Biphasic Nanofibrous Constructs with Seeded Cell Layers for Osteochondral Repair

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Biphasic scaffolds have gained increasing attention for the regeneration of osteochondral interfacial tissue because they are expected to effectively define the interfacial structure of tissue that comprises stratified cartilage with a degree of calcification. Here, we propose a biphasic nanofiber construct made of poly(lactide-co-caprolactone) (PLCL) and its mineralized form (mPLCL) populated with cells. Primary rat articular chondrocytes (ACs) and bone marrow-derived mesenchymal stem cells (MSCs) were cultured on the layers of bare PLCL and mPLCL nanofibers, respectively, for 7 days, and the biphasic cell-nanofiber construct was investigated at 4 weeks after implantation into nude mice. Before implantation, the ACs and MSCs grown on each layer of PLCL and mPLCL nanofibers exhibited phenotypes typical of chondrocytes and osteoblasts, respectively, under proper culture conditions, as analyzed by electron microscopy, histological staining, cell growth kinetics, and real-time polymerase chain reaction. The biphasic constructs also showed the development of a possible formation of cartilage and bone tissue *in vivo*. Results demonstrated that the cell-laden biphasic nanofiber constructs may be useful for the repair of osteochondral interfacial tissue structure.

Introduction

OSTEOCHONDRAL TISSUE REGENERATION at the interfacial region between cartilage and bone has been challenged due to the stratified tissue structure with a degree of calcified cartilages. Among other things, scaffolds with biphasic structures have primarily been developed and considered to be effective for the reconstruction of cartilage and bone.¹ The structural and physical feature of biphasic scaffolds has been implicated to significantly affect cellular responses and secretion of extracellular matrices (ECMs) of osteochondral tissues.² For example, biodegradable polymers such as collagen³ and polyesters⁴ were functionally constructed with inorganic hydroxyapatite (HA), calcium phosphate, or bioactive glasses for the chondral and osseous phase, respectively. Furthermore, when a scaffold is nanostructured, mimicking the native tissue ECMs, cells recognize better and behave more actively on the artificial biomaterial.

Among the scaffolds, electrospun nanofibers have been widely used in the field of tissue engineering, because the nanofibrous structures largely mimic the native ECMs of collagenous tissues, thus supporting the adhesion, growth, and functions of cells.⁵ For the osteochondral recovery, nanofibrous scaffolds have also been used to promote protein

adsorption, cell adhesion, and osteoblastic differentiation.^{6,7} Besides scaffolds, the choice of appropriate cells is also crucial to effectively repair the osteochondral tissues. Among the cell sources, self-renewing mesenchymal stem cells (MSCs) and primary chondrocytes have been used intensively for osteochondral tissue engineering.⁸⁻¹¹ MSCs have been shown to successfully differentiate into an osteogenic lineage *in vitro* and calcified bone formation *in vivo*.^{12,13} Moreover, primary chondrocytes have also been demonstrated to preserve the chondrogenic phenotypes *in vitro* and to successfully form cartilage tissues *in vivo*.^{14,15}

In this study, we propose a novel scaffold-cell construct for osteochondral repair. Biphasic nanofiber scaffolds composed of poly(lactide-co-caprolactone) (PLCL) and its mineralized form (mPLCL) were used as the matrix for the culture of chondrocytes and MSCs, respectively. The efficacy of the biphasic scaffold-cell constructs was investigated *in vitro* and *in vivo* after implantation in nude mice.

Materials and Methods

Fabrication and characterization of nanofibers

PLCL nanofibrous scaffolds were fabricated as described previously.¹⁶ PLCL (12.5% w/v, Boehringer Ingelheim) was

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dissolved in a cosolvent (20% ethanol + 80% dichloromethane). The polymer solution was subjected to electrospinning at an injection rate of 0.4 mL/h and a distance of 15 cm under a voltage of 13 kV. After collecting on a metal collector, fibers were dried under vacuum overnight to evaporate the remnant solvent.

The mineralization of PLCL nanofibrous scaffolds was carried out according to the procedure as described previously.¹⁷ First, the surface of nanofibers was activated by dipping the nanofiber into a 2-N NaOH solution and incubating for 6 h at room temperature. Next, the specimen was dipped alternately into an individual 150 mM calcium solution and 150 mM phosphate solution prepared from CaCl_2 and Na_2HPO_4 , respectively. This process was repeated six times. Finally, for mineralization, the nanofiber was incubated in a simulated body fluid at 37°C for 7 days.

The morphology and phase of the mineralized nanofiber were evaluated by scanning electron microscopy (SEM, Hitachi S-3000H) and X-ray diffraction (XRD; Rigaku), respectively. The atomic element and chemical status of the mineralized nanofiber were analyzed by energy dispersive spectroscopy (EDS) in conjunction with SEM and by Fourier transformed infrared (FT-IR; Perkin-Elmer) spectroscopy, respectively.

The hydrolytic degradation of nanofibers was carried out for up to 6 weeks. Each specimen (40 × 40 mm) was placed in a vial containing 25 mL of phosphate-buffered saline (PBS) and incubated at 37°C for up to 6 weeks with refreshing the medium every 2 weeks. After incubation for 2, 4, and 6 weeks, samples were taken out, dried completely, and the weight change was recorded. Three specimens were tested for each condition.

Cell isolation and expansion

All protocols involving animals were approved by the Animal Ethics Committee, Dankook University School of Medicine. Rat primary articular chondrocytes (ACs) and bone marrow-derived MSCs used in this study were harvested from articular cartilage of the knees and from the excised proximal and distal epiphyses of the femora and tibiae of a Sprague Dawley rat, respectively. These cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose; Gibco-BRL) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator containing 5% CO_2 at 37°C. Both cells were used after four passages for the following experiments.

In vitro cell differentiation study of chondrocytes and MSCs on the scaffolds

Chondrocytes (2×10^4 cells in 200 µL DMEM) were seeded on each sheet of the PLCL nanofiber (5 mm in diameter), which was contained in each well of 96-well plates. Likewise, MSCs (2×10^4 cells in 200 µL α -minimum essential medium [α -MEM]) were seeded on each sheet of the mPLCL nanofiber (5 mm in diameter). The cell-nanofiber sheets were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h, during which the cells reached almost 70% confluence. Thereafter, each medium was replaced with the chondrogenic- or osteogenic-defined medium. The chondrogenic medium consists of the normal medium (DMEM + 100 U/mL

penicillin/100 µg/mL streptomycin + 10% FBS) + 1% insulin-transferrin-selenium (PAA Laboratories, Inc.) + 37.5 µg/mL ascorbic acid + 100 nM dexamethasone + 10 ng/mL transforming growth factor- β 1 (PeproTech), and the osteogenic medium is made of the normal medium (α -MEM + 100 U/mL penicillin/100 µg/mL streptomycin + 10% FBS) + 50 µg ascorbic acid + 10 mM β -glycerophosphate + 100 nM dexamethasone.¹⁸ Within each medium, the cell-nanofiber constructs were cultured for up to 14 days, and the medium was refreshed every 2–3 days. In case of cells without having undergone specific differentiation, the normal medium (as described above) was used for each type of cells.

Scanning electron microscopy

Cell-layered constructs were fixed in 2.5% glutaraldehyde for 10 min at room temperature at 7 days after culture *in vitro*. The fixed samples were then dehydrated for 5 min in increasing concentrations of ethanol (75%, 95%, and 100%), after which they were critical-point dried. Finally, the samples were coated with a thin layer of gold, and the morphology of the construct was examined and photographed using SEM (Hitachi 3000) at an accelerating voltage of 15–20 kV.

F-actin observation

Cell-layered constructs fixed with 4% paraformaldehyde were incubated for 30 min with 20 nM Alexa Fluor 546-conjugated phalloidin (Invitrogen). An inverted microscope (IX-71; Olympus) was used for image processing.

Cellular proliferation assay

Cell proliferation was assessed using a cell counting kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo Molecular Technologies, Inc.). At the end point of each culture time, 10 µL of CCK-8 solution was added to each well, and the plates were then incubated for 3 h at 37°C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices).

Histological analysis of in vitro specimens

After 7 days of culture, chondrocyte-layered constructs were stained with Safranin O (Sigma-Aldrich) for glycosaminoglycans (GAGs) visualized in red and Alcian blue for extracellular GAGs in blue to analyze chondrocyte differentiation. Under the same conditions, MSC-layered constructs were also stained with alkaline phosphatase (ALP) to analyze the osteogenic differentiation. The level of ALP expression was observed by staining the cell-layered constructs with a chemical reagent (Cat.# MK300; Takara). The samples stained in purple-blue color were observed using optical microscopy.

Gene expression by real-time polymerase chain reaction

After 7 days of culture, the cells were lysed with TRIzol, RNA was extracted using the standard phenol/chloroform method, and first-strand cDNA was synthesized from the total RNA (1 µg) using a SuperScript first strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using SYBR GreenER qPCR

TABLE 1. PRIMER SEQUENCES OF THE GENES FOR THE REAL-TIME POLYMERASE CHAIN REACTION

Gene	Primer sequence
ALP	F: 5'-ACTGGTACTCGGACAATGAG-3' R: 5'-ATCGATGTCCTTGATGTTGT-3'
SOX9	F: 5'-CGTCAACGGCTCCAGCA-3' R: 5'-TGCGCCACACCATGA-3'
Collagen II	F: 5'-GAGTGGAAAGAGCGGAGACTACTG-3' R: 5'-CTCCATGTTGCAGAAGACTTTCA-3'
Aggrecan	F: 5'-CTAGCTGCTTAGCAGGGATAACG-3' R: 5'-TGACCCGCAGAGTCACAAAG-3'
β -Actin	F: 5'-ACGTTGACATCCGTAAAGAC-3' R: 5'-TAATCTCCTTCTGCATCCTG-3'

PLCL, poly(lactide-co-caprolactone); mPLCL, mineralized form; PBS, phosphate-buffered saline.

SuperMix reagents (Invitrogen). The relative transcript quantities were calculated using the $\Delta\Delta C_t$ method with β -actin as the endogenous reference. The primer sequences of the bone- and cartilage-associated genes used in real-time PCR are presented in Table 1.

In vivo transplantation

Eight-week-old male athymic mice were randomly divided into two groups (three mice per group). For the *in vivo* tests, samples were prepared as follows: First, each cell-nanofiber construct was maintained in the chondrogenic and osteogenic medium for 7 days, respectively. After this, the cultured five sheets of MSC-mPLCL were layer-by-layer stacked manually. Likewise, five sheets of chondrocyte-PLCL constructs were also stacked separately. Last, the MSC-mPLCL-layered constructs were put together with the chondrocyte-PLCL layered constructs to produce 10 layers of the cell-nanofiber constructs (5 chondrocyte-PLCL layers over 5 MSC-mPLCL layers). During the layer-by-layer stacking process, each cell-nanofiber layer was examined to be integrated well without being separated, and this might be driven by the high surface tension of the nanofibrous matrix.

The animals were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg; Rompun[®]; Bayer Korea, Ltd.) and ketamine (70 mg/kg; Ketara[®]; Yuhan Corp.). Small incisions were made on the dorsal skin. Two pouches per animal were created by blunt dissection of subcutaneous sites. Cell-layered scaffold constructs were immediately implanted into one pouch with a direction of PLCL-up, and subsequently, acellular scaffold constructs were implanted into the other pouch. After 4 weeks, samples were harvested, fixed in formalin, embedded in paraffin, cut into about 5- μ m-thick sections, and then stained with hematoxylin and eosin (H&E), Safranin O, and Alizarin red S. Furthermore, immunostaining of the harvested samples was carried out to detect the expression of type II collagen. The slides were incubated with 5% normal goat serum (Vector Laboratories) in PBS for 30 min to suppress nonspecific staining, and then incubated with a primary antibody, anti-collagen type II (1:150 dilution, sc-52658; Santa Cruz Biotechnology), for 24 h at 4°C. The specimens were subsequently incubated with the FITC-conjugated antibody against mouse IgG (1:100 dilution, 115-095-003; Jackson Immunoresearch) for

30 min at room temperature. The nuclei of the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The slides were examined with an inverted fluorescence microscope equipped with a DP-72 digital camera (Olympus Co.).

Statistical analysis

For statistical comparison of the means between the groups of interest, a Student's *t*-test was performed. Statistically significant levels were considered at $p < 0.05$ and $p < 0.01$.

Results and Discussion

Concept of biphasic scaffold-cell constructs for osteochondral repair

PLCL with a highly elastic property has been extensively used as scaffold material in the field of tissue engineering for peripheral nerves, meniscus, blood vessels, and cartilage.¹⁹ Honda *et al.* first recognized a cartilage-like structure and cell morphology in chondrocyte-inoculated PLCL sponges subcutaneously implanted into nude mice.²⁰ To the best of our knowledge, we report for the first time the possibility of individual tissue regeneration in chondrocyte- and MSC-constructed PLCL biphasic nanofibers subcutaneously implanted into nude mice. This study focused on the design of tissue-engineered PLCL nanofibers for simultaneous regeneration of bone and cartilage. Figure 1 depicts our experimental scheme, where PLCL nanofibers served as an ECM for the selective culture of ACs and of rat MSCs on the bare and mineralized surface of the nanofibers, respectively, for 7 days under each differentiation condition. The cell-constructed nanofibers were then layered and subcutaneously implanted into nude mice to investigate each tissue-associated formation. First, we confirmed the appropriate differentiation of each cell type under the differentiating conditions in comparison with undifferentiating conditions by examining phenotypic expressions of specific cell lineages. Next, the *in vivo* test was performed with the differentiated cell-scaffold constructs to find the efficacy of the biphasic tissue-engineered design, in direct comparison with the cell-free biphasic scaffold group.

Characteristics of scaffolds

Figure 2 shows morphologies of electrospun bare PLCL (Fig. 2A) and mPLCL (Fig. 2B) nanofibers. Bare PLCL nanofibers exhibit sleek surfaces, while the morphology of bead-shaped attached nanofibers is observed in mPLCL nanofibers. The feature of nanofibrous morphology was well preserved with the presence of highly interconnected pores. Fiber diameters of bare PLCL ranged from 400 to 600 nm, but the mPLCL nanofibers were approximately twofold thicker than the bare PLCL nanofibers due to homogeneously deposited mineral crystals on mPLCL nanofibers (Fig. 2C). In fact, the fiber diameter should influence the adhesion and proliferation of cells, which also was dependent on cell types.^{21–23} Although it would be better to optimize the fiber diameter for each cell type (MSCs or chondrocytes), we consider this issue as another interesting study to follow. In this study, we thus used the fabricated range of fiber diameters, and the two cell types were

FIG. 1. Schematic showing the overall experimental procedures of the present study. (A) Chondrocytes were plated onto poly(lactide-co-caprolactone) (PLCL) nanofibrous scaffold; (B) chondrocyte-PLCL constructs were incubated in chondrogenic medium by layer-by-layer assembly for 7 days; (C) mineralized form (mPLCL) nanofibrous scaffold was prepared after incubation in simulated body fluid for 7 days; (D) mesenchymal stem cells (MSCs) were seeded onto mPLCL sheet; (E) MSC-mPLCL constructs were incubated in osteogenic medium by layer-by-layer assembly for 7 days; (F) biphasic construct was made by interfacing chondrogenic chondrocyte- and osteogenic MSC-seeded layers; (G) biphasic constructs were implanted into the dorsal subcutaneous sites of the athymic nude mice. Image of sample for *in vivo* tests (10-layered cell-nanofiber constructs with 5 mm diameter) is shown in inset. Color images available online at www.liebertpub.com/tec

cultured separately. Figure 2D shows EDS analyses of major atomic elements in bare PLCL and mPLCL nanofibers. The EDS spectrum in mPLCL nanofibers, contrasted to that in bare PLCL, shows additional peaks of calcium and phosphorus elements.²⁴ The physicochemical properties of bare PLCL and mPLCL nanofibers were further characterized by FT-IR (Fig. 2E) and XRD (Fig. 2F) analyses. The FT-IR spectrum of mPLCL nanofibers revealed that the vibration bands relating phosphate groups are shown at wave numbers of 1029 and 563 cm^{-1} , and other unique bands corresponding to PLCL molecules were concealed due to covered mineral molecules in comparison with the spectrum presenting bare PLCL. The XRD pattern of mPLCL shows peaks at 26° and 32°, which corresponds to the HA while the amorphous XRD pattern is observed in bare PLCL. The result indicates that tiny crystallites constituting bone mineral-like HA completely covered the PLCL nanofiber surface. Taken together, the produced mineral phase is considered to mimic the native bone mineral phase, which possibly favors the cellular reactions mainly involved in the osteogenic differentiation and bone formation. Last, we examined the degradation behavior of the nanofibers for a long period of up to 6 weeks, as presented in Table 2. The degradation of both nanofiber samples gradually increased with time, and the mPLCL showed a higher degradation rate than PLCL; 2.6% (PLCL) and 4.2% (mPLCL) for 2 weeks, and 6.6% (PLCL) and 12.3% (mPLCL) for 6 weeks. Although the nanofiber samples clearly show a level of degradation with time, the degradation rate is not considered high, suggesting that the nanofibers might preserve the structural and morphological integrity for a long period, possibly during the cell culture and *in vivo* implantation periods.

The *in vitro* cell differentiation studies

After culture for 7 days on each nanofiber scaffold, cells were harvested for the *in vitro* studies. Figure 3A shows the morphology of chondrocytes grown on bare PLCL nanofibers. The chondrocytes almost completely covered the nanofibers and were highly flattened. The fluorescence image of cells with cytoskeletons in yellow and with nuclei in blue showed the highly active and viable status of cells (Fig. 3B). The chondrocyte growth kinetics on PLCL nanofibers was measured during the culture period (Fig. 3C). The culture plate was used as positive control. Cells grew actively on PLCL nanofibers, exhibiting an on-going increase in growth kinetics. Although the initial level was lower on PLCL than on the culture plate, the cell growth on PLCL at 7 days became almost comparable to that on a culture dish. Results indicate that viable chondrocytes might be continuously working on the nanofibers up to the culture period.

The chondrogenic differentiation marker staining of cells was further carried out at 7 days of culture on PLCL nanofibers under the chondrogenic differentiation medium. The stains of cells cultured on PLCL with Safranin O (in red, Fig. 3D) and Alcian blue (in blue, Fig. 3E) showed dark positive signals for both markers, demonstrating the production of abundant sulfated GAGs, characteristic ECM molecules of cartilage, when compared to the stains of acellular PLCL nanofibers used as a negative control. The results indicate that chondrocytes grown on bare PLCL nanofibers were able to produce chondrogenic ECMs. The quantitative real-time PCR analyses of the chondrocyte-related genes (SOX9, collagen type II, and aggrecan) expressed at 7 days also demonstrated that significant chondrogenic effects

FIG. 2. Scanning electron microscopy (SEM) morphologies of the nanofiber samples at different magnifications: (A) PLCL and (B) mPLCL. Characteristics of nanofibers examined: (C) size distribution, (D) energy dispersive spectroscopy atomic signal, (E) Fourier transformed infrared chemical bond structure, and (F) X-ray diffraction phase development. Color images available online at www.liebertpub.com/tec

occurred (Fig. 3F). Much stronger gene expressions of *SOX9*, type II collagen, and aggrecan in the differentiated group than in the undifferentiated control group at the 7-day culture strongly indicate that the upregulated mRNA of these genes responded to the dynamic physiological environment in which the cells grew.

The MSCs cultured on the mPLCL nanofibers were also examined. Figure 4A shows the SEM image of MSCs cultured on mPLCL nanofibers for 7 days. Cells highly elongated along the mPLCL nanofibers, favoring the underlying substrate condition. Although not covering the entire surface

of the mPLCL nanofibers, a number of MSCs seemed to be in contact with each other. Supported by an SEM analysis, the fluorescence image also shows the elongated growth morphology at large numbers as analyzed by F-actin (yellow) distribution along the nanofibers (Fig. 4B). The MSC growth kinetics on mPLCL nanofibers was also monitored (Fig. 4C). MSCs grew actively on the mPLCL, exhibiting an on-going increase in growth kinetics. Although the initial level on mPLCL was lower than that on a culture plate, the cell growth at 7 days became comparable to that on the culture dish. As with the analysis of chondrocyte growth,

TABLE 2. HYDROLYTIC DEGRADATION OF THE NANOFIBER SAMPLES, MEASURED BY THE WEIGHT LOSS IN PBS FOR UP TO 6 WEEKS

	PLCL	mPLCL
2 weeks	2.6% $\pm$ 0.07%	4.2% $\pm$ 0.15%
4 weeks	4.3% $\pm$ 0.05%	8.5% $\pm$ 0.09%
6 weeks	6.6% $\pm$ 0.09%	12.3% $\pm$ 0.09%

PLCL, poly(lactide-co-caprolactone); mPLCL, mineralized form; PBS, phosphate-buffered saline.

MSCs are viable up to 7 days, during which, MSCs are considered to undergo a relatively early stage of osteogenic differentiation. We thus confirmed this early osteogenesis by determining the ALP level of the cells (Fig. 4D). A dark violet colored image is shown only in the ALP-stained

MSCs cultured on mPLCL nanofibers at a 7-day culture, indicating osteogenic differentiation, compared to that of acellular mPLCL nanofibers. Furthermore, *ALP* gene expression quantified by real-time PCR also showed that its significant upregulation was observed in the MSC-layered mPLCL nanofibers for up to 14 days when compared to the undifferentiated control (Fig. 4E), indicating that the cells were better committed to an osteogenic lineage under the osteogenic medium and with increasing culture period. Although this culture period of 14 days showed higher osteogenic differentiation of MSCs, we chose 7 days of culture for the *in vivo* implantation. This was based on the previous works, where the preculture effects of MSCs on *in vivo* bone regeneration ability were reported. Previous studies demonstrated that MSCs, derived from bone marrow, adipose, or dental pulp, when precultured for short-term periods (mostly within a week), played more effective roles in the bone

FIG. 3. (A) SEM and (B) fluorescence images of the chondrocytes grown on PLCL nanofibers for 7 days. Yellow, actin cytoskeleton stained with Alexa Fluor 546-conjugated phalloidin; blue, nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI). (C) Chondrocyte growth kinetics on PLCL nanofibers during culture for up to 7 days, showing an ongoing increase with culture time. Culture plate was used as positive control. (D, E) Immunocytochemical staining for sulfated glycosaminoglycan (GAG) at 7 days of chondrocyte culture on nanofibers under differentiation condition: (D) Safranin O (in red) and (E) Alcian blue (in blue) positively stained the cells cultured on PLCL nanofibers, contrasting to the acellular PLCL nanofibers. (F) Quantitative real-time polymerase chain reaction (PCR) analyses of the gene expression levels at 7 days of chondrocytes cultured in chondrogenic medium and normal medium. Cartilage-associated genes, including *SOX9*, collagen type II, and aggrecan, were upregulated under chondrogenic medium conditions. * $p < 0.05$ and ** $p < 0.01$, by a Student's *t*-test. Color images available online at www.liebertpub.com/tec

FIG. 4. (A) SEM and (B) fluorescence images of the MSCs grown on mPLCL nanofibers for 7 days. Yellow, actin cytoskeleton stained with Alexa Fluor 546-conjugated phalloidin; blue, nuclei counterstained with DAPI. (C) Cell growth kinetics on mPLCL nanofibers observed for up to 7 days, showing an on-going increase with culture time. Culture plate was used as positive control. (D) Early osteogenic differentiation was confirmed by alkaline phosphatase (ALP) staining of MSC-mPLCL constructs with culture for 7 and 14 days in osteogenic medium. ALP staining of acellular mPLCL was shown for comparison. (E) Quantitative real-time PCR analyses of the *ALP* gene expression level of MSCs cultured for up to 14 days in osteogenic medium. The *ALP* level of cells cultured in normal medium for 7 days was used for comparison. * $p < 0.05$ and ** $p < 0.01$, by a Student's *t*-test. Color images available online at www.liebertpub.com/tec

formation *in vivo*, with respect to the relatively long-term (over 7 to 16 days) precultured MSCs.^{25–28}

Taken together, it is considered that PLCL and mPLCL nanofibers are effectively permitted to react with chondrocytes and MSCs, favoring ECM secretion of cartilage and bone, respectively. Consequently, we prepared two types of cell-layered constructs, an AC layer of PLCL nanofibers and an early differentiated MSC layer of mPLCL nanofibers. Each construct was layered to prepare a 10-layered cell-nanofiber construct, combining five layers of chondrocyte-PLCL with five layers of MSC-mPLCL.

In vivo findings

The *in vivo* niche can provide decisive cues with exogenously implanted cells to construct a stable ectopic tissue.²⁹ However, an inappropriate *in vitro* treatment can direct the undesirable fate of exogenous cells in a subcutaneous environment, although these cells were *in vitro* induced to express specific phenotypes.^{30,31} Therefore, we confirmed that the cells on the nanofibers were properly driven to each lineage of tissue desirable cells under the appropriate conditions before implantation. The biphasic cell-layered constructs engineered for 7 days of *in vitro* induction were subcutaneously implanted at the dorsum part of athymic nude

mice. At 4 weeks after implantation, samples were gathered and stained by H&E (Fig. 5A, B), Safranin O (Fig. 5C, E), and Alizarin red S (Fig. 5D, F) for histological observation, which were then divided into an acellular control group (Fig. 5A, C, D) and a cell-layered construct group (Fig. 5B, E, F). Cell-layered constructs clearly show more vivid colors than the acellular control group. H&E-stained histological view of cross-sectioned cell-layered constructs clearly revealed that cells and ECMs increased in both the cartilage and bone layers compared to cell-free scaffolds. The enlarged image of cell-free scaffolds (Fig. 5A') at the interfacial region showed well integration of the layered structure and a number of cells migrated and populated within the scaffolds during the 4 weeks of implantation. For the case of cell-layered constructs, enlarged images revealed that a large number of cells populated on both sides of the constructs. Based on the intensity of stain signals, more cells were present in the cell-layered group with respect to those in the cell-free case, which might be due to the fact that the cells delivered initially were present together with the cells migrated *in vivo* during implantation. Safranin O-stained images (Fig. 5C, E) of the chondral part revealed much darker pink colored stains in the chondrocyte-PLCL layers than in the acellular PLCL layers, indicating more abundant accumulation of sulfated GAGs as a typical cartilage ECM was produced by chondrocyte-layered

FIG. 5. Histological analyses of subcutaneously implanted constructs at the dorsum part of athymic nude mice. At 4 weeks after implantation, the *in vivo* constructs demonstrated significant histological characteristics of bone and cartilage in terms of extracellular matrix production when compared to acellular scaffolds (A, C, D, acellular scaffolds; and B, E, F, cell-layered constructs). (A, B) Hematoxylin and eosin-stained images showing the presence of cells: (A') and (B', B'') are the enlarged images of (A, B), respectively, showing the layered structure and the cells either migrated or seeded initially. (C, E) Safranin O staining in the chondral part, and (D, F) Alizarin red S staining in the osseous part, showing clear dark stains only noticed in the cell-layered constructs. Dashed lines indicate the interfacial region between chondrocyte-PLCL layers and MSC-mPLCL layers. Star symbols in (A', B', B'') point out each layer of the constructs. Color images available online at www.liebertpub.com/tec

constructs than by cell-free scaffolds. The Alizarin red S-stained images (Fig. 5D, F) of the osseous part also showed much darker red colored stains in the MSC-mPLCL layer than in the acellular case, indicating that calcium phosphate minerals accumulated more in the implanted area of the MSC-layered constructs than in the acellular scaffold. An additional experiment was performed to examine the specific expression of type II collagen in the chondrocyte-PLCL constructs, as shown in Figure 6. Strong positive green signals were clearly revealed on the chondrocyte-PLCL constructs, suggesting that the engineered cell constructs functioned properly to secrete major ECM components of

cartilage tissue. The weak signals found in the MSC-mPLCL part was possibly due to the side action of cells in the ectopic site.

The *in vivo* findings demonstrated that the cell-PLCL and cell-mPLCL layers showed favorable tissue reactions through the implantation period and that each layer was effective to a great degree in developing specific tissue ECMs. Although in this study we showed the efficacy of the biphasic cell-nanofiber constructs in forming *in vivo* osteochondral tissues in ectopic environments, and the model used herein is widely applied to prove the performance of engineered materials and cells for osteochondral tissues,³²⁻³⁴ it is, however, not

FIG. 6. Immunohistochemical staining of cell-layered *in vivo* samples, showing fluorescence signals: (A) FITC-conjugated type II collagen (green), (B) DAPI-stained nuclei of cells (blue), and (C) merged one. Dashed lines indicate interfascial region between chondrocyte-PLCL layers and MSC-mPLCL layers. Color images available online at www.liebertpub.com/tec

considered to be a clinically relevant model. The environments where osteochondral defects are engaged in and the repair processes are necessitated should be different from the ectopic environments, as thus, the cellular differentiation would be dissimilar, which possibly results in different out-

comes. Therefore, further study remains as to the *in vivo* efficacy of the cell-nanofiber constructs using more clinically relevant osteochondral defect models.

Conclusion

In this study, we proposed cell-layered PLCL/mPLCL biphasic nanofiber constructs for osteochondral repair. The ACs and MSCs cultured on the nanofibers successfully exhibited the phenotypes of chondrocytes and osteoblasts, respectively. Furthermore, new cartilage and bone tissues were formed in the implanted area of cell-layered constructs at 4 weeks in nude mice. Although more study is needed with clinically relevant animal models, the biphasic cell-layered nanofiber constructs may be importantly considered for the repair of osteochondral interfascial tissues.

Disclosure Statement

No competing financial interests exist.

Acknowledgment

This study was supported by grants from the Priority Research Centers Program (2009-0093829), National Research Foundation, Republic of Korea.

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Received: August 28, 2013

Accepted: February 20, 2014

Online Publication Date: September 12, 2014