PD98059-Impregnated Functional PLGA Scaffold for Direct Tissue Engineering Promotes Chondrogenesis and Prevents Hypertrophy from Mesenchymal Stem Cells

Jong Min Lee, PhD,¹ Jong Dae Kim, MD,¹ Eun Jo Oh, MS,² Se Heang Oh, PhD,³
Jin Ho Lee, PhD,² and Gun-Il Im, MD¹

In cartilage tissue engineering from mesenchymal stem cells, it is important to suppress hypertrophy to produce a neocartilage with stable phenotypes of hyaline articular cartilage (AC). The aim of this study was to develop and test the usefulness of functional chondrogenic scaffolds that serve the purpose of hypertrophy suppression. PD98059-impregnated poly(lactic-co-glycolic acid) (PLGA) scaffold is fabricated and compared with transforming growth factor (TGF)- β_2 -immobilized scaffold. The PD98059 is continuously released from the scaffolds over 140 days in contrast to the rapid release in TGF- β_2 -immobilized scaffold. The *in vitro* culture results show that the PD98059-impregnated scaffold is more effective in suppressing hypertrophy than the TGF- β_2 -immobilized scaffold while both scaffolds enhance chondrogenesis from human mesenchymal stem cells. After 10 weeks of *in vivo* implantation in rabbits, the osteochondral defects is successfully repaired in both PD98059-impregnated and TGF- β_2 -immobilized scaffold seeded with rabbit mesenchymal stem cells when evaluated grossly and microscopically. However, type X collagen is not observed from regenerated cartilage in PD98059-impregnated scaffold, whereas it is detected around chondrocytes in the TGF- β_2 -impregnated scaffolds. In addition, the PD98059-impregnated scaffold has better reconstitution of the subchondral plate. These results suggest that the use of the PD98059-impregnated scaffold leads to AC regeneration of better quality and prevents hypertrophy when implanted in the osteochondral defects.

Introduction

ARTICULAR CARTILAGE (AC) has very limited potential for self-repair. Injuries to AC usually progress to osteoarthritic changes.^{1,2} Of late, tissue engineering approaches combining the use of an appropriate cell source, biocompatible and biodegradable scaffolds, and growth factors have been applied to restore the functional cartilage.^{3–5} Mesenchymal stem cells (MSCs) from adults have been investigated as an important cell source for cartilage repair.^{3,5,6} The conventional concept of cartilage tissue engineering using stem cells includes the prolonged *in vitro* culture of cells within a carrier scaffold. The considerable period of time, from harvesting the cells to their implantation on the defect, has negative effects on patient care. A recent concept involves the direct seeding of stem cells, isolated from patients, into a functional scaffold and the immediate placement of the stem cell-functional scaffold hybrid into the defect.⁷

In cartilage tissue engineering from MSCs, hypertrophy poses formidable challenges.⁸ Hypertrophy itself is a land-

mark for correct chondrocyte differentiation, representing the normal fate of chondrocytes during the developmental process with the exception of the AC.⁹ Nevertheless, it is still important to suppress hypertrophy if a neocartilage bearing the characteristics and stable phenotypes of the hyaline AC of a normal joint is to be produced. Molecules that affect intracellular events downstream of a growth factor signal can be used to enhance chondrogenesis and control hypertrophy from MSCs. Chondrogenic growth factors such as transforming growth factor (TGF)- β and bone morphogenetic protein initiate signals in MAPK (mitogen-activated protein kinase) pathway, which play a key role in a variety of cellular responses, including proliferation, differentiation, and apoptosis.¹⁰ The inhibition of extracellular signal-regulated kinase (ERK)-1 and ERK-2, two MAPK subtypes, enhanced chondrogenesis by up to 1.7-fold in micromass cultures of chick embryo mesenchyme.¹¹ PD98059 is one of the ERK inhibitors that indirectly inhibit ERK1/2 by acting on its upstream kinase.¹² We have previously demonstrated that PD98059 suppressed hypertrophy in the chondrogenesis

¹Department of Orthopaedics, Dongguk University Ilsan Hospital, Goyang, Republic of Korea.

²Department of Advanced Materials, Hannam University, Daejeon, Republic of Korea.

³Department of Nanobiomedical Science, WCU Center, Dankook University, Cheonan, Republic of Korea.

from human mesenchymal stem cells (hMSCs).¹³ As this small molecule is more stable and easier to apply than peptide growth factors, PD98059 can be impregnated in the porous scaffolds, and further evaluated as a chondrogenic and antihypertrophic factor in the direct tissue engineering from MSCs.

Poly(lactic-co-glycolic acid) (PLGA) porous scaffolds are satisfactory candidates for the PD98059 delivery because of their favored mechanical properties and degradability.¹⁴ The PLGA scaffold hydrophilized by adding Pluronic F127 would not only provide an enhanced PD98059 release profile from the scaffold but also allow effective convective transport of oxygen/nutrients and metabolic waste, which is essential effective 3D tissue (e.g., cartilage) regeneration. The aim of this study was to develop and test the usefulness of the PD98059-impregnated functional chondrogenic scaffold that serves as a successful carrier of MSCs and suppress hypertrophy in direct cartilage tissue engineering. The effectiveness of this functional scaffold was compared with a scaffold immobilized with TGF- β_2 , which has been known to induce chondrogenic differentiation from MSCs.^{15,16}

Materials and Methods

Materials

PLGA (lactic to glycolic acid mole ratio, 75:25; M_w 120,000 Da) and Pluronic F127 (F127; EG99PG65EG99; M_w 12,500 Da) were purchased from Lakeshore Biomaterials (Birmingham, AL) and BASF (Parsippany, NJ), respectively, to prepare the 3D porous scaffold. Heparin was purchased from Celsus Laboratories (Cincinnati, OH). TGF- β_2 and PD98059 were purchased from R&D Systems (Minneapolis, MN) and Merck Millipore (Billerica, MA), respectively. All other chemicals were of analytical grade and were used as received. Ultrapure grade water ($>18\text{ m}\Omega$) was purified using a Milli-Q purification system (Merck Millipore).

MSC sample collection, isolation, and cultivation

The bone marrow samples used to isolate hMSCs were obtained from four patients (mean age: 43 years, range: 30–63 years) undergoing total hip replacement due to osteoarthritis. hMSCs from each patients were pooled separately. Informed consent was obtained from all donors. Rabbit mesenchymal stem cells (rMSCs) were isolated from the bone marrow of New Zealand White rabbits (16 weeks old, weighing 3.0–3.4 kg; Samtako Bio Korea, Osan, Korea). To accomplish this, bone marrow was obtained from the right femurs by aspiration with a 16-gauge needle and a 10-mL syringe containing 1 mL of heparin (3000/mL). hMSCs or rMSCs were expanded as described previously.¹⁷ All MSCs at passage 3 were used for all subsequent experiments. Chondrogenic, osteogenic, and adipogenic potentials of MSCs were confirmed (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

Fabrication of PLGA/F127 scaffold

PLGA/F127 scaffold was fabricated by a melt-molding particulate-leaching method developed in our laboratory.¹⁸ Briefly, PLGA pellets were dissolved in methylene chloride (MC; Junsei, Kyoto, Japan) to a 10 wt% solution, and the Pluronic F127 powder was added in the PLGA solution

(10 wt%, PLGA base). The prepared mixture solution (20 mL) was cast on a glass Petri dish (diameter, 10 cm), and the solvent was slowly evaporated in a desiccator cabinet at room temperature for 3 days followed by vacuum drying overnight to remove the residual solvent. The thickness of the prepared PLGA/F127 film was $\sim 200\text{ }\mu\text{m}$. The film was cut into discs of 6 mm diameter, and each disc was placed in a brass mold (diameter, 18 mm; thickness, 2.5 mm) sandwiched between two layers of sodium chloride particles (sieved to sizes between 200 and 250 μm). The mold was then thermally compressed. The porous scaffold was then obtained after washing in excess water to leach out the salts and after the following freeze-drying process. The dimensions of the prepared scaffold were $\sim 7\text{ mm}$ diameter and $\sim 2\text{ mm}$ thickness.

Loading and release profile of PD98059

PD98059-loaded PLGA/F127 scaffolds were also fabricated by a melt-molding particulate-leaching method. To accomplish this, PD98059 powder was evenly suspended in the PLGA/F127 mixture solutions (0.3, 0.5, and 1 wt%, polymer base) prepared as described previously. Surface and cross-sectional morphologies of the scaffold were observed by a scanning electron microscope (SEM, Model S-3000N; Hitachi, Tokyo, Japan). The average pore size was measured from the SEM images using an image analysis program (*i*-solution; IMT, Uiwang, Korea). The PD98059-impregnated scaffolds were prepared using the same method as that described previously. The loading amount of PD98059 in the scaffold was quantified by a reversed-phase high-performance liquid chromatography [RP-HPLC; pump (A3920-040; Thermo Separation Products, Waltham, MA), an autosampler (AS-3000; Thermo Separation Products), and a UV/VIS detector (UV 1000; Thermo Separation Products)] using the total scaffold solutions dissolved in MC. The HPLC conditions were as follows: column, Shodex C18M 4D 150 $\times$ 4.6 mm, 5 μm ; mobile phase, methanol/water (5/5, v/v with 0.1% trifluoroacetic acid); flow rate, 1 mL/min; UV/Vis detector; λ_{max} , 254 nm. To investigate the release profile of the PD98059 from the scaffolds with different PD98059 compositions, the PD98059-loaded PLGA/F127 porous scaffolds were incubated in 1 mL phosphate-buffered saline (PBS) at 37°C for up to 24 weeks under mild shaking ($\sim 50\text{ rpm}$). At preset time intervals, the incubation medium was collected and replaced with fresh PBS. The amount of released PD98059 in the collected medium was determined using an RP-HPLC with the same HPLC condition as described previously.¹⁹

Immobilization and release profile of TGF- β_2

TGF- β_2 was incorporated onto the pore surfaces of the PLGA/F127 scaffold via heparin immobilization. To immobilize heparin onto the pore surface of the prepared PLGA/F127 scaffold, the scaffold was soaked in the heparin solution [1 mg/mL (in 2 wt% NaCl solution); 4°C for 3 h]. The heparin-immobilized PLGA/F127 scaffold was rinsed with 2 wt% NaCl solution and water successively and then freeze-dried. The amount of immobilized heparin on the PLGA/F127 scaffold was determined using toluidine blue assay.²⁰ To incorporate the TGF- β_2 onto the heparin-immobilized scaffold, the scaffold was immersed in TGF- β_2 solution (200 ng/mL) for 3 h. The TGF- β_2 -immobilized scaffold was washed with PBS (pH 7.4) three times, and the amount of

immobilized TGF- β_2 was quantified using the direct ELISA technique.²¹ The TGF- β_2 -adsorbed PLGA/F127 scaffold without the heparin immobilization was also prepared using the same method to investigate the role of heparin immobilized onto the pore surfaces of the PCL/F127 scaffold for the TGF- β_2 binding and release profile. TGF- β_2 -immobilized (or adsorbed) scaffolds were incubated in 1 mL PBS supplemented with 1% bovine serum albumin at 37°C for a period of up to 3 weeks under mild shaking (50 rpm) to perform the release study. At preset time intervals, the incubation medium was collected and replaced with fresh PBS. The amount of released TGF- β_2 in the collected medium was determined using an ELISA kit.

Induction of in vitro chondrogenic differentiation

The chondrogenesis of hMSCs from TGF- β_2 -immobilized or PD98059-impregnated PLGA scaffold was compared with that of the PLGA scaffolds without any growth factor as a control. After three passages, the hMSCs were seeded onto the scaffolds at a density of 5×10^5 cells per scaffold ($\varnothing \approx 7$ mm, thickness ≈ 2 mm) and placed in a 24-well PS plate (Corning, Inc., Corning, NY). The scaffolds were then maintained at 37°C for 2 h in an incubator with a humidified 5% CO₂ atmosphere for cell adhesion to the scaffolds. The chondrogenic medium [Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture: DMEM/F-12 (Life Technologies, Carlsbad, CA) supplemented with 1% insulin-transferrin-sodium selenite (ITS), 1×10^{-7} M dexamethasone, 50 μ M ascorbate-2-phosphate, 50 μ M L-proline, and 1 mM sodium pyruvate] was added to the plate wells, and the cells in each scaffold group were cultured in the incubator for up to 4 weeks. The chondrogenic medium was changed for a fresh one every third day during the cell culture periods.

DNA quantitation and glycosaminoglycan contents analysis

After grinding in liquid nitrogen, each PLGA scaffold was digested overnight in a cell lysis buffer containing proteinase K of a GeneAll Tissue SV mini Kit (GeneAll, Seoul, Korea) at 37°C. Genomic DNA from each PLGA scaffold was prepared according to the manufacturer's instructions. The DNA contents were determined using the Quanti-iTTM dsDNA BR assay kit and Qubit fluorometer (Invitrogen, Carlsbad, CA). For analysis of glycosaminoglycan (GAG) contents, the ho-

mogenized PLGA scaffolds were digested in a papain buffer at 60°C for 7 h and then transferred to 1.5-mL microcentrifuge tubes. GAG production was determined using a Blyscan kit (Biocolor, Carrickfergus, Northern Ireland, United Kingdom). This assay is based on the specific binding of the cationic dye DMMB to the sulfated GAG (s-GAG) chains of proteoglycans and protein-free s-GAG chains. The procedure was carried out according to the manufacturer's instructions. Briefly, standard solutions (0, 1.0, 2.0, 3.0, and 5.0 μ g of chondroitin-4-sulfate in 100 μ L) and test samples (100 μ L) were mixed with 300 μ L of Blyscan dye reagent for 30 min at room temperature. s-GAG-dye complex was recovered by centrifugation for 20 min at 15,000 rpm, and the pellets were resuspended in 300 μ L of dissociation buffer. Absorbances were measured at 656 nm in a Spectra maxplus 384 apparatus (Molecular Devices, Sunnyvale, CA). GAG contents were expressed as micrograms of GAG per microgram of DNA.

Reverse transcription and real-time polymerase chain reaction analysis

The total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified using the Quant-iT RNA assay kit and Qubit Fluorometer system (Life Technologies). Isolated RNA samples were converted to cDNA using a PrimeScriptTM 1st strand cDNA synthesis kit (Takara Bio., Shiga, Japan). All polymerase chain reactions (PCRs) were performed on the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) in a standard 15 μ L reaction volume. The expressions of the following genes were examined: COL2A1, SOX9, COL1A1, COL10A1, and ALP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The primers used for amplification are listed in Table 1. After polymerase activation (95°C for 15 s), 50 amplification cycles were run (5 s denaturation at 95°C, 15 s annealing at 60°C, and 15 s extension at 72°C). Melt-curve analysis was performed immediately after the amplification protocol using the following conditions: 5 s at 95°C (holding time on reaching temperature), 1 min at 65°C, and 1 s at 97°C. The temperature change rate was 20°C/s (except for the final step, during which the temperature change rate was 0.1°C/s). The peak melting temperatures obtained were considered to be those of the specific amplified products.

TABLE 1. PRIMERS USED FOR REAL-TIME POLYMERASE CHAIN REACTION

Gene symbol	Sequences (5' to 3')	Accession no.
COL2A1	F-AACCAGATTGAGAGCATCCG R-ACCTTCATGGCGTCCAAG	NM_033150
SOX9	F-GGAGCTCGAAACTGACTGGAA R-GAGGCGAATTGGAGAGGAGGA	NM_000346
COL10A1	F-CAGTCATGCCTGAGGGTTTT R-GGGTCATAATGCTGTTGCCT	NM_000493
COL1A1	F-CCCCTGGAAAGAATGGAGATG R-TCCAAACCACTGAAACCTCTG	NM_000088
ALP	F-TCTTGGTGTGTTGGTGTGGATG R-ATTGCTGATGGACGTGAGG	NM_001083607
GAPDH	F-CACATGGCCTCCAAGGAGTAA R-GTACATGACAAGGTGCGGCTC	NM_002046

To guarantee the reliability of the results obtained, all samples were processed in triplicate. Each assay was performed using positive and negative controls. The threshold cycle (Ct) value of each gene was measured for each reverse transcript sample. The Ct value of GAPDH was used as an endogenous reference for normalization purposes (User bulletin #2; Applied Biosystems, Roche Molecular System, Alameda, CA). The values thus obtained were normalized versus the negative control and expressed as fold changes.

Western blotting

For total protein extraction, each PLGA scaffold was washed twice with cold PBS and homogenized by grinding in liquid nitrogen and incubated with RIPA buffer (ThermoFisher Scientific, Inc., Waltham, MA) containing a protease inhibitor cocktail (ThermoFisher Scientific, Inc.) for 30 min on ice and were then centrifuged at 15,000 rpm for 20 min at 4°C. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted, and probed using the appropriate antibody. Proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane and blocked for 1 h in 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBST). Thereafter, the membranes were incubated overnight at 4°C with primary antibody against types II collagen (1/500; Merck Millipore), SOX9 (1/1000; Abcam, Cambridge, United Kingdom), type I collagen (1/1000; Abcam), ALP (1/1000; Abcam), types X collagen (1/1000; Abcam), and β -actin (1/1000; Cell Signaling Technology, Inc., Danvers, MA). Next, membranes were then washed three times with PBST and then incubated with horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG (1:3000; Abcam) for 1 h at room temperature. After a further washing step, bands were visualized by using ECL+Plus™ Western blotting system (GE Healthcare Biosciences, Piscataway, NJ).

In vivo implantation of the PLGA scaffolds or autologous rMSCs-PLGA scaffold composite

After autologous rMSCs adhesion to each PLGA scaffold, the cell-scaffold composites were implanted in a large osteochondral defect that was created in the patellar groove of the distal femur of the left knee. Twenty New Zealand White rabbits (20-week-old, weighing 3.4–3.8 kg) were anesthetized with zoletile (50 mg/kg) and xylazine (4.5 mg/kg). A 5-cm medial parapatellar incision was made over the left knee and the patella was everted. We made an osteochondral defect (diameter: 7 mm, depth: 2 mm) on the patellar groove of the left distal femur using an osteochondral autograft transfer system trephine. The defects were managed using one of the following methods: filling with the PLGA scaffold only (group 1), filling with the PLGA scaffold seeded with rMSCs (group 2), filling with the PD98059-impregnated PLGA scaffold seeded with rMSCs (group 3), and filling with the TGF- β_2 -immobilized PLGA scaffold seeded with rMSCs (group 4). MSCs from the same rabbit were seeded to scaffolds and incubated at 37°C 2 h before implantation. Five rabbits were allocated to each group. The patella was reduced, and the wounds were closed in layers. The rabbits were allowed to move freely as soon as they recovered from anesthesia. Gentamycin (4 mg/kg) was injected intramuscularly once a day for 3 days after the sur-

gery. The animal study procedures were approved by the Institutional Animal Care and Use Committee at the Dongguk University.

Histological and immunohistochemical analyses

Ten weeks after the surgery, the distal femurs were dissected and embedded in a frozen section compound (aqueous embedding medium within a mold) (Leica Microsystems, Wetzlar, Germany), frozen in a metal pan over a bath of liquid nitrogen. All frozen tissue blocks were cryo-sectioned to a nominal thickness of 10 μ m and mounted on gelatin-coated slides. Before staining, each slide was fixed in ice-cold acetone for 5 min and dried for 30 min at room temperature. Hematoxylin–eosin and Safranin-O staining were performed. The regenerated cartilage was grossly examined using the International Cartilage Repair Society (ICRS) macroscopic score,²² which evaluates the degree of defect repair, integration to the border zone, and the macroscopic appearance. Wakitani Histological Grading Scale²³ was used to evaluate the quality of the repaired tissue microscopically (Supplementary Table S1). For immunohistochemistry, we used Dako cytation LSAB2 System HRP kits (Dako, Hamburg, Germany). After acetone fixation, each slide was treated with Pepsin Soluble® (Fine Life Science, Seoul, Korea) for 15 min and washed in a 1× wash buffer (Dako). Sections were incubated for 5 min with a peroxidase-blocking solution and reacted with primary antibodies against type II collagen (Merck Millipore), type I collagen (Abcam), and type X collagen (Abcam) diluted 1:100 in goat serum overnight at 4°C. After three washes in 1× wash buffer, the slides were incubated with HRP-labeled secondary antibody for 1 h, extensively washed, reacted with substrate buffer and diaminobenzidine (DAB) chromogen (50:1; Dako) for 5 min, and then mounted.

Statistical analysis

Descriptive statistics were used to determine the group means and standard deviations for numerical data, and the analysis was performed using the Mann–Whitney *U*-test. A *p*-value < 0.05 was considered significant.

Results and Discussion

Loading and release profile of PD98059

The prepared PLGA/F127 scaffolds showed highly porous and interconnective pore structures, which can allow high cell–cell interactions and thus can enhance the chondrogenesis of stem cells. The pore size in the scaffold was almost the same as that of added salt particles (sizes range from 200 to 250 μ m; Fig. 1A). The porosity of the scaffolds was around 90%. It has been well established that the porosity of a scaffold should be at least 90% to provide a sufficient space for extracellular matrix production and effective diffusion of the cell culture medium into a scaffold during *in vitro* cell culture.²⁰

Figure 1B shows the release behavior of the PD98059 from the PLGA/F127 scaffold with time. The loading amount of the PD98059 in each scaffold was determined as follows: $14.7 \pm 0.6 \mu$ g for 0.3 wt% PD98059 scaffold, $22.5 \pm 0.8 \mu$ g for 0.5 wt% PD98059 scaffold, and $44.8 \pm 3.7 \mu$ g for 1.0 wt% PD98059 scaffold. The PD98059 was continuously released from the scaffolds over 140 days without a plateau,

FIG. 1. Loading and release profile of PD98059. Representative scanning electron microscope images of poly(lactic-co-glycolic acid) (PLGA)/F127 scaffolds (A). Cumulative release profiles of PD98059 from the PD98059-loaded PLGA/F127 scaffolds with different PD98059 compositions (B) ($n=3$). The PD98059 was continuously released from the scaffolds over 140 days, regardless of the PD98059 composition.

regardless of the PD98059 compositions, which may effectively promote chondrogenesis and inhibit hypertrophy from MSCs.¹³ The sustained release of PD98059 from the scaffold can be explained by the degradation of the polymeric matrix through water diffusion into the matrix.²⁴ In our previous study, we demonstrated that the PD98059 of 0.03–0.30 $\mu\text{g/mL/day}$ in the culture medium can effectively prevent the hypertrophy during the chondrogenesis of the MSCs.¹³ On the basis of this finding, we recognized that the scaffold with 1.0 wt% PD98059 may be appropriate for the hypertrophy inhibition and chondrogenesis of MSCs as a 3D porous matrix (release rate, $\sim 0.12 \mu\text{g/mL/day}$). Therefore, the PD98059-loaded scaffold (1 wt% PD98059) was used for further experiments.

Immobilization and release profile of TGF- β_2

The amount of heparin immobilized on the PLGA and PLGA/F127 scaffolds was determined by the toluidine blue assay. The PLGA/F127 scaffold allowed significantly greater heparin immobilization on the scaffold ($5.48 \pm 0.02 \mu\text{g/scaffold}$) than the PLGA scaffold ($1.21 \pm 0.16 \mu\text{g/scaffold}$; Fig. 2A). This observation can be explained by a specific interaction (hydrogen bonding) between the ether oxygen of Pluronic F127 exposed on the pore surface of the scaffold and the carboxylic acid group of heparin.²⁵ The result suggests that the Pluronic F127 chains were appropriately exposed onto the surfaces of the porous scaffold to interact with the heparin. The quantitative amount of TGF- β_2 immobilized on the scaffolds was determined using the direct ELISA tech-

FIG. 2. Immobilization and release profile of transforming growth factor (TGF)- β_2 . Amount of heparin immobilized onto the PLGA and PLGA/F127 scaffolds (A), loading amount of TGF- β_2 onto the PLGA/F127 scaffold with and without heparin immobilization (B), and their cumulative release profile (C). The PLGA/F127 scaffold allowed significantly greater heparin immobilization on the scaffold than PLGA scaffold. The heparin-immobilized scaffold had significantly larger TGF- β_2 loading amount compared to the scaffold without the heparin. Also, the heparin-immobilized scaffold showed the continuous release of TGF- β_2 up to $\sim 80\%$ of the loading amount over 21 days. Bar represents mean $\pm$ SD. $n=3$, $*p<0.05$.

nique. The heparin-immobilized scaffold had a significantly larger TGF- β_2 loading amount (+Hep; $30.18 \pm 0.63 \text{ ng/scaffold}$) compared to the scaffold without the heparin (-Hep; $15.87 \pm 2.41 \text{ ng/scaffold}$; Fig. 2B), due to the ionic interaction between heparin (O-sulfate and N-sulfate groups) and

growth factor (certain lysine and arginine residues).²⁶ The TGF- β_2 observed on the scaffold without heparin can be explained by the physical adsorption on the pore surfaces of the scaffold, which may lead to the initial burst release in the medium. The release behaviors of the TGF- β_2 from the porous scaffolds are shown in Figure 2C. The heparin-immobilized scaffold showed the continuous release of TGF- β_2 up to ~80% of loading amount over 21 days. The slow release of the growth factor bound via ionic interactions on the porous scaffold may be understood by the gradual dissociation of ionic bonds in PBS solution. On the basis of our previous study,¹⁶ we recognized that the release profile of TGF- β_2 for 3 weeks can provide an appropriate environment for chondrogenesis of hMSCs. On the other hand, the scaffold without heparin immobilization showed a greater initial burst for the 3 days (over 60% of loading amount) because of the desorption of TGF- β_2 simply adsorbed onto the porous scaffold. The sustained release of TGF- β_2 from the heparin-immobilized porous scaffolds can prolong their biological effects.

It is well known that the chondrogenesis of stem cells is usually determined within 3 weeks after the treatment of

differentiation agents^{13,16} while hypertrophy continues (>3 weeks) even thereafter. Mackay *et al.* also reported that the TGF- β_2 can act as an important factor to induce the chondrogenesis of MSCs at initial stage (~3 weeks), but long-term TGF- β_2 treatment rather increase the hypertrophy of chondro-induced cells.²⁷ So, we expected that the relatively fast release of the TGF- β_2 (for up to 3 weeks, as a differentiation agent) may provide an appropriate environment for chondrogenesis, whereas long-term release of the PD98059 (~24 weeks, as a differentiation agent and hypertrophy inhibitor) from our scaffolds may be beneficial for chondrogenesis and hypertrophy suppression in the process of cartilage regeneration.

In vitro chondrogenesis from MSCs in the functional scaffolds

After fabrication of factor-impregnated scaffolds and identification of the release profile, the *in vitro* chondrogenic potential of each scaffold was tested by seeding hMSCs in the scaffold and culturing for 28 days. The results

FIG. 3. *In vitro* chondrogenesis from mesenchymal stem cells in the functional scaffolds. The *in vitro* chondrogenic potential of each scaffold was tested by seeding human mesenchymal stem cells (hMSCs) in the scaffold and culturing for 28 days. DNA amounts and glycosaminoglycan (GAG) contents measured in each scaffold normalized against total DNA (**A**), gene expression by real-time polymerase chain reaction (**B**), and western blotting (**C**). All values are shown as percentages over the control (hMSCs seeded in plain PLGA scaffold). PD98059 and TGF- β_2 represent hMSCs seeded in PD98059-impregnated scaffold group and TGF- β_2 -impregnated scaffold group, respectively. Bar represents mean $\pm$ SD. $n=3$, $*p<0.05$; NS, not significant.

demonstrated that the GAG per DNA amount was 6-fold greater ($*p < 0.05$) in the PD98059-impregnated scaffold and 4.5-fold greater ($*p < 0.05$) in the TGF- β_2 -immobilized scaffold compared with the control PLGA scaffold (no loaded factor). The GAG per DNA amount in the PD98059-impregnated scaffold was, in turn, significantly greater ($*p < 0.05$) than that in the TGF- β_2 -loaded scaffold (Fig. 3A). However, this difference was not significant when GAG contents in each scaffold were compared between the two groups (Supplementary Fig. S2). The results from reverse

transcription and quantitative polymerase chain reaction (RT-qPCR) demonstrated that the chondrogenic marker type II collagen (COL2A1) was increased by 3.8- and 3.5-fold ($*p < 0.05$) in the PD98059-impregnated scaffold and the TGF- β_2 -immobilized scaffold, respectively, compared with the control. Sex-determining region Y (SRY)-box 9 protein (SOX9), another chondrogenic marker, also significantly increased in both functional scaffolds. Type I collagen, detected in undifferentiated MSCs as well as osteoblasts, did not change significantly. Type X collagen (COL10A1), the typical

FIG. 4. *In vivo* effect of the functional scaffolds on the healing of rabbit osteochondral defect. Four types of constructs were implanted in the osteochondral defects created in the patellar groove of rabbits: group 1, PLGA scaffold only; group 2, PLGA scaffold seeded with rabbit mesenchymal stem cells (rMSCs); group 3, PD98059-impregnated PLGA scaffold seeded with rMSCs; group 4, TGF- β_2 -immobilized PLGA scaffold seeded with rMSCs (A). Gross appearances of the osteochondral defect and International Cartilage Repair Society (ICRS) macroscopic scores after 10 weeks of implantation: margins of defects are indicated by the yellow arrows (B). The histological findings from Safranin-O staining and Wakitani histological score after 10 weeks of implantation: red arrows denote the margin of the defects (C). Bar represents mean $\pm$ SD. $n = 5$, $*p < 0.05$, $**p < 0.01$; NS, not significant. Color images available online at www.liebertpub.com/tea

hypertrophy marker, increased fivefold ($*p < 0.05$) in TGF- β_2 -immobilized scaffold, whereas it did not change in PD98059-impregnated scaffold. Alkaline phosphatase (ALP), a marker of osteoblastic differentiation and hypertrophy, significantly decreased ($*p < 0.05$) in both functional scaffolds, more so in the PD98059-impregnated scaffold than the TGF- β_2 -immobilized scaffolds ($*p < 0.05$) (Fig. 3B). The results from western blotting were all in line with the results from RT-qPCR, and no mismatch between gene and protein expression was detectable (Fig. 3C). These results suggest that while chondrogenic differentiation of seeded hMSCs was enhanced in both PD98059-impregnated scaffold and TGF- β_2 -immobilized scaffold, the PD98059-impregnated scaffold was more effective in suppressing hypertrophy than the TGF- β_2 -immobilized scaffold. Although the results from TGF- β_2 -immobilized scaffold concur with our previous studies in which TGF- β_2 was continuously treated to

induce chondrogenesis from MSCs in a pellet culture, the finding that impregnated PD98059 alone without any growth factor can work comparably to or better than TGF- β_2 when applied for direct tissue engineering is very promising. We had also tried to obtain the histology of the chondro-induced hMSC-PLGA hybrid several times for months, but it was not possible unfortunately because the prepared sections were dissolved away during the washing process. It is attributed to the hydrophilic nature of Pluronic F127-added PLGA scaffold.

Although the PD98059-impregnated scaffold did not reduce type X collagen to a level lower than that of the undifferentiated control, even the "no increase" is a meaningful finding in view of the fact that type X collagen always increases in the chondrogenesis of MSCs.^{8,17,27,28} These additional properties of the PD98059-impregnated scaffold make it more suitable for *in vivo* application.

FIG. 5. Immunohistochemistry for COL2A1, COL1A1, and COL10A1 of regenerated cartilage. Type II collagen (A–C), type I collagen (D–F), and type X collagen (G–I). The lower rows (a–i) are magnified view of boxed area in the upper row (A–I). The red arrows indicate type I collagen expression (f) and type X collagen (i) within and around chondrocytes in group 4, while group 3 is essentially free from type I collagen and type X collagen. Scale bar = 200 μ m. Color images available online at www.liebertpub.com/tea

In vivo promotion of chondrogenesis and suppression of hypertrophy in a rabbit osteochondral model

To test the *in vivo* effectiveness of the factor-impregnated functional scaffolds to enhance the chondrogenesis and suppress hypertrophy from seeded MSCs, four types of constructs were implanted in the osteochondral defects created in the patellar groove of rabbits: group 1, PLGA scaffold only; group 2, PLGA scaffold seeded with MSCs; group 3, PD98059-impregnated PLGA scaffold seeded with MSCs; group 4, TGF- β_2 -immobilized PLGA scaffolds seeded with MSCs (Fig. 4A). The rabbits were sacrificed after 10 weeks.

Gross appearance of the defect showed good quality of repair in groups 3 and 4, which showed smooth, firm, and glistening articular surface with good integration into the adjacent normal cartilage. Group 2 defects were also filled with firm glistening tissue, but the border with the adjacent normal cartilage was distinct. The group 1 defect showed a persistent depression filled with friable tissue. The ICRS macroscopic scores of the repaired defects were significantly higher in groups 3 and 4 than in group 1 ($*p < 0.01$; statistical power > 0.95 , Fig. 4B).

The histological findings from Safranin-O staining showed that the AC was well regenerated in groups 3 and 4 with a smooth surface, abundant matrix and good integration with adjacent cartilage. However, the reconstitution of subchondral bone was better in group 3 than in group 4. While group 2 defects also showed reasonable restoration of the AC, there were signs of degeneration including fibrillation and irregularity of surface as well as decreased amount of cartilaginous matrix. Group 1 defects showed a persistent depression of defect. Although there were some areas with thin regenerated cartilage, mostly bare bone surface was exposed in group 1 defects. The histological score was significantly better in groups 3 and 4 than in group 1 ($*p < 0.05$; statistical power > 0.95). Group 3 also had a significantly higher score than group 2 or 4 ($*p < 0.05$; statistical power > 0.95 , Fig. 4C). These *in vivo* results confirmed that while the chondrogenic differentiation was successfully induced by both PD98059-impregnated and TGF- β_2 -immobilized scaffold when evaluated grossly and microscopically, the overall quality of cartilage regeneration was better with the PD98059-impregnated scaffold.

To further analyze the quality of repair and confirm if the functional scaffold suppressed hypertrophy *in vivo*, immunohistochemistry of the regenerated cartilage was performed for type II collagen, type I collagen, and type X collagen. Immunostaining in groups 1 and 2 showed irregular protein expressions because the defects were incompletely reconstituted (Supplementary Fig. S3). While type II collagen was abundantly expressed in the matrix of normal AC, the degree of type II collagen expression was less marked in groups 3 and 4 than in normal cartilage. Safranin-O reacts to acidic proteoglycan (aggrecan, type II collagen) within AC. While metachromasia in Safranin-O staining and detection of type II collagen are hallmarks of cartilaginous tissue, the finding from Safranin-O staining does not always correspond with type II collagen expression in regenerated tissue. However, the longer regeneration time might have led to the stronger type II collagen expression in groups 3 and 4. Type I collagen and type X collagen were not observed in normal AC and group 3, while they were detected within and around chondrocytes in group 4 (Fig. 5).

These results suggest that the use of the PD98059-impregnated scaffold led to AC regeneration of better quality than the TGF- β_2 -immobilized scaffold and prevented hypertrophy when implanted in the osteochondral defects of rabbits. It can be asserted that the PD98059-impregnated functional scaffold is very effective for cartilage regeneration using MSCs and holds considerable promise as a tool for direct cartilage tissue engineering.

Conclusions

PD98059-impregnated and TGF- β_2 -immobilized PLGA/F127 scaffolds were fabricated using a melt-molding particulate-leaching method. The PD98059 was continuously released from the scaffolds over 140 days without a plateau in contrast to the rapid release of TGF- β_2 over 21 days. When the *in vitro* chondrogenic potential of each scaffold was tested by seeding hMSCs in the scaffold and culturing for 28 days, the PD98059-impregnated scaffold was more effective in suppressing hypertrophy than the TGF- β_2 -immobilized scaffold while chondrogenic differentiation of the seeded MSCs was enhanced in both scaffolds. The *in vivo* effectiveness of the factor-impregnated scaffold to enhance the chondrogenesis and suppress hypertrophy from seeded MSCs was tested by implanting a functional scaffold/rMSC hybrid in the osteochondral defects created in the patellar groove of rabbits. While the defects were successfully repaired in both PD98059-impregnated and TGF- β_2 -impregnated scaffolds seeded with rMSCs when evaluated grossly and microscopically, the use of the PD98059-impregnated scaffold provided AC regeneration of better quality and prevented hypertrophy when implanted in the osteochondral defects of rabbits.

Acknowledgment

This study was supported by a grant from the National Research Foundation of Korea (2009-0092196).

Disclosure Statement

The authors declare no competing financial interest.

References

1. Buckwalter, J.A., and Mankin, H.J. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr Course Lect* **47**, 487, 1998.
2. Buckwalter, J.A., Mankin, H.J., and Grodzinsky, A.J. Articular cartilage and osteoarthritis. *Instr Course Lect* **54**, 465, 2005.
3. Caplan, A.I. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng* **11**, 1198, 2005.
4. Caplan, A.I., and Goldberg, V.M. Principles of tissue engineered regeneration of skeletal tissues. *Clin Orthop Relat Res* **367 Suppl**, S12, 1999.
5. Tuan, R.S., Boland, G., and Tuli, R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther* **5**, 32, 2003.
6. Chen, F.H., Rousche, K.T., and Tuan, R.S. Technology Insight: adult stem cells in cartilage regeneration and tissue engineering. *Nat Clin Pract Rheumatol* **2**, 373, 2006.
7. Im, G.I., and Lee, J.H. Repair of osteochondral defects with adipose stem cells and a dual growth factor-releasing

- scaffold in rabbits. *J Biomed Mater Res B Appl Biomater* **92**, 552, 2010.
8. Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B.G., Aigner, T., and Richter, W. Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* **54**, 3254, 2006.
 9. Bohme, K., Winterhalter, K.H., and Bruckner, P. Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-beta 2 and basic fibroblast growth factor in synergy. *Exp Cell Res* **216**, 191, 1995.
 10. Stanton, L.A., Underhill, T.M., and Beier, F. MAP kinases in chondrocyte differentiation. *Dev Biol* **263**, 165, 2003.
 11. Oh, C.D., Chang, S.H., Yoon, Y.M., Lee, S.J., Lee, Y.S., Kang, S.S., and Chun, J.S. Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymal cells. *J Biol Chem* **275**, 5613, 2000.
 12. Hwang, N.S., Varghese, S., Puleo, C., Zhang, Z.J., and Elisseeff, J. Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells. *J Cell Physiol* **212**, 281, 2007.
 13. Kim, H.J., and Im, G.I. The effects of ERK1/2 inhibitor on the chondrogenesis of bone marrow- and adipose tissue-derived multipotent mesenchymal stromal cells. *Tissue Eng Part A* **16**, 851, 2010.
 14. Chung, C., and Burdick, J.A. Engineering cartilage tissue. *Adv Drug Deliv Rev* **60**, 243, 2008.
 15. Im, G.I., Jung, N.H., and Tae, S.K. Chondrogenic differentiation of mesenchymal stem cells isolated from patients in late adulthood: the optimal conditions of growth factors. *Tissue Eng* **12**, 527, 2006.
 16. Kim, H.J., Kim, Y.J., and Im, G.I. Is continuous treatment with transforming growth factor-beta necessary to induce chondrogenic differentiation in mesenchymal stem cells? *Cells Tissues Organs* **190**, 1, 2008.
 17. Kim, Y.J., Kim, H.J., and Im, G.I. PTHrP promotes chondrogenesis and suppresses hypertrophy from both bone marrow-derived and adipose tissue-derived MSCs. *Biochem Biophys Res Commun* **373**, 104, 2008.
 18. Lee, K.H., Oh, S.H., Choi, S.W., and Lee, J.H. *In-situ* mechanical behavior of porous biodegradable polymer scaffolds fabricated by melt molding compression method. *Biomater Res* **5**, 17, 2001.
 19. Lee, L.M., Davison, Z., and Heard, C.M. *In vitro* delivery of anti-breast cancer agents directly via the mammary papilla (nipple). *Int J Pharm* **387**, 161, 2010.
 20. Shen, Y.H., Shoichet, M.S., and Radisic, M. Vascular endothelial growth factor immobilized in collagen scaffold promotes penetration and proliferation of endothelial cells. *Acta Biomater* **4**, 477, 2008.
 21. Oh, S.H., Kim, J.H., Song, K.S., Jeon, B.H., Yoon, J.H., Seo, T.B., Namkung, U., Lee, I.W., and Lee, J.H. Peripheral nerve regeneration within an asymmetrically porous PLGA/Pluronic F127 nerve guide conduit. *Biomaterials* **29**, 1601, 2008.
 22. van den Borne, M.P., Raijmakers, N.J., Vanlauwe, J., Victor, J., de Jong, S.N., Bellemans, J., and Saris, D.B. International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in autologous chondrocyte implantation (ACI) and microfracture. *Osteoarthritis Cartilage* **15**, 1397, 2007.
 23. Wakitani, S., Goto, T., Pineda, S.J., Young, R.G., Mansour, J.M., Caplan, A.L., and Goldberg, V.M. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am* **76**, 579, 1994.
 24. Oh, S.H., Kim, T.H., Jang, S.H., Im, G.I., and Lee, J.H. Hydrophilized 3D porous scaffold for effective plasmid DNA delivery. *J Biomed Mater Res A* **97**, 441, 2010.
 25. Chung, Y.I., Tae, G., and Hong, Y.S. A facile method to prepare heparin-functionalized nanoparticles for controlled release of growth factors. *Biomaterials* **27**, 2621, 2006.
 26. Lyon, M., Rushton, G., and Gallagher, J.T. The interaction of the transforming growth factor-betas with heparin/heparan sulfate is isoform-specific. *J Biol Chem* **272**, 18000, 1997.
 27. Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* **4**, 415, 1998.
 28. Johnstone, B., Hering, T.M., Caplan, A.L., Goldberg, V.M., and Yoo, J.U. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* **238**, 265, 1998.

Address correspondence to:

Gun-Il Im, MD

Department of Orthopaedics

Dongguk University Ilsan Hospital

Goyang 410-773

Republic of Korea

E-mail: gunil@duih.org

Received: May 15, 2013

Accepted: October 10, 2013

Online Publication Date: December 10, 2013