In Vitro and In Vivo Chondrogenesis of Rabbit Bone Marrow–Derived Stromal Cells in Fibrin Matrix Mixed with Growth Factor Loaded in Nanoparticles

Ji Sun Park, M.S., Han Na Yang, M.S., Dae Gyun Woo, M.S., Hyung-Min Chung, Ph.D., and Keun-Hong Park, Ph.D.

The effects of growth factor loaded in nanoparticles mixed in fibrin constructs on chondrogenic differentiation were investigated by evaluating the specific cartilage extracellular matrix components in vitro and in vivo using a special cell source of bone marrow–derived stromal cells (BMSCs). The proliferation of cultured and transplanted BMSCs was found to be greater in fibrin constructs that contained TGF-β3–loaded nanoparticles and TGF-β3 alone than in constructs that contained unloaded nanoparticles or in fibrin hydrogel alone. Further, reverse transcriptase–polymerase chain reaction revealed that BMSCs cultured in the presence of TGF-β3 in vitro and in vivo expressed high levels of aggrecan, cartilage oligomer matrix protein, SOX9, and type II collagen. However, a decrease in type I collagen expression was observed from 1 to 4 weeks in the presence of TGF-β3. Moreover, histological and immunohistochemical assays revealed that large amounts of type II and proteoglycan were released from BMSCs embedded in fibrin constructs, while decreased levels of collagen type I were observed in BMSCs cultured in constructs that contained nanoparticles that were loaded with TGF-β both in vitro and in vivo. These findings indicate that use of fibrin constructs that contained BMSCs and were provided with sustained levels of growth factors for a long period of time enabled the formation of hyaline cartilage tissue in vitro and in vivo. Overall, these results indicate that the system evaluated here may be useful for minimally invasive transplantation, BMSC differentiation, and engineering of composite tissue structures with multiple cellular phenotypes.

Introduction

It is well known that defective and damaged cartilage is not repaired by natural healing. Therefore, many methods designed to aid the regeneration of cartilage have been evaluated. Among these methods, cell-based therapies have been actively studied via evaluation of successful trials of primary cell implantation. However, such methods have drawbacks such as the isolation of undamaged cartilage cells and expansion of isolated cells for neocartilage regeneration. To overcome these drawbacks, stem cell therapy has been investigated as a method to facilitate regenerative tissue repair. Accordingly, bone marrow–derived stromal cells (BMSCs) have been investigated as suitable cell sources for specific tissue regeneration. BMSCs are pluripotent cells that are known to easily differentiate into cartilage, bone, muscle, fat, marrow stroma, and other tissue types when induced by several types of stimulants in vitro and in vivo. It is well known that stem cells (SCs) have the potential to change the development of specific target cell phenotype based on the environment they are cultured or transplanted in. This property enables the use of SCs in conjunction with synthetic or natural scaffolds. Indeed, a biodegradable porous scaffold, hydrogel, or microscaffold is often capable of inducing the chondrogenesis of SCs. Of these methods of induction, fibrin is commonly used to create hydrogel scaffolds that mimic certain aspects of the structure and function of natural extracellular matrix (ECM). However, the employment of scaffold alone has a limited ability to differentiate fully transplanted SCs.

To induce the differentiation of transplanted SCs, several methods of carrying the growth factors to the delivery sites have been evaluated. However, such procedures have been plagued by drawbacks such as undesired delivery resulting in an initial burst release of growth and increasing the half-life of growth factors. As a result, the use of heparin for the regulation of delivery using growth factors has emerged as a solution to the problems associated with transplanted cell differentiation. Specifically, due to its electrostatic nature and specific affinity for growth factors, heparin enables the
delivery of growth factors in a controlled manner.28–30 In addition, our group previously fabricated a new type of carrier that is coated on nanoparticles or blended with hydrogel.31,32 The results of studies evaluating these carriers have revealed that a variety of proteins have heparin-binding domains that enable the growth factors to crosslink their receptors and be protected from proteolytic degradation.33–35

The purpose of the present study was to evaluate the stabilized bioactivity and long-term release of growth factor loaded in nanoparticles mixed with fibrin hydrogel and used to construct scaffolds to determine if this system could be utilized to create suitable carriers of rBMSCs in vitro and in vivo. In vivo analyses play a key role in the evaluation of transplanted SCs by enabling identification of the cause of differentiation. In many cases, SCs that are meant to undergo chondrogenic differentiation in vitro lose their phenotype and do not undergo transformation into cartilage in vivo. Therefore, it is essential to develop methods that enable the administration of growth factors in a localized environment at low dose, so that they can function symbiotically without interference. Accordingly, implantable systems containing nanoparticles loaded with growth factors can be used for local delivery to enable site-specific pharmacological effects such as the induction of cell proliferation and differentiation. By optimizing the transplantation of MSCs in fibrin scaffolds that contain growth factors, transplanted MSCs can be used to generate constructs with substantial cartilaginous properties that are similar to those produced by differentiated chondrocytes when they are used in clinical trials.

Materials and Methods

Cell harvesting and culture

Rabbit BMSCs were isolated and harvested using a previously described method.20 Briefly, rabbit bone marrow stromal cells (rBMSCs) were harvested from 3-month-old New Zealand White rabbits. To accomplish this, bone marrow was obtained from the rabbit tibias and femurs via aspiration or flushing with a 16-gauge needle and a 10-mL syringe containing 1 mL of heparin (3000 U/mL). The bone marrow was then placed in a 50-mL tube containing 5 mL of low-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco–BRL, Grand Island, NY) and then centrifuged for 10 min at 600 g to obtain a cell pellet. The supernatant was then removed, after which the cells were resuspended in 10 mL of low-glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% antibiotics. Next, 10^5 cells/dish were plated on culture dishes and cultured in 10-cm dishes that were incubated at 37°C in a humidified atmosphere comprised of 5% CO2 and 95% air. Nonadherent cells were removed by changing the culture medium after 5 days of incubation. After 2 weeks of primary culturing, each dish of cells was passaged into three 10-cm culture dishes at 7-day intervals. rBMSCs at passage 3 were used for all subsequent experiments conducted in this study.

Preparation of growth factor–loaded heparinized nanoparticles

The nanoparticles were fabricated using previously described methods.20 Briefly, the heparin solution was mixed with 1, 10, 100, or 1000 ng/mL TGF-β3, after which cationic poly(L-lysine) solution was added with constant vortexing for 20 s. The solution was then incubated for 30 min at room temperature. In this study, the optimal concentration of heparin and poly(L-lysine) (1:0.5) was chosen due to its low particle size and measured zeta potential.

Preparation of fibrin hydrogel constructs

To prepare the fibrin constructs, BMSCs were collected by centrifugation and then suspended in a solution of fibrinogen (9–18 mg/mL; Mokam Research Center, Suwon, Korea) with TGF-β3 alone or TGF-β3–loaded nanoparticles (n = 6). Each suspension of 2×10^5 cells/mL in fibrinogen was then homogeneously mixed with aprotinin (Mokam Research Center), 60 U/mL thrombin (1000 U/mg protein; Sigma, St. Louis, MO), fibrin-stabilizing Factor XIII, and 50 mM CaCl2. Next, 250 μL of the fibrin constructs were dropped into 15 mL polypropylene round tubes to form a gel. Each construct was then transferred to another 15 mL polypropylene round tube and cultured in α-MEM supplemented with 1% antibiotics (streptomycin at 100 mg/mL and penicillin at 100 IU/mL).

Growth factor released from fibrin hydrogel constructs

The release of TGF-β3 (100 ng/mL) and TGF-β3 (100 ng/mL)–loaded nanoparticles from the fibrin hydrogels were evaluated. To accomplish this, the fibrin constructs containing the TGF-β3 (100 ng/mL) and TGF-β3 (100 ng/mL)–loaded nanoparticles were relocated to conical polypropylene tubes that contained 1 mL of α-MEM, and then placed on an orbital shaker and incubated at 37°C. The suspension of cultured media containing the TGF-β3 was then removed, frozen, and replenished at 1, 3, 5, 7, 10, 14, 21, and 28 days. Enzyme-linked immunosorbent assay (ELISA) for TGF-β3 was then performed to determine the concentration of growth factors that were released from the fibrin hydrogel.

Circular dichroism spectroscopy analysis

To determine the secondary structure of the released growth factors, circular dichroism (CD) spectroscopy was used. The CD spectra of the released growth factors were measured at 4°C on a Jasco J-750 spectropolarimeter (Jasco, Easton, MD). Each sample was equilibrated at the desired temperature for 30 min before analysis, with equilibration being indicated by the absence of further changes in the CD signal with increased equilibration times. All CD spectra were taken in a quartz cuvette with a 1 mm path length at wavelengths ranging from 190 to 260 nm. Data points were recorded at every nanometer with a 4.0 s response time. The concentrations of TGF-β3 and TGF-β3–loaded nanoparticles were then determined via amino acid analysis and calculation of the mean residue ellipticities.

Biochemical assays for MSC proliferation and glycosaminoglycan production

At each time point, the samples and negative controls were extracted, rinsed in 2.5 mL of phosphate-buffered saline (PBS), homogenized with a pellet grinder (Fisher Scientific, Hampton, NH), and digested in 500 mL of proteinase K solution (1 mg/mL proteinase K, 10 mg/mL pepstatin A, and 185 mg/mL iodoacetamide) in PBE buffer (6.055 mg/mL
Tris(hydroxymethyl aminomethane), 0.372 mg/mL EDTA, pH 7.6, adjusted by HCl) at 60°C for 16 h. After collection and digestion of all samples and controls, the DNA was extracted from the specimens by subjecting the samples to three repetitions of freeze/thaw/sonication (30 min at -80°C, 30 min at room temperature, and 30 min of sonication). The DNA and glycosaminoglycan (GAG) assays were run in triplicate for each experimental and control group at each time point. The number of cells was determined by measuring the double-stranded DNA content using a PicoGreen assay (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. The fluorescence of the negative, cell-free controls was subtracted from the fluorescence values of the experimental groups to determine the fluorescence of the material alone.

In addition, the GAG content was determined using the dimethylmethane blue dye assay. In this assay, a red color is produced upon the binding of dimethylmethane blue to GAG, which allows the GAG to be quantified by measuring the absorbance at 520 nm. The GAG content in the hydrogels was calculated based on comparison with a calibration curve generated from standards containing known amounts of chondroitin sulfate. A microplate reader (BIO-TEK Instrument, Winooski, VT) was used to measure both the absorbance and fluorescence. The total amount of GAG was normalized against the total amount of DNA.

Nude mouse transplantation

Five-week-old NOD mice were purchased from Clea Japan (Tokyo, Japan). All animal experiments were approved by the Animal Care Committee of Pochon CHA University. As a control group (n = 12), fibrin hydrogel constructs containing only cells were transplanted into the back subcutis of mice. For the treatment groups, heparinized nanoparticle-loaded fibrin hydrogel constructs (n = 12) with BMSCs, TGF-β3–loaded fibrin hydrogel constructs with BMSCs (n = 12), or TGF-β3–loaded nanoparticles embedded in fibrin hydrogel constructs with BMSCs (n = 12) were transplanted into the back subcutis of mice. The chondrogenic differentiation was then tested for each of these fibrin constructs. Four weeks after transplantation, the mice were sacrificed (n = 8 for each time point) using an overdose of anesthetic, and an area of skin that included the transplanted site was then carefully removed for subsequent biological examination.

RNA extraction and reverse transcriptase–polymerase chain reaction

RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The RNA pellets were then dissolved in 20 µL of RNase- and DNase-free water, after which the RNA yields were estimated based on the A260. First-strand cDNA was then reverse transcribed from 0.5 µg of total RNA using a SuperScript First-Strand Synthesis System kit. Gene-specific amplicons were then amplified by polymerase chain reaction (PCR) using oligonucleotide primers (Table 1). The PCR conditions were as follows: 4 min at 95°C, followed by cycles of denaturation at 95°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 45 s. The PCR products were then confirmed by electrophoreses in 1.5% agarose gel and subsequent visualization under UV light after ethidium bromide staining.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’)</th>
<th>Size (bp)</th>
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<tr>
<td>Col I</td>
<td>(S) CGGTACCCTCGGCATATTG</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td>(AS) TCCAGTAGCCACCCTCITT</td>
<td>366</td>
</tr>
<tr>
<td>Col II</td>
<td>(S) GCACCCAAGACATTGAGGG</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>(AS) GACACGAGTAGCAACCAGTC</td>
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</tr>
<tr>
<td>COMP</td>
<td>(S) CAGGACGACTTITGATCAGA</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>(AS) AACGTGACGCTCTCCTGCTA</td>
<td>293</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>(S) CCTTGGAGGCTCGTGTAAGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(AS) AGGTAACCTTCCTGCGCAGTG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>(S) TCACAATCTCCAGGAGCGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(AS) CACACTGCCAGTGGTCTCGT</td>
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COMP, cartilage oligomer matrix protein.

Immunohistochemistry

BMSCs embedded in fibrin hydrogel sections were fixed in 4% paraformaldehyde solution, dehydrated, and then embedded in paraffin. After deparaffinization, 6-µm sections were processed and stained with hematoxylin and eosin (H&E). The deparaffinized sections were then incubated with 0.5% Triton X-100/PBS solution for 30 min and washed three times with PBS. The nonspecific binding sites were then blocked with normal horse serum that was diluted 1:10 in 0.3% bovine serum albumin for 30 to 60 min, after which the samples were incubated for 2 h at 4°C in mouse antiserum against collagen type I and II (Chemicon, Billerica, MA) at 1:1000 in a humid environment. Next, the samples were rinsed in Triton-buffered saline, after which the sections were incubated in peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min. The peroxidase activity was then visualized using 3'-3'-diaminobenzidine (DAB) as the substrate. To accomplish this, the sections were incubated with 0.06% DAB in 0.1 M Tris-HCl (pH 7.5) containing 0.03% H2O2 in a humidified chamber. Direct dual staining was then conducted by incubating the samples for 1 h at 4°C with a mixture of FITC- and TRITC-conjugated mouse monoclonal antibodies against collagens I and II. Next, the samples were rinsed in Triton-buffered saline and then incubated in FITC-conjugated rabbit anti-mouse immunoglobulin G (Amersham Pharmacia Biotech), after which secondary antibody was applied. The samples were then washed with PBS three times, after which they were incubated with 1:100 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) stain for 5 min. Finally, the sections were examined by confocal laser scanning microscopy.

Statistical analysis

Differences between the experimental groups were assessed using a two-tailed Student’s t-test. As shown in Figures 1, 2, and 3, multiple comparisons were made using the Bonferroni adjustment to calculate the p-values. A p-value <0.05 was considered to indicate statistical significance.

Results

Gross mass, release profiles, and CD analysis

To evaluate the effect of growth factors on new tissue generation in the fibrin constructs, differences in the gross
FIG. 1. Glycosaminoglycan (GAG) content (A) and collagen content (B) per DNA analysis by a different condition of chondrogenic differentiation. BMSCs were cultured in 3D hydrogels for up to 1 week or 4 weeks in basal medium. Error bars represent standard error of the mean (*p < 0.05).

FIG. 2. Gene expression profiles of COMP, SOX9, aggrecan, and Col II as analyzed by RT-PCR after 4 weeks (A); quantification of Col II, SOX9, COMP, and aggrecan (B); and H&E blue staining of BMSCs embedded on the 3D matrix in vivo (C). (a, e), control; (b, f), NP; (c, g), TGF-β3; and (d, h), NP-TGF-β3. Error bars represent standard error of the mean (*p < 0.05). Scale bar: 100 µm. Color images available online at www.liebertonline.com/ten.
mass of fibrin constructs that contained BMSCs and TGF-β3-loaded nanoparticles (NP-TGF-β3) were evaluated at 1 and 4 weeks after in vitro culture (Fig. 4A). During the cultivation, the BMSCs embedded in the fibrin constructs easily formed a solid matrix in response to the addition of TGF-β3, while the fibrin constructs that contained BMSCs alone disappeared during cultivation (Fig. 4A). Further, BMSCs in the fibrin constructs that contained the TGF-β3 and NP-TGF-β3 were maintained at constant volumes or decreased slightly during the culture periods, even through the fibrin was easily degraded (1 and 4 weeks). In contrast, the fibrin constructs containing nanoparticles without TGF-β3 decreased by approximately 25% throughout the culture period (Fig. 4A). These findings indicate that the TGF-β3 stimulated the BMSCs to promote formation of an ECM, which then replaced the degraded fibrin constructs and replenished the original constructs.

Growth factor release profiles were generated by analyzing the release of growth factors from constructs that contained TGF-β3 or TGF-β3–loaded nanoparticles using ELISA (Fig. 4B). The release of growth factors from the nanoparticles in the fibrin hydrogel was sustained throughout the 4-week culture period. However, the release of TGF-β3 from the constructs that did not contain the nanoparticles occurred in a burst and was not sustained. These results indicate that loading the growth factors into nanoparticles may help induce cultured cell proliferation and differentiation by enabling their prolonged circulation and release from the constructs.

Figure 4C shows the change in the secondary structure of the TGF-β3 and NP-TGF-β3 that was released from the fibrin hydrogel constructs. The results of the CD analysis revealed the gradual loss of the α-helical structure of the released growth factors when both the native TGF-β3 and the NP-TGF-β3 were used. However, the α-helical structure of the NP-TGF-β3 released from the fibrin constructs was more stable than that of the native TGF-β3. Taken together, these findings indicate that the growth factors loaded in the NPs were more stable than those added in their native state.

Measurement of GAG and collagen content

To determine if the growth factors showed activity toward the production of specific ECMs by BMSCs, the GAG released from the BMSCs was evaluated. Among the groups evaluated, the constructs containing the NP-TGF-β3 showed a much higher level of GAG production in BMSCs embedded in the hydrogel constructs than was observed in the
other constructs. Further, the level of GAG released from BMSCs embedded in fibrin constructs after 4 weeks of culture was significantly higher than the amount of GAG released after 1 week of culture. These findings indicate that the addition of TGF-β3 had a significant impact on the GAG production by BMSCs. Additionally, more potent GAG production was observed in response to the addition of the TGF-β3–loaded nanoparticles.

The collagen production by BMSCs was also evaluated in the different fibrin constructs. It is well known that collagen is very important in native cartilage as an ECM for cell adhesion and proliferation. As shown in Figure 1B, BMSCs embedded in the fibrin hydrogel constructs containing TGF-β3 or NP-TGF-β3 produced a much higher amount of collagen than those in the hydrogel constructs that did not contain TGF-β3 or in the cultures that contained cells alone. These results indicate that the TGF-β3 is essential for chondrogenic differentiation. Specially, fibrin constructs containing NP-TGF-β3 are a suitable matrix for the delivery of cells intended to form neocartilage.

RT-PCR and histological analysis

Collagen type II, aggrecan, SOX9, and cartilage oligomer matrix protein (COMP) are well-known chondrogenic markers in native cartilage tissues. Therefore, to evaluate the chondrogenic differentiation, the expression of collagen type II, aggrecan, COMP, and collagen type I mRNA was evaluated by reverse transcriptase (RT)–PCR (Fig. 5a). As shown in Figure 5a, collagen type II expression was strong in the fibrin constructs that contained NP-TGF-β3, and its expression was enhanced as the chondrocytes differentiated. In addition, chondrocyte-specific proteins (COMP) and aggrecan were also strongly expressed in the fibrin constructs that contained the NP-TGF-β3, although they were not clearly shown in the fibrin constructs that did not contain TGF-β3. Moreover, the expression of the osteogenic marker, collagen type I, was not observed in the constructs that contained TGF-β3.

Changes in the expression of specific marker genes were also evaluated over time. The cultures containing the TGF-β3–loaded nanoparticles showed increased collagen type II mRNA levels after 3 days (Fig. 5b). However, by day 28, the levels of collagen type II mRNA in fibrin-cultured cells that contained NP-TGF-β3 were higher than those in the controls or in cultures that contained TGF-β3 alone in hydrogel, although a small amount of mRNA expression was observed in the fibrin hydrogel that did not contain TGF-β3–cultured cells (Fig. 5b). Further, expression of aggrecan mRNA cultured in cells that were cultured with NP-TGF-β3 increased over the 28-day culture period.
Cell proliferation, cell density, and polysaccharide and proteoglycan deposition were evaluated using hematoxylin and eosin (H&E), Safranin-O, and Alcian Blue staining. The results revealed clear evidence of cell proliferation and chondrogenic differentiation (Fig. 5c). For example, at 4 weeks, H&E staining revealed the presence of hyaline cartilage cells and lacunae in the fibrin constructs that contained NP-TGF-β3, while there were no lacunae in the fibrin constructs that did not contain the growth factor. This pattern was even greater after 4 weeks. In addition, the location of the lacunae was increased and fulfilled in the fibrin constructs, while a mixed population of lacunae and undifferentiated BMSCs (fibroblast-like cells) were observed in the fibrin constructs that did not contain TGF-β3.

Safranin-O and Alcian Blue staining were used to confirm the polysaccharide and proteoglycan deposition in the fibrin constructs. These analyses revealed that the BMSCs embedded in the fibrin constructs that contained TGF-β3 and TGF-β3–loaded nanoparticles contained a large amount of proteoglycan and polysaccharide deposits. Conversely, the ECMs produced by BMSCs that were embedded in the fibrin constructs that did not contain TGF-β3 were only stained in the areas surrounding the embedded cells. After 4 weeks, differences in the morphologies and ECM secretions of BMSCs cultured in the fibrin constructs in the presence and absence of growth factor were more clearly seen, with lacunae formation being observed in the former.

**FIG. 5.** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of gene expression of BMSCs. Total RNA was prepared from fibrin gels as 3D cultures of BMSCs at culture day 28, grown in NP, TGF-β3 100 ng/mL, or NP-TGF-β3 100 ng/mL. RT-PCR analysis was performed for Col II, Col I, aggrecan, and COMP, with GAPDH as an internal control (a). Total RNA was prepared from fibrin gels as 3D cultures of rabbit mesenchymal SCs at culture day 3, day 7, day 14, day 21, and day 28, grown in fibrin gels containing TGF-β3 (100 ng/mL) encapsulated nanoparticles (b). Histological sections of neocartilage formed by TGF-β3–loaded nanoparticles in fibrin hydrogel culture for 4 weeks by hematoxylin and eosin (H&E) (A–C), Safranin-O (D–F), and Alcian Blue staining (G–I) (c). Scale bar: 100 μm. Color images available online at www.liebertonline.com/ten.

Masson’s trichrome, DAB, and immunohistochemical staining revealed a release of total collagens and specific collagen isoforms from BMSCs in the fibrin constructs. As shown in Figure 6, a lower amount of collagen secretions

**Total collagen and immunohistochemical assay of collagen type I and II in vitro**

Masson’s trichrome, DAB, and immunohistochemical staining revealed a release of total collagens and specific collagen isoforms from BMSCs in the fibrin constructs. As shown in Figure 6, a lower amount of collagen secretions
from BMSCs was observed in samples collected from cultures of BMSCs alone than in samples obtained from cultures that contained BMSCs plus the nanoparticles. Moreover, a large amount of collagen deposition from BMSCs was observed in the fibrin constructs containing the NP-TGF-β3 after 1 week, and the collagen levels in this culture had increased further after 4 weeks. These findings indicate that TGF-β3 is essential for the enhancement of ECM secretion from BMSCs embedded in fibrin constructs.

To confirm the specific markers of chondrogenesis, the collagen type I and II phenotype expression of BMSCs that had been differentiated in response to the addition of TGF-β3 was tested by immunostaining with collagen type I and II antibody and DAPI staining. The results of these analyses revealed a high level of stained collagen type II in the fibrin constructs that contained the TGF-β3. In addition, the fibrin constructs that contained the TGF-β3 were stained diffusely positive for collagen type II in several portions of the constructs, not only in areas near the embedded cells. Conversely, collagen type I was not visible in the fibrin constructs that contained NP-TGF-β3. However, fibrin constructs containing nanoparticles without TGF-β3 showed a greater degree of collagen type I staining than those that contained nanoparticles with TGF-β3 (Fig. 6). These findings indicate that TGF-β3 induced chondrogenesis in the BMSCs that were embedded in the fibrin constructs.

**RT-PCR and H&E staining to determine if chondrogenesis occurs in vivo**

RT-PCR analysis was used to evaluate the expression of collagen type II, aggrecan, SOX9, and COMP mRNA in vivo. As shown in Figure 2A, specific markers of collagen type II, an important component in native cartilage tissues, were expressed in the transplanted cells after 1 week. Further, the expression of these markers was strongly enhanced as differentiated BMSCs became embedded in the fibrin constructs that contained NP-TGF-β3. This expression was long lasting and increased with time. When the chondrogenic-specific proteins were evaluated, COMP and aggrecan were strongly expressed in the constructs that contained NP-TGF-β3.

Collagen type II, aggrecan, SOX9, and COMP are major marker proteins in hyaline cartilage tissue. To certify the expression of quantitative levels of mRNA encoding these markers, samples were evaluated by semi RT-PCR. As shown in Figure 2B, the levels of COMP, collagen type II, and aggrecan expression in the BMSCs in the fibrin constructs that contained NP-TGF-β3 were significantly higher than the levels in the fibrin constructs themselves (Fig. 2A). Interestingly, when the collagen type II level was evaluated, the expression of mRNA from BMSCs stimulated by NP-TGF-β3 present in the fibrin constructs was much higher than the expression of mRNA from BMSCs stimulated by TGF-β3 in the fibrin constructs in vivo.

Hematoxylin and eosin (H&E) staining revealed orderly cell distribution and differentiation of the cells in the fibrin hydrogel constructs (Fig. 2C). Four weeks after transplantation into nude mice, the fibrin constructs that contained NP-TGF-β3 had fulfilled with lacunae cells covering the entire construct. However, there was no lacunae formation observed in fibrin constructs that did not contain TGF-β3 and a mixture of lacunae cells and undifferentiated cells of BMSCs (Fig. 2C).

**Discussion**

The results of the analysis of the specific gene expression revealed that it was necessary to evaluate the GAG released from BMSCs embedded in the fibrin constructs. Therefore, the fibrin constructs were evaluated by Alcian Blue staining to confirm that specific changes in the ECM released from the BMSCs had occurred. The results of these analyses suggested that the BMSCs embedded in the fibrin constructs that contained NP-TGF-β3 stored a large amount of ECMs that were associated with polysaccharides (Fig. 7A–D). Conversely, BMSCs embedded in the fibrin constructs that did not contain the growth factors only produced an ECM when the cells were transplanted (Fig. 7A, B).

In addition, Safranin-O staining revealed deeply stained orange-colored cells and surroundings when BMSCs embedded in the fibrin constructs that contained NP-TGF-β3 were evaluated. As seen in Figure 7E–H, BMSCs that were transplanted into nude mice were homogeneously distributed and contained ECMs that were secreted from BMSCs embedded in the fibrin constructs. Moreover, differentiated cells from the transplanted BMSCs had a round shape and formed cell aggregates during the transplantation periods. Further, many distinct cell populations were observed when the histological assay of fibrin constructs containing NP-TGF-β3 was compared to the assay of BMSCs in fibrin constructs that did not contain TGF-β3 or that contained nanoparticles alone.

Histology using von Kossa staining 4 weeks after implantation revealed mineral deposition (brownish yellow and black precipitates) throughout the fibrin hydrogel constructs, which indicated that osteogenesis had occurred (Fig. 7I–L). Specifically, when the control constructs that contained cells alone were evaluated, several portions containing differentiated osteogenic cells were revealed by von Kossa staining (Fig. 7I). In addition, some calcium deposition was observed in the fibrin hydrogel constructs that contained nanoparticles alone, while no calcium deposition was observed in the fibrin hydrogel constructs that contained NP-TGF-β3 (Fig. 7L).

To confirm the presence of the specific marker of chondrogenesis, collagen type II, immunohistological analysis, and DAB staining were evaluated using collagen type I and II antibodies and staining with hematoxylin (Fig. 3A, B). The formation of cartilage tissue within the fibrin constructs was observed in both cases at 4 weeks after transplantation. Further, collagen type II was primarily expressed by the BMSCs embedded in the fibrin hydrogel constructs that contained NP-TGF-β3.

To quantify the released collagen, the immunofluorescence in pictures of the samples that were stained with collagen type I and II was calculated using confocal laser microscopy. The results revealed that a higher amount of collagen type II protein expression was present in the fibrin constructs containing the NP-TGF-β3 than in any of the other fibrin constructs (Fig. 3C). However, the fibrin constructs that contained NP-TGF-β3 contained the lowest amount of collagen type I when compared to the other fibrin constructs.
FIG. 6. Histological sections of neocartilage formed by TGF-β3–loaded nanoparticles in fibrin hydrogel culture for 4 weeks by Masson’s trichrome and immunohistochemical staining for type I and type II collagen of BMSCs cultured in fibrin gels containing TGF-β3 loaded in nanoparticles at day 28. Collagen type II (A–C) and collagen type I (D–F) DAB staining and collagen type II (J–L) and collagen type I (M–O) immunofluorescence staining of BMSCs embedded in fibrin gels containing TGF-β3–encapsulated nanoparticles. (A, D, G), NP; (B, E, H), TGF-β3; and (C, F, I), NP-TGF-β3. Scale bar: 100 μm. Color images available online at www.liebertonline.com/ten.
use of systems in which encapsulated cells are directly injected into the sites of disease, where they form novel fabricated constructs that eliminate the need for surgery. Such hydrogels, which include hyaluronate (HA), fibrin, collagen, alginate, and chitosan, are of particular interest for the treatment of joint defects.36–40 Of the media used to create hydrogels, fibrin has recently been developed as a cell carrier.23–25 This biomaterial has satisfied the conditions of biocompatibility, cytotoxicity, biodegradability, and the ability to be injected into and fixed in desired areas such as subchondral bone and host cartilage.

In this study, we examined the ability of fibrin hydrogel to support the chondrogenic differentiation of BMSCs in response to growth factors. The ability to deliver the growth and sustain the administration of specific tissue differentiated growth factors in combination with SCs in scaffolds offers a wealth of therapeutic opportunities in regenerative medicine. Here, we examined the cartilage regenerative potential of embedded TGF-β3–loaded nanoparticles in biodegradable fibrin scaffolds for the release of solvent-sensitive and thermolabile growth factors in combination with BMSCs cultured in vitro and transplanted in nude mice for 4 weeks. An important finding of this study was that the fibrin hydrogel constructs containing BMSCs grown without additional chondrogenic supplements exhibited a similar biochemical composition and immunohistochemical staining for cartilage-specific matrix molecules as constructs grown with chondrogenic supplements.

Previously, we demonstrated the accumulation of a cartilaginous matrix produced by BMSCs mixed with TGF-β3 in a thermo-reversible hydrogel.41 Taken together, the results of the present study and these previous findings suggest that the TGF-β3 interacts with rMSCs in a manner that promotes the expression of the chondrocytic phenotype and potentially eliminates the need for external supplements. Further, these results are similar to the results of previous studies, which have reported that the effects of TGF-β3 on rMSCs depend on the context in which the factor is provided.41,42

In the present study, we fabricated scaffolds containing growth factors for chondrogenesis and BMSCs. The reason why the volume of fibrin hydrogel including TGF-β3 is different to that of control group might be because of proliferated BMSCs and secreted cartilage ECM by growth factors.
In the case of differentiated BMSCs, production of the cartilage matrix results in the production of proteins such as collagens and GAG in the differentiated materials. In the present study, the levels of these products were higher in the treatment groups than in the control group. Further, in the group that did not contain TGF-β3, BMSCs did not proliferate well, possibly due to apoptosis. Moreover, after 4 weeks of culture, the GAG content per sample was significantly higher in constructs that contained the TGF-β3 than in those that did not. Finally, the GAG content in the constructs containing TGF-β3 was significantly higher for the remainder of the culture period than that of the constructs containing TGF-β3 (Fig. 4).

It is well known that cell condensation occurs in joint development \textit{in vivo}, and that this leads to the formation of cartilage tissue.\textsuperscript{43} Therefore, cell–cell contact may be important in promoting the chondrogenic differentiation of BMSCs in this system. Additionally, the relative expression of collagen type II, aggrecan, and collagen type I was evaluated by RT-PCR as an indicator for chondrogenic differentiation (Fig. 1). The results revealed a significant increase in collagen type II expression at day 21 and in aggrecan expression beginning at day 7 in groups that contained TGF-β3-loaded heparinized nanoparticles. These findings indicate that TGF-β3–loaded heparinized nanoparticles promote chondrogenic differentiation of BMSCs. These results may have occurred because the BMSCs encapsulated in the hydrogels maintained a round morphology during the culture period. This change in shape from a spread morphology during preculture to a round morphology after encapsulation may also have induced the decrease in collagen I expression that was observed the first 1 week and maintained throughout the remaining culture period. This finding was further confirmed by the histological and immunohistochemical studies, which demonstrated that the fibrin hydrogel constructs that contained TGF-β3 produced a cartilage-specific matrix that included SOX-9, type II collagen, and, importantly, no evidence of type I collagen.

\textit{In vitro} and \textit{in vivo} histological analysis of samples revealed that BMSCs exhibited a round shape that was similar to differentiated chondrocyte-like cells in the hydrogel constructs, and that they also formed cell aggregates in the hydrogel composites. To confirm the specific morphological changes, the cultured constructs were examined by Safranin-O and Alcian Blue staining. These methods indicated that BMSCs that had been encapsulated in the fibrin hydrogels that contained the TGF-β3 loaded in nanoparticles accumulated a significant number of cells as a result of proliferation. Further, the histological analyses revealed that the BMSCs cultured in these fibrin hydrogels produced an abundant ECM that was rich in proteoglycans and polysaccharides (Figs. 5 and 7).

\textit{In vitro} studies of articular cartilage explants have shown that lysis of GAG precedes catabolism of type II collagen during the progression of osteoarthritis. To be consistent with these previous reports, the results of the present study should have revealed type II collagen in the same regions found to contain GAG. However, type II collagen was reliably restricted to only one location, while GAG was partitioned within a disconnected space, and both of these proteins were maintained independently of each other. It is possible that this apparent dissociation was an artifact related to epitope exposure or integrity within the tissue. While this finding does not correspond precisely to prior observations, these results do offer a basis for speculation. Both experimental formulations and, to a lesser extent, the untreated sham specimens produced promising, but imperfect, hyaline-like repair tissue. It is conceivable that the cells populating these tissues were newly differentiated cells that had not propagated through the repaired cartilage and, at 24 weeks, were in the process of remodeling the repair site. Our present observation may represent an imbalance in the regeneration or degradation of GAG and type II collagen. It is clear that cells populating both experimental formulations produced biological markers unique to the chondrocyte phenotype. Taken together, these findings indicate that utilizing devices that contain high concentrations of chondrocytes or pluripotent cells at high concentrations and/or appropriate morphogens and growth factors is likely to produce self-sustaining hyaline-like cartilage repair tissues. Ongoing research is currently being conducted to evaluate the ability of such implants to serve as delivery vehicles for cells and growth factors. While the results obtained in this study are promising, the potential for resurfacing larger defects of articular cartilage must be examined before discussing whether the implant has clinical applications in humans. Investigation of later time points will also be necessary to assess the long-term survival of the repair.

**Conclusion**

In summary, the results of this study suggest that rBMSCs in fibrin constructs that contain TGF-β3–loaded nanoparticles may be useful for cartilage tissue regeneration. Indeed, the histology and RT-PCR results revealed that TGF-β3–loaded nanoparticles induced a greater amount of chondrogenic differentiation of BMSCs embedded in the constructs. Further, the presence of TGF-β3–loaded nanoparticles in fibrin constructs stimulated cell proliferation and differentiation \textit{in vitro} and \textit{in vivo}. This cell carrier system will likely help stimulate SC proliferation and differentiation, thereby inducing differentiation cascade events \textit{in vitro} and \textit{in vivo}.

**Acknowledgment**

This work was supported by a grant (SC2190) from the Stem Cell Research Center of the 21C Frontier R&D Program funded by the Ministry of Science and Technology, Korea.

**Disclosure Statement**

No competing financial interests exist.

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Received: September 24, 2008
Accepted: December 12, 2008
Online Publication Date: April 29, 2009