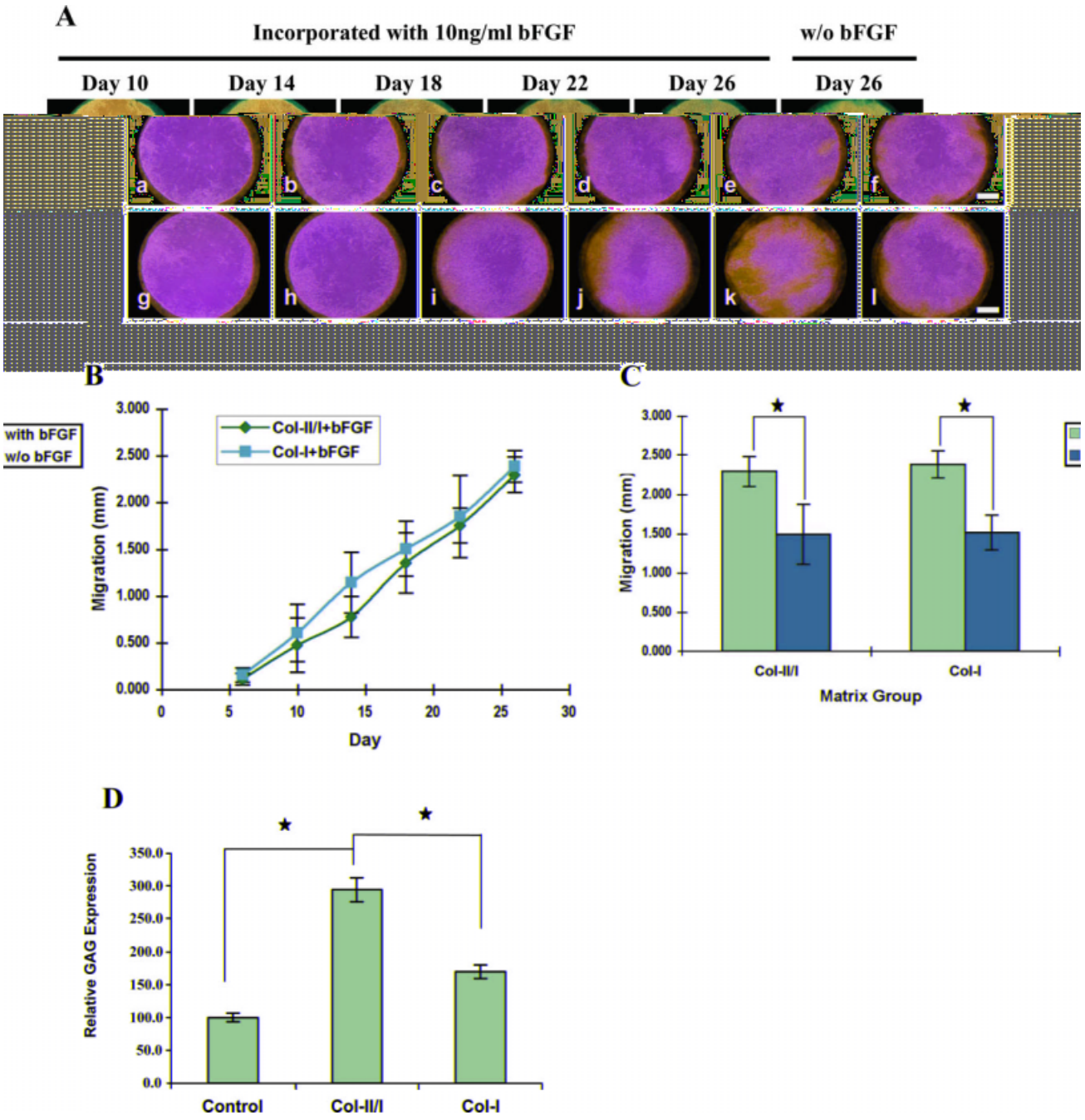

Figure 3. Effects of collagen and basic fibroblast growth factor (bFGF) on migration, proliferation, and differentiation of chondrocytes in a 3D cartilage defect model. A: Microscopic observation of cell migration, proliferation, and GAG synthesis with and without bFGF in the 3D cartilage wound healing model. In the presence of type I collagen with bFGF, migration and proliferation were observed on days 10, 14, 18, 22, and 26 (a to e) and without bFGF on day 26 (f). In the presence of type II/I collagen with bFGF data were shown on days 10, 14, 18, 22, and 26 (g to k), and without bFGF on day 26 (l). GAG synthesis of chondrocytes in the defect areas were observed by microscopy after Alcian-blue staining (green color). B: Effects of type I and type II/I collagen on chondrocyte migration in the presence of bFGF. No significant difference in chondrocyte migration was found on day 26 between the 2 types of collagen matrices with bFGF. C: Effects of bFGF on chondrocyte migration in type I and II/I collagen matrices. Note that incorporation with bFGF induced more-rapid migration of chondrocytes in both types of collagen matrices compared with those without bFGF. No significant difference was seen between type I and type II/I collagen matrices. D: Incorporation with bFGF led to a significant increase in glycosaminoglycan (GAG) synthesis of chondrocytes observed in the type II/I collagen matrix, compared with the type I collagen matrix on day 26. Type I collagen matrix on day 18 was used as the control. Mean ± SD; (n = 21, $p < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

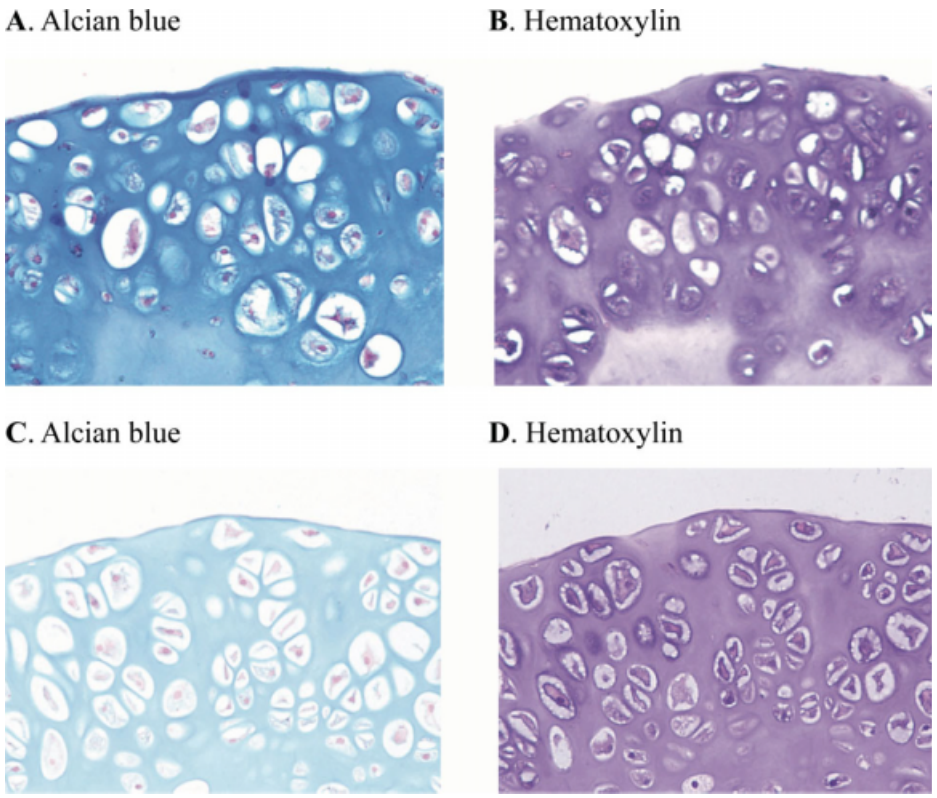

Figure 4. Histological evaluation of glycosaminoglycan (GAG) synthesis and chondrogenesis in type I and type II/I collagen matrix-chondrocyte constructs on day 26. Significant hypertrophic differentiation and GAG synthesis were found in the type II/I collagen matrix (A, B). Note less degree of GAG synthesis and hypertropic differentiation were shown in the type I collagen matrix on day 26 (C, D). (A) and (C) are stained with Alcian blue, and (B) and (D) with Hematoxylin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Limited GAG synthesis was noted in the type I collagen matrix-chondrocyte constructs from days 10 to 26 in the presence of bFGF. In contrast, type II/I collagen matrix-chondrocyte construct exhibited a significant increase in GAG synthesis in the presence of bFGF [Fig. 3(A)]. The areas of chondrocyte migration and proliferation as determined by GAG level in type II/I collagen compared with type I collagen constructs were ~1.7 fold larger [Fig. 3(D)]. GAG synthesis was noticeable on day 18 and increased significantly on day 26 [Fig. 3(A)]. In comparison, no significant difference was detected prior to day 18 between cells embedded in either type I or type II/I collagens [Fig. 3(A)].

Histological evaluation

Significant hypertrophic differentiation of chondrocytes and GAG accumulation were shown in the cartilaginous lacuna in type II/I collagen matrix on day 26, indicating the endogenous synthesis of the cartilaginous matrix from chondrocytes [Fig. 4(A,B)]. However, at the same time period, the type I collagen matrix-chondrocyte constructs underwent less chondrogenic differentiation compared with those in the type II/I collagen matrix. GAG was also found to accumulate around the lacuna of chondrocytes in the type I collagen matrix [Fig. 4(C,D)].

3D cartilage defect model and cartilage regeneration

Four stages can be identified in the chronological sequence of the 3D cartilage wound-healing model (Fig. 5). In the first stage (days 0–6), chondroblasts represent a lag phase, and display an undifferentiated appearance [Fig. 5(A)]. From days 6–18 (stage II), chondroblasts begin proliferation and migration [Fig. 5(B)]. In stage III (days18–26), cells show redifferentiation and GAG expression [Fig. 5(C)]. In stage IV (days 26–34), a neocartilage structure can be identified [Fig. 5(D)]. Abundant GAG was found in the cartilage-specific lacuna.

DISCUSSION

Current therapies for osteoarthritis include periosteal and perichondrial tissue grafting, chondrogenic cell transplantation, and growth factors. Results are

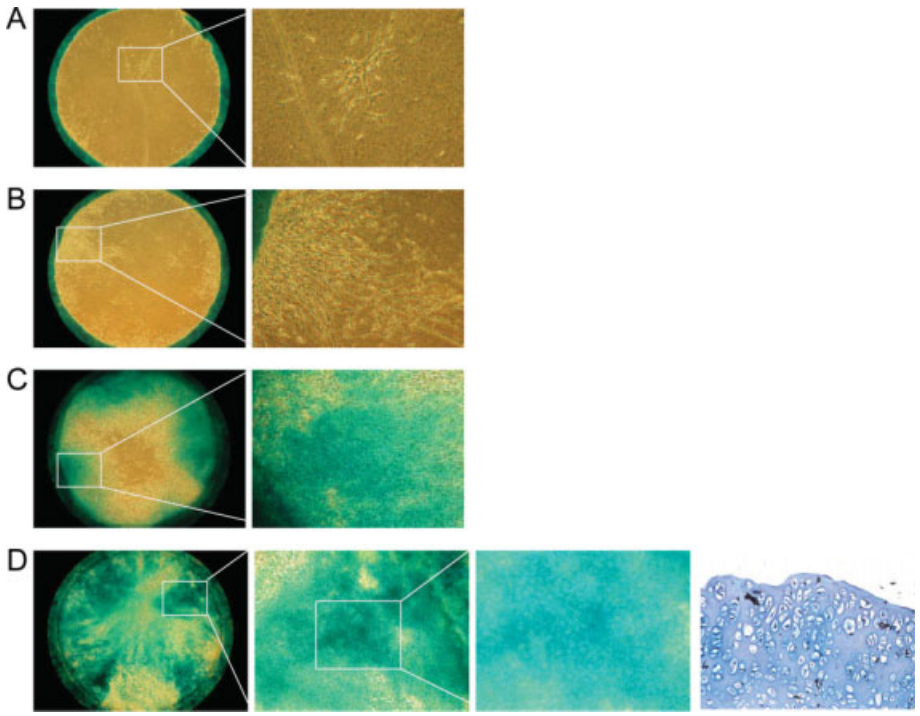

Figure 5. A 3D cartilage defect model for cartilage regeneration showing the four stages of the chronological sequence of the 3D cartilage wound healing model. A: Stage I: High-power photomicrograph of chondrocytes near the center of the 5-mm-diameter defect repaired with type II/I collagen in the presence of bFGF on day 6. B: Stage II: High-power photomicrograph of chondrocytes around the periphery of the 5-mm defect repaired with type II/I collagen in the presence of bFGF on day18. C: Stage III: Chondrocyte proliferation around the periphery of the 5-mm defect repaired with type II/I collagen in the presence of bFGF on day 26. D: Stage IV: Chondrocyte proliferation around the periphery of the 5-mm defect repaired with type II/I collagen in the presence of bFGF on day 34.

still unpredictable,[29] however, primarily due to unsuccessful regeneration of cartilaginous ECMs and the stress-bearing microstructures.[30] A dense matrix system appears essential for providing mechanical strength for cartilage growth.

3D scaffolds used previously were constructed with both cubic and ellipsoidal pore architecture using polypropylene fumarate. To increase seeding efficiency and cellular retention, hydrogels were used to deliver cells into the scaffolds to evaluate the hydrogel best able to stimulate proteoglycan synthesis in porcine chondrocytes in both *in vitro* and *in vivo* models.[31]

Our *in vitro* cartilage wound healing model uses reconstructed cartilage in the form of a 3D scaffold. This neocartilage model closely resembles the real human joint cartilage when compared with other artificial scaffolds. However, it still contains less extracellular matrix than real joint matrix.

This 3D scaffold, designed to mimic the ECM, enables chondrocytes to remain differentiated. This model is also able to characterize the behaviors of both chondrocytes and fibroblasts. Differentiated chondrocytes are capable of locomotion and production of type II collagen, which are both necessary for cartilage healing.

In vivo or *in vitro* models developed in other laboratories retained the individual variations of the animals and the area difference even within one piece of cartilage.[12–15] More recently, larger discs from bovine have been cultured as ex vivo models to study mechanisms of disc degeneration. Discs were incubated with different concentrations of the proteolytic enzymes.[18] However, because the proteolytic enzymes may damage experimental cells such as chondrocytes, this model may not be suitable for studying cell migration and differentiation. In vitro culture of rabbit articular cartilage explants was also used as a model to generate a collagenase-induced matrix degradation.[19] However, this model could not be used to study the repair mechanism of articular damage nor to investigate the role of chondrocytes in wound healing. A recent report combined adult human chondrocytes (hCHs) with 3D-aqueous-derived porous silk fibroin scaffolds for in vitro cartilage tissue engineering.[32] Although it may shed some light on autologous cell-based skeletal tissue engineering, the silk fibroin scaffold after all is not a natural extracellular matrix.

The cartilage defect model in our study provides a homogeneous cartilage system with the same geno-

type constructs. Our model has the potential for use in drug and biomaterial screening as two of our model's positive factors, namely type II collagen and bFGF, may prove useful in promoting the efficiency and exploring the mechanisms of cartilage repair.

Type II collagen is cartilage-specific, while type I collagen is broadly distributed in almost all kinds of tissues. The interactions between the cartilage-specific type of collagen and chondrocytes have been shown to modulate the spread, migration, and differentiation of chondrocytes.[33] Treated with extracellular type II collagen, chondrocytes upregulated the mRNA level of type II collagen and aggrecan in a 3D alginate system.[34] The interactions between the collagen and cells were also implicated in the distinct structure of the collagen molecule. Type I and type II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells has been reported.[33,34] In addition, previous studies suggest that both positive (Interleukin-1(IL-1)) and negative (prostaglandin (PGE_2)) signals may control collagen synthesis at the transcriptional level resulting in modulation of matrix turnover in cartilage, synovium, and skin.[4] In particular, Interleukin-1 (IL-1) also has profound effects on the synthesis of extracellular matrix components such as type I collagen and is mediated by prostaglandins at the transcriptional level.[5]

Chondrocytes are commonly embedded in the ECM. They are responsible for catabolism and anabolism of cartilage. Chondrocytes are differentiated from mesenchymal stem cells under appropriate cellular signals. The types of cells reaching injured sites affect cartilage wound healing. Fibroblasts exhibited faster migration than chondrocytes in our study. This may indicate that deposition of scar tissue takes place shortly after trauma and forms faster than the repair of cartilage can occur. In an attempt to enhance cartilage repair, scar tissue deposition was reported to have been minimized and postponed.[35]

Our results show that type II/I collagen support the migration of chondrocytes as do the type I collagen matrix. However, type II/I collagen is shown to enhance GAG accumulation around chondrocytes and facilitate neocartilage formation, which is in agreement with our previous study.[36] There was significant re-differentiation and GAG expression noted within the defects 22 days after employment of the type II/I collagen matrix. Elevated GAG expression was found on day 26. Meanwhile, the type I collagen matrix appeared to lack the ability to promote GAG synthesis by fibroblastic chondrocytes.

In the presence of bFGF, chondrocytes increased the migration rate in both the type I, and II/I collagen matrices. Nevertheless, the cartilaginous phenotypes such as round to polygonal morphology and GAG accumulation are enhanced by bFGF only in the type II/I collagen matrix. Our data showed that bFGF alone did not enhance GAG synthesis, in the sense of not directly inducing re-differentiation of chondrocytes. This may indicate that bFGF in the type II/I collagen matrix contributes to the healing processes through increasing proliferation and differentiation of chondrocytes, resulting in an increase in cell density at the defect site, which augments the re-differentiation of chondrocytes. The combination of the type II/I collagen matrix and bFGF may provide a new therapeutic method for treating cartilage defects.

Our study evaluates the early biological activity of chondrocytes and fibroblasts in a 3D model of cartilage wound healing. The novel 3D model of *in vitro* cartilage wound healing limits the diversities caused by cell sources and tissue compositions. This study supports the need to screen other cytokines and matrices for cartilage repair. After the evaluation of migration, proliferation, and differentiation of chondrocytes, the degree of cartilage repair may be evaluated by measuring mechanical properties such as shear stress and compression which influence cartilage tissue engineering.[37]

In our study, type I collagen, type II collagen, and bFGF were used to demonstrate the mechanisms of wound healing. Additional cytokines and cartilaginous ECMs will be investigated in the future, using this *in vitro* 3D model.

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