Published in final edited form as:
Acta Biomater. 2010 August ; 6(8): 2920–2931. doi:10.1016/j.actbio.2010.02.046.

The Effects of TGF-β3 and Preculture Period of Osteogenic Cells on the Chondrogenic Differentiation of Rabbit Marrow Mesenchymal Stem Cells Encapsulated in a Bilayered Hydrogel Composite

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Abstract

In this work, injectable, biodegradable hydrogel composites of crosslinked oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) were utilized to fabricate a bilayered osteochondral construct. Rabbit marrow mesenchymal stem cells (MSCs) were encapsulated with transforming growth factor-β3 (TGF-β3)-loaded MPs in the chondrogenic layer and cocultured with cells of different periods of osteogenic preculture (0, 3, 6 and 12 days) in the osteogenic layer to investigate the effects of TGF-β3 delivery and coculture on the proliferation and differentiation of cells in both layers. The results showed that, in the chondrogenic layer, TGF-β3 significantly stimulated chondrogenic differentiation of MSCs. Additionally, cells of various osteogenic preculture periods in the osteogenic layer, along with TGF-β3, enhanced gene expression for MSC chondrogenic markers to different extents. In the osteogenic layer, cells maintained their alkaline phosphatase activity during the coculture; however, mineralization was delayed by the presence of TGF-β3. Overall, this study demonstrated the fabrication of bilayered hydrogel composites that mimic the structure and function of osteochondral tissue, along with the application of these composites as cell and growth factor carriers, while illustrating that encapsulated cells of different degrees of osteogenic differentiation can significantly influence the chondrogenic differentiation of cocultured progenitor cells in both the presence and absence of chondrogenic growth factors.

Keywords

bilayered hydrogel composites; mesenchymal stem cell; cell differentiation; coculture

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Introduction

Articular cartilage is an avascular tissue with limited ability to self-repair once injured. Injured articular cartilage can lead to degeneration of subchondral bone, resulting in severe pain and loss of mobility of the affected joint [1,2]. Therefore, for long-term repair of articular cartilage, it is necessary to develop a construct consisting of both articular cartilage and subchondral bone to mimic the structure and fulfill the functions of native osteochondral tissue [3–5].

Mesenchymal stem cells (MSCs) are progenitor cells that reside in many tissues, including bone marrow [6,7]. MSCs can be easily isolated and expanded without losing their ability to differentiate into different connective tissue cell types, such as chondrocytes and osteoblasts. Therefore, for the generation of an osteochondral construct, MSCs are a suitable candidate cell population, which circumvents the need for invasive isolation of chondrocytes and osteoblasts from limited sources [4]. However, investigation of the chondrogenic and osteogenic differentiation of MSCs, especially in a coculture environment, stands as a fundamental aspect of the development of a cellular construct for osteochondral tissue repair.

A three-dimensional network is known to be important for MSC proliferation and differentiation, since it mimics the *in vivo* environment [8]. Among the numerous scaffold materials available for tissue engineering approaches for osteochondral repair, hydrogels possess the key ability to allow for ample intake of nutrients and removal of wastes to support the viability of encapsulated cells [9,10]. Our laboratory has developed a biodegradable and injectable oligomer, oligo(poly(ethylene glycol) fumarate) (OPF) [11]. The double bonds in the OPF backbone allow it to crosslink to form a hydrogel network under physiological conditions; and the crosslinked material may degrade via ester hydrolysis. Additionally, gelatin microparticles (MPs) have been incorporated in OPF hydrogels as a digestable porogen and a drug delivery vehicle [12,13]. The resulting hydrogel composites containing growth-factor-loaded MPs have been shown to support both chondrogenic and osteogenic differentiation of encapsulated MSCs [14–16].

In addition to a three-dimensional scaffold, chemical supplements and growth factors are also found to be crucial for guiding cell differentiation *in vitro* [17,18]. Osteogenic differentiation of MSCs *in vitro* usually occurs with osteogenic supplements in a culture medium, including dexamethasone, ascorbic acid, and β-glycerophosphate [18]; whereas chondrogenic differentiation requires not only a chemically-defined, serum-free medium but also the presence of chondrogenic growth factors, especially members of the transforming growth factor-β (TGF-β) family [17], such as TGF-βs and bone morphogenetic proteins (BMPs).

Another strategy to enhance chondrogenesis is via coculture. Several studies have investigated the coculture of chondrocytes and osteoblasts *in vitro*, and they found that the proliferation and differentiation of chondrocytes were regulated by cell-cell contact and by soluble factors secreted by the osteoblasts [19,20]. Similarly, the coculture of MSCs or embryonic stem cells (ES) with other cells types, such as chondrocytes and hepatic cells, also demonstrated enhanced levels of chondrogenic differentiation of the MSCs [7,21]. Recently, a bilayered hydrogel coculture system has been developed in our laboratory for osteochondral tissue engineering applications [22]. The study found that TGF-β1-loaded MPs in the chondrogenic layer of the hydrogel composites and MSC-derived osteogenic cells in the osteogenic layer synergistically enhanced MSC chondrogenesis in the chondrogenic layer.

In the present work, we fabricated bilayered OPF hydrogel composites encapsulating MPs and MSCs in each layer; and we specifically asked the questions: (1) whether MSCs can undergo chondrogenic and osteogenic differentiation in the respective layers of a bilayered hydrogel composite; (2) whether TGF-β3-loaded MPs affect MSC chondrogenic differentiation in the chondrogenic layer; (3) whether TGF-β3-loaded MPs and cells of different periods of

osteogenic preculture encapsulated in the osteogenic layer have a combined effect on MSC chondrogenic differentiation in the chondrogenic layer.

Materials and Methods

Experimental Design

In this study, bilayered hydrogel composites consisting of a chondrogenic layer at top and an osteogenic layer at bottom were fabricated, as shown in Figure 1. More specifically, in the top layer, MSCs were encapsulated in OPF hydrogels with either blank MPs (noted with −) or TGF-β3-loaded MPs (noted with +), while in the bottom layer, OPF hydrogel composites with blank MPs were utilized to encapsulate MSCs, 3-day osteogenically precultured cells (OS3 cells), 6-day osteogenically precultured cells (OS6 cells), or 12-day osteogenically precultured cells (OS12 cells). Eight formulations of bilayered constructs (groups OS0±, OS3±, OS6±, and OS12± in Figure 1) were cultured in chondrogenic medium (CM) supplemented with 10 mM β-glycerophosphate for 28 days. Samples from the top and bottom layers were analyzed to evaluate chondrogenic and osteogenic differentiation of the cells, respectively. Similar hydrogel composites containing TGF-β1-loaded MPs in the top layer were also fabricated for comparison.

OPF Synthesis and Characterization

OPF was synthesized from fumaryl chloride and poly(ethylene glycol) (PEG) with a nominal molecular weight of 10,000 g/mol (Sigma, St. Louis, MO) according to a previously established method [11], and sterilized prior to use by exposure to ethylene oxide.

Gelatin Microparticle Fabrication and Growth Factor

Loading Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) and crosslinked with 10 mM glutaraldehyde (Sigma, St. Louis, MO) following established procedures [23]. After drying, MPs were sieved to obtain particles of 50–100 μm in diameter and sterilized with ethylene oxide.

Before encapsulation, sterile MPs were loaded with TGF-β3 (R&D Systems, Minneapolis, MN) by swelling in an aqueous solution of the growth factor at pH 7.4 for 15 h according to a previously reported method [24]. The volume of TGF-β3 loading solution needed for equilibrium swelling of the MPs (110 μl) was combined with 22 mg of MPs. The loading solution had a concentration of 3.6 μg TGF-β3/ml phosphate buffered saline (PBS; Gibco) to achieve a final loading of 600 ng TGF-β3/ml in the crosslinked scaffolds. This growth factor amount has been shown to promote the chondrogenic differentiation of rabbit MSCs encapsulated in hydrogel composites *in vitro* [14]. TGF-β1-loaded MPs were prepared in a similar fashion using a solution of the same growth factor concentration. Blank MPs were loaded with PBS alone.

Rabbit Marrow MSC Isolation and Preculture

Rabbit marrow MSCs were isolated from the tibiae of six 4-month-old New Zealand white rabbits as previously described [14]. The bone marrow was cultured in general medium (GM) containing Dulbecco's modified Eagle's medium (DMEM), 10% v/v fetal bovine serum (FBS; Gemini, Calabasas, CA), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin for 2 weeks, and then cryopreserved as described previously [22]. For MSC expansion, the cryopreserved cells were thawed at 37°C and cultured in T-75 flasks with GM up to passage three (12 days), as shown in Figure 1. Osteogenic cells were cultured from the same batch of cryopreserved MSCs for 12 days, as shown in Figure 1, with both GM and osteogenic medium (OM), which was DMEM supplemented with 10% v/v FBS, 50 mg/l ascorbic acid, 10mM β-

glycerophosphate, 10^{-8} M dexamethasone, 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin (all from Sigma). Specifically, osteogenic cells for the bottom layer of OS3±, OS6± and OS12± groups were exposed to OM for 3, 6 and 12 days immediately prior to encapsulation, respectively.

Fabrication of Bilayered Hydrogel Composites

Bilayered hydrogel composites were fabricated via a two-step crosslinking procedure as described previously [22]. The desired composition for the osteogenic (bottom) layer was first prepared. Specifically, 0.1 g of sterile OPF and 0.05 g of sterile poly(ethylene glycol) diacrylate (PEG-DA; 4,000 Da nominal molecular weight, Monomer-Polymer & Dajac Labs, Feasterville, PA) were first dissolved in 300 μl of PBS and mixed with 110 μl of swelled MP solution (blank MPs). The mixture was then added to equal volumes (46.8 μl) of the thermal radical initiator solutions, 0.3 M ammonium persulfate (APS) and 0.3 M N,N,N′,N′-tetramethylethylenediamine (TEMED) in PBS. A proper cell suspension (6.7 million cells in 168 μl of PBS) was subsequently added to the polymer solution to achieve a concentration of 10 million cells/ml final suspension. After gentle mixing, the suspension for the osteogenic layer was quickly injected into the bottom 1 mm of Teflon molds (6 mm diameter, 2 mm thickness) and incubated for 4 min, allowing for partial crosslinking. Meanwhile, another polymer-cell suspension was prepared, and then injected into the partially filled Teflon molds to form the chondrogenic layer. The resulting bilayered constructs were then incubated at 37° C for 8 min to achieve crosslinking.

Each hydrogel construct was then cultured with 2.5 ml chondrogenic medium, which was DMEM supplemented with ITS+ Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.35 μg/ml linoleic acid and 1.25 μg/ml bovine serum albumin) (BD Biosciences, Franklin Lakes, NJ), 1 mM sodium pyruvate (Sigma), 50 mg/l ascorbic acid, 10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 250 mg/l fungizone, 100 mg/l ampicillin and 50 mg/l gentamicin. The medium was changed every 3 days during a 28-day culture period.

At various time points (days 0, 7, 14, and 28), samples were collected for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4), biochemical assays (n=4), live/dead assay (n=1) and histological analysis (n=1). Bilayered samples for RT-PCR and biochemical assays were dissected with a blade to separate the chondrogenic layer and osteogenic layer [22]; samples from each layer were stored for analysis. Samples loaded with TGF-β1 were analyzed only for RT-PCR (n=4) at day 28.

Real Time RT-PCR

Samples from the chondrogenic layer of the osteochondral constructs were subjected to RT-PCR analysis to quantify MSC chondrogenic differentiation as described previously [14]. Gene expression for collagen type II, aggrecan and collagen type I was determined, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method as reported previously [25,26]. All gene expression data were normalized to the expression of a house-keeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) and expressed as the fold ratio compared to baseline gene expression of a control group at day 0, as previously reported by our group and others for cell-laden hydrogels [14,16,22,27–29]. In this study, the control group contained four top-layer hydrogels encapsulating MSCs that were collected immediately after encapsulation.

Biochemical Assays

Samples from the osteogenic layer of the osteochondral constructs were analyzed for DNA, alkaline phosphatase (ALP) enzyme activity, and calcium content using microplate readers (BIO-TEK Instrument, Winooski, VT) to characterize osteogenic differentiation of the cells as described previously [22]. Bottom-layer samples containing MSCs, 3-day, 6-day and 12-

day osteogenically precultured cells (n=4) were collected after encapsulation to represent day 0 values for the OS0±, OS3±, OS6± and OS12± groups respectively.

Similarly, samples from the chondrogenic layer of the constructs were digested in a proteinase K solution and subjected to DNA, glycosaminoglycan (GAG), and calcium assays to evaluate chondrogenic differentiation as previously described [22],[30]. Four samples from top layer hydrogels containing MSCs and blank MPs were collected immediately after encapsulation and analyzed to represent day 0 values for all the groups.

Confocal Fluorescence Microscopy

A live/dead assay was performed at days 0 and 28 after encapsulation as reported previously [29]. After rinsing with PBS, each layer of the construct was then placed in dye solutions containing 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein acetoxymethyl ester (Calcein AM) (Invitrogen) for 30 min. Cells encapsulated in the hydrogel composites were examined by confocal fluorescence microscopy (Zeiss LSM 510, Thornwood, NY). Using argon laser excitation at 488 nm, the cells were imaged under a 10x objective, and the emitted light was collected using 505–526 nm and 612–644 nm filters.

Histology

At the various time points, bilayered hydrogel composites were fixed, dehydrated, and embedded in paraffin. Cross sections of 20 μm were prepared for Von Kossa staining using a method reported previously [15]. Briefly, the sections were exposed to 5% silver nitrate solution, exposed to UV light for 30 minutes to visualize calcium deposition, and counterstained with 0.1% Safranin O solution.

Statistical Analysis

DNA, GAG and calcium content, ALP activity and gene expression data of the eight treatments (in Figure 1) at different time points are reported as means ± standard deviation. Repetitive ANOVA and Tukey's multiple comparison tests were used to determine possible significant differences ($p < 0.05$) between groups. Additionally, gene expression data of hydrogels containing TGF-β3- and TGF-β1-loaded MPs at day 28 were compared similarly with repetitive ANOVA and Tukey's multiple comparison tests ($p < 0.05$). Statistical significance symbols for Figure 2, 3 and 4 are defined in Table 1.

Results

Cellularity in Both Chondrogenic and Osteogenic Layers

DNA content of the chondrogenic and osteogenic layers of the hydrogel composites are depicted in Figure 2(A) and Figure 3(A), respectively. For both layers of all the treatments, the DNA amount decreased from day 0 to day 7.

In the osteogenic layer [Figure 3(A)], samples containing osteogenically precultured cells (the OS3±, OS6±, and OS12± groups) generally showed higher DNA values compared to those containing plain MSCs (the OS0± groups), either in the presence or absence of TGF-β3. Specifically, with blank MPs in the chondrogenic layer, a significantly higher DNA value was observed in the osteogenic layer of the hydrogels containing 6-day osteogenically precultured cells (the OS6− group) than in those encapsulating MSCs (the OS0− group) at day 28. Similarly, with TGF-β3-loaded MPs in the chondrogenic layer, significantly more DNA was found in the osteogenic layer of samples of the OS3+ and OS6+ groups than in those of the OS0+ group at day 28.

As in the osteogenic layer, TGF-β3 did not show an effect on cell number in the chondrogenic layer [Figure 2(A)]. However, cells in the osteogenic layer significantly influenced DNA content in the chondrogenic layer of the hydrogels. For example, MSCs in the chondrogenic layer that were cocultured with 3-day osteogenically precultured cells (the OS3± groups) demonstrated a significantly higher DNA amount than those cocultured with MSCs (the OS0 ± groups) at day 28, either with or without TGF-β3-loaded MPs. MSCs that were cocultured with 6-day osteogenically precultured cells (the OS6± groups) exhibited the highest DNA content compared to all the other groups (the OS0±, OS3±, and OS12± groups) during the culture with or without TGF-β3. However, the coculture with 12-day osteogenically precultured cells (the OS12± groups) did not result in a significant increase in DNA content in the chondrogenic layer when compared to the OS0± groups.

Chondrogenic Differentiation of MSCs in the Chondrogenic Layer

The chondrogenic differentiation of MSCs in the chondrogenic layer of the bilayered hydrogels was determined via gene expression of two chondrogenic markers, collagen type II and aggrecan, as well as the gene expression of an MSC-associated marker, collagen type I. Gene expression data are shown in Figure 4(A–C).

For all the treatments, collagen type II gene expression was upregulated over time [Figure 4 (A)]. The presence of TGF-β3-loaded MPs enhanced collagen type II gene expression of MSCs encapsulated in the chondrogenic layer. At day 28, significantly higher levels of gene expression were observed in the groups with TGF-β3 (the OS0+, OS3+, OS6+ and OS12+ groups) than in the corresponding groups without TGF-β3 (the OS0−, OS3−, OS6− and OS12 − groups, respectively). Additionally, 12-day osteogenically precultured cells had a combined effect with TGF-β3 (the OS12+ group), resulting in the highest level of collagen type II gene expression among all the treatments at day 28. In similar hydrogel composites, where the same amount of TGF-β1 was loaded in the chondrogenic layer, a combined effect of osteogenic cells and the growth factor was also observed. Collagen type II gene expression of MSCs exposed to TGF-β1 increased with the duration of osteogenic preculture of the cocultured osteogenic cells. However, with plain MSCs in the osteogenic layer, TGF-β1 alone only resulted in a 357 ± 211 fold increase in collagen type II gene expression at day 28, which was significantly lower than a 927 ± 148 fold increase caused by TGF-β3 alone.

Aggrecan gene expression levels also increased over time for all the treatments [Figure 4(B)]. For the groups that were cultured with either osteogenic cells alone (the OS3−, OS6− groups) or with TGF-β3 alone (the OS0+ group), significant increases compared to a day 0 baseline were observed no earlier than day 28. However, when MSCs were cultured with both osteogenic cells and TGF-β3, significant increases relative to the day 0 baseline were observed as early as day 7 or 14 (day 14 for the OS3+ group, day 7 for the OS6+ group, and day 14 for the OS12+ group). Cells of 3-day and 6-day osteogenic preculture durations (the OS3± and OS6± groups) seemed to result in more overall aggrecan gene expression of the MSCs in the chondrogenic layer than plain MSCs (the OS0± groups) or 12-day osteogenically precultured cells (the OS12± groups), with either TGF-β3-loaded or blank MPs in the top layer.

Collagen type I gene expression was found to decrease significantly at day 28 relative to day 0, when MSCs in the chondrogenic layer were cultured with either TGF-β3, or with osteogenic cells, or with both. It should be noted that samples treated with TGF-β1 alone exhibited a significantly higher collagen type I gene expression than those treated with TGF-β3 alone.

GAG content, indicative of the chondrogenic phenotype, was measured for the chondrogenic layer of the bilayered hydrogels to further investigate chondrogenic differentiation of the encapsulated MSCs, as shown in Figure 2(B). For all the formulations, significantly higher GAG/DNA levels were observed at days 14 and 28 compared to day 0. At day 28, significant

differences in GAG/DNA values among groups were found between the OS0− group and the OS6− group; as well as between the OS0+ group and the OS3+, OS6+ and OS12+ groups.

Calcium deposition was determined for the chondrogenic layer of the hydrogels to ensure that no bone upgrowth to the chondrogenic layer occurred with the combined effects of TGF-β3 and β-glycerophosphate in the coculture medium. As illustrated in Figure 2(C), all the formulations had a calcium content of less than 1.1 μg during the culture. No statistically significant difference was observed over time or among groups.

Osteogenic Differentiation of Cells in the Osteogenic Layer

ALP enzyme activity and calcium content were measured for the osteogenic layer of the hydrogels to evaluate the osteogenic differentiation of the encapsulated cells. ALP results over time are shown in Figure 3(B). Immediately after encapsulation, ALP activity of cells in the osteogenic layer of the hydrogels was found to be higher in samples containing cells of a longer osteogenic preculture period (not significant). During the subsequent hydrogel culture, ALP values of the osteogenic cells (the OS3±, OS6± and OS12± groups) slightly increased or remained unchanged, either with or without TGF-β3 in the top layer. In contrast, the OS0± groups containing MSCs in the osteogenic layer both exhibited significant increases in ALP activity at later time points compared to day 0. In particular, in the OS0− group, ALP activity of the cells significantly increased from day 0 to day 7, and then significantly decreased by day 28 relative to day 7. The OS0+ group had a significantly higher ALP activity at days 7, 14 and 28 compared to day 0, with a peak at day 14. Additionally, the ALP values of the OS0+ group were found to be significantly higher than those of all the other treatments at days 7, 14 and 28.

Calcium deposition in the osteogenic layer of the hydrogels for all the treatments is shown in Figure 3(C). Although statistical analysis did not reveal any significant differences among the groups at each time point, significant increases in calcium deposition compared to day 0 values were found in some groups containing osteogenically precultured cells, for example, in the OS3− group at days 14 and 28, in the OS12− group at days 7, 14 and 28, and in the OS12+ group at days 7 and 14.

Confocal Fluorescence Microscopy

Confocal fluorescence microscopy images of cells encapsulated in both layers of the hydrogels at day 0 and day 28 are presented in Figure 5. The cells were stained with a live/dead assay kit, where calcein AM stained the live cells with a green fluorescence and ethidium homodimer-1 (EthD-1) stained dead cells with a red fluorescence, to provide a qualitative indication of cell viability and distribution within the hydrogels. Immediately after encapsulation, an overwhelming majority of the cells encapsulated in each layer were alive, and they were well distributed within the hydrogel composites, as qualitatively illustrated in Figure 5(A). After 28 days of culture, cells in each layer of the hydrogel composites retained their viability in all the treatments [Figure 5(B–C)]. However, cells from different groups showed a different distribution in the top (chondrogenic) layer of the hydrogels. As shown in Figure 5(B), cells of the OS0− group were sparsely distributed in the top layer; whereas in the groups treated with TGF-β3, more cell aggregation was observed in the top layer of the hydrogels [noted with small arrows in Figure 5(C)]. No difference in cell distribution was observed for cells encapsulated in the bottom (osteogenic) layer of the hydrogels [Figure 5(B–C)].

Light Microscopy and Histology

A light microscopy image of a cross section of a bilayered hydrogel composite containing MSCs and MPs in both layers immediately after encapsulation is shown in Figure 6(B). In the

sample, cells (shown as small dots) and MPs (noted with a big arrow) were well distributed. No apparent border can be recognized between the layers, indicative of a good integration between layers.

Figure 6(A, C–J) depicts histological cross sections of the hydrogel composites after 0 and 28 days of culture. The sections were prepared with Von Kossa staining and counter stained with Safranin O, which collectively stain mineralized matrix with a black color and GAG, a cartilaginous matrix component, with a red color. MPs (noted with big arrows) and cells (aggregation noted with small arrows) in the samples appear in dark red in the images. Similar to the results from confocal fluorescence images [Figure 5(A)] and the light microscopy image [Figure 6(B)], MPs and cells were found to distribute evenly in both layers of the hydrogel composites after encapsulation [Figure 6(A)]. At day 28, most of the MPs were degraded, while cells maintained their rounded phenotype. There was a trend of increasing cell aggregation [noted with small arrows in Figure 6(D, F, H)] in the TGF-β3-treated groups, especially with osteogenic cells cocultured in the bottom (osteogenic) layer of the hydrogels. Although no significant difference was observed for GAG staining among the samples, a more intense staining for mineralized matrix was noticed in the blank MP treatments [Figure 6(C, E, G, I)] than in the TGF-β3 loaded MP treatments [Figure 6(D, F, H, J)] at day 28. The hydrogel composites also seemed to have more calcium deposition in the center than in the top or bottom surfaces [Figure 6(E, G, I)].

Discussion

In the current work, bilayered composites consisting of a chondrogenic layer and an osteogenic layer were fabricated using OPF hydrogel networks containing gelatin MPs and MSCs. Specifically, we investigated: (1) the proliferation and differentiation of cells in both layers of the bilayered hydrogel composites; (2) the effect of TGF-β3-loaded MPs on the chondrogenic differentiation of MSCs in the chondrogenic layer; and (3) the combined effects of the duration of the osteogenic preculture of the cells in the osteogenic layer and TGF-β3-loaded-MPs on MSC chondrogenesis in the chondrogenic layer.

Previously, our laboratory has fabricated a similar bilayered OPF construct containing TGF-β1-loaded MPs and MSCs in the chondrogenic layer, and containing 6-day osteogenically precultured cells in the osteogenic layer. The culture of the bilayered construct showed that TGF-β1-loaded MPs in the chondrogenic layer and osteogenic cells in the osteogenic layer synergistically promoted chondrogenesis of MSCs encapsulated in the chondrogenic layer [22].

TGF-β3 was chosen for the current study because a previous study has shown that TGF-β3 was more effective than TGF-β1 in terms of promoting the chondrogenesis of human MSCs in pellet culture, which was evidenced by an earlier and greater deposition of GAG and type II collagen [31]. Additionally, TGF-β3 was also reported to have a biphasic stimulatory effect on DNA synthesis of osteoblasts, and to inhibit osteogenic differentiation of MSCs or MSC-derived osteoblasts [32,33].

In vitro osteogenic culture period of MSCs has been shown to significantly affect their osteogenic gene expression and extracellular matrix production levels [34,35], and even to influence their osteogenic potential when implanted *in vivo* [35–37]. Based on these findings, we hypothesized that cells of different durations of osteogenic preculture can provide different signaling to the cocultured MSCs in a bilayered OPF hydrogel composite. Therefore, this study investigated how the osteogenic preculture duration of cells in the osteogenic layer in combination with TGF-β3-loaded MPs in the chondrogenic layer would affect cell proliferation and differentiation in both layers of a bilayered hydrogel composite.

Cellularity in Both Chondrogenic and Osteogenic Layers

DNA content of both the chondrogenic layer and the osteogenic layer was examined at different time points, as shown in Figure 2(A) and Figure 3(A), respectively. In both layers, there was a significant decrease in DNA content from day 0 to day 7 for all the treatments. Previous studies using a similar single-layer hydrogel composite for MSC chondrogenic and osteogenic differentiation have indicated that the encapsulation process may lead to some cell loss, especially at early time points [14, 15]. However, images of the live/dead assay in Figure 5 qualitatively demonstrated that the vast majority of cells in both layers were alive after encapsulation [Figure 5(A)] and that they maintained their viability during the subsequent culture [Figure 5(B–C)], demonstrating the cytocompatibility of the macromer, crosslinker and initiators under the conditions explored in this study.

Statistical analysis of the DNA assay data revealed quantitatively that TGF-β3 did not influence the cellularity of either layer. However, the cells encapsulated in the osteogenic layer significantly affected the DNA content of both layers. In particular, more DNA was seen in both layers in the treatments with osteogenically precultured cells (the OS3±, OS6±, and OS12 ± groups) than the treatments with plain MSCs (the OS0± groups). This was in accordance with our previous findings that osteogenic cells (6-days of osteogenic preculture) were more resilient than plain MSCs when encapsulated in the osteogenic layer of an OPF hydrogel composite and that they supported the proliferation of the cells in the chondrogenic layer, possibly through a paracrine effect [22]. Furthermore, in this study, we found that osteogenic cells of various osteogenic preculture periods in the osteogenic layer supported the proliferation of MSCs in the chondrogenic layer to different extents. As shown in Figure 2(A), MSCs in the chondrogenic layer that were cocultured with 3-day osteogenically precultured cells (the OS3 ± groups) demonstrated a significantly higher DNA content compared to those cultured with plain MSCs (the OS0± groups) in the osteogenic layer at day 28; while MSCs that were cocultured with 6-day osteogenically precultured cells (the OS6+ groups) demonstrated the highest levels of DNA, which were significantly higher than all the other treatments (the OS0 +, OS3+ and OS12+ groups) at days 14 and 28 in the presence of TGF-β3. In contrast to the OS3± and OS6± groups, MSCs cocultured with 12-day osteogenically precultured cells (the OS12± groups) did not show a significant increase in DNA content compared to the control groups (OS0±) at any time point.

The difference in the ability of osteogenic cells to promote the proliferation of the cocultured MSCs may be related to their osteogenic differentiation levels. It is know that during the three stages of osteogenic differentiation (proliferation, matrix maturation, and mineralization), there is a reciprocal relationship between cell proliferation and the subsequent matrix maturation and mineralization [34,35]. In this study, 3-day and 6-day osteogenically precultured cells can be considered as immature osteoblasts, which possess a great ability to proliferate. A previous review on osteogenic differentiation of osteoblasts indicated that immature osteoblasts actively express cell-cycle and cell-growth- regulated genes, which encode proteins that support proliferation [34], whereas 12-day osteogenically precultured cells represented more extensively differentiated osteoblasts, which express genes or ECM proteins associated with extracellular matrix maturation or mineralization and downregulate genes for proliferation [34]. Compared to 12-day osteogenically precultured cells, 3-day and 6-day osteogenically precultured cells may secrete more bioactive factors to support cell proliferation, thus resulting in a higher DNA amount of both layers due to autocrine and paracrine signaling.

Chondrogenic Differentiation of MSCs in the Chondrogenic Layer

It has been shown that when MSCs undergo chondrogenic differentiation, they start to express chondrocyte-specific marker genes such as collagen type II and aggrecan, along with downregulating expression of the gene for collagen type I, which is produced by

undifferentiated MSCs [25]. Therefore, chondrogenic differentiation of MSCs encapsulated in the top (chondrogenic) layer of the constructs was determined by gene expression for collagen type II, aggrecan, and collagen type I. Gene expression data shown in Figure 4(A–C) demonstrated upregulation of collagen type II and aggrecan gene expression and downregulation of collagen type I gene expression over time for all the treatments, indicating the chondrogenic differentiation of the MSCs encapsulated in the chondrogenic layer of the hydrogel constructs in the coculture environment. Similar trends have been reported by our group and others in previous studies characterizing the chondrogenic gene expression of MSCs encapsulated in hydrogel constructs under various conditions, relative to the gene expression of encapsulated cells at day 0 [14,16,22,28,29]. However, differences in the conditions explored in the various studies preclude direct comparison of the data with the present study.

The stimulatory effect of TGF-β3 on MSC chondrogenic differentiation was apparent, as higher levels of collagen type II and aggrecan gene expression were observed in the TGF-β3-treated groups (the OS0+, OS3+, OS6+ and OS12+ groups) than in the corresponding groups without TGF-β3 (the OS0−, OS3−, OS6− and OS12− groups, respectively). Similar results have been reported in the literature, showing the important roles of TGF-β3 for MSC chondrogenic differentiation [31,38]. Comparing the results of TGF-β3 and TGF-β1 treatments at day 28 [Figure 4(A–C)], where the same amount of TGF-β3 or TGF-β1 was loaded on MPs and encapsulated with MSCs in the chondrogenic layer of the bilayered hydrogel composites, we found that TGF-β3 was more efficient than TGF-β1 in enhancing MSC chondrogenic differentiation, when no osteogenic cells were involved in the bottom (osteogenic) layer. TGF-β3 resulted in higher levels of gene expression for collagen type II and aggrecan, as well as a lower level of gene expression for collagen type I, than TGF-β1 at day 28. The results agreed with the findings from some previous studies. For example, Barry et al. have compared the chondrogenic potential of three TGF-β isoforms on human bone marrow derived MSCs [31]. They found that TGF-β2 and TGF-β3 are more effective than TGF-β1 in promoting chondrogenesis, as evidence by an earlier and greater deposition of GAG and type II collagen.

The incorporation of osteogenic cells of three different osteogenic preculture periods in the osteogenic layer of the hydrogels all resulted in an increase in chondrocytic gene expression of the chondrogenic-layer cells (not significant) [Figure 4(A–B)], suggesting their stimulatory effect on MSC chondrogenesis. The results were expected, since studies in our laboratory have shown that MSCs cultured in osteogenic medium either express genes for or secrete many bioactive factors related to cartilage and bone formation, including TGF-β1, BMP-2, fibroblastic growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1) [39, 40]. Many studies including ours have suggested these paracrine signals could have contributed to the chondrogenic differentiation of the cocultured MSCs or chodnrocytes [19, 21, 22].

Osteogenic cells demonstrated a combined effect with both TGF-β1 and with TGF-β3 on enhancing MSC chondrogenic differentiation in this study. It should be noted that the combined effects were more apparent in the TGF-β1 treatments than the TGF-β3 treatments at day 28, however, it was probably due to a more efficient potential of TGF-β3 alone for MSC chondrogenesis. Osteogenic cells of various osteogenic preculture periods, in combination with TGF-β3- (or TGF-β1-) loaded MPs, enhanced collagen type II and aggrecan gene expression of the MSCs at different levels. For collagen type II gene expression, there was a trend that longer osteogenic preculture periods of cells in the osteogenic layer resulted in a higher collagen type II and aggrecan gene expression of the cocultured MSCs in the chondrogenic layer in the presence of TGF-β3-loaded MPs. A more apparent trend was seen in similar hydrogel composites containing TGF-β1. In contrast, for aggrecan gene expression, 3-day and 6-day osteogenically precultured cells in the bottom (osteogenic) layer resulted in a more rapid and greater upregulation in gene expression of the top-layer MSCs compared to plain MSCs or 12-

day osteogenically precultured cells in the bottom layer. This was true in all the cases, with blank MPs, TGF-β3-loaded MPs, or TGF-β1-loaded MPs in the chondrogenic layer. For collagen type I gene expression, although no difference was observed among the osteogenic treatments, all the values decreased at day 28 compared to day 0. Our previous study indicated that the synergistic effect of TGF-β1 and osteogenic cells on MSC chondrogenic differentiation could be attributed to the cross-talk between the growth factor released from MPs and other growth factors secreted by osteogenic cells [22]. Therefore, the results of this study suggest possible differences in the soluble growth factors secreted by cells of various osteogenic preculture periods.

In another study, Nakaoka et al. cocultured chondrocytes and osteoblasts in a culture medium with or without osteogenic supplements [19]. Their results also indicated that the differentiation level of osteoblasts (caused by the presence of osteogenic supplements) influenced collagen type II and aggrecan gene expression levels of the chondrocytes. Additionally the chondrocytes showed a significant increase in collagen type X gene expression, indicative of differentiation into a hypertrophic phenotype, under the combined effects of osteoblasts and osteogenic supplements in the culture medium. Although the results are not directly comparable with the present study due to the differences in the cells types, culture medium, and the coculture system selected, both studies underscore the importance of the investigation of the cell differentiation stages and on the cell signaling in osteochondral tissue.

GAG production, associated with the chondrogenic phenotype of the cultured cells, exhibited higher values for all the groups at later time points than at day 0 [Figure 2(B)], which provided additional evidence of MSC chondrogenic differentiation. However, GAG production was comparatively low, and no apparent difference was observed in the cross sections stained with Safranin O [Figure 6 (top layer of C, E, G, I)]. As previously reported, the tight polymer network may have limited the deposition of matrix [14]. Calcium content was low in the chondrogenic layer of bilayered hydrogels for all the treatments at all the time points [Figure 2(C)], confirming that MSCs in the chondrogenic layer did not undergo osteogenic differentiation under the influence of both TGF-β3 and β-glycerophosphate in the medium.

Confocal microscopy images [Figure 5(B–C)] and histological images [Figure 6(C–J)] each showed differences in cell distribution in the top (chondrogenic) layer of hydrogels at day 28. Compared to the OS0− (control) group [Figure 5(B) and Figure 6(C)], where the cells were sparsely distributed, more cell aggregates were observed in the TGF-β3 treatments [noted with small arrows in Figure 5(C) and Figure 6(D, F, H)] at day 28. Previous studies have shown that MSC condensation is important for inducing chondrogenesis during joint development [4]. Thus, a higher extent of chondrogenic differentiation of MSCs in the presence of TGF-β3 in the current study may be related to a better cell-cell contact.

Osteogenic Differentiation of Cells in the Osteogenic Layer

The osteogenic differentiation of the cells encapsulated in the bottom (osteogenic) layer was assessed by their ALP enzyme activity and calcium deposition. ALP activity has been regarded as an early marker for the osteogenic differentiation of MSCs or osteoblasts [15,34,35]. Many previous studies investigating osteogenic differentiation of cells have reported that ALP activity of the cells increased during their proliferation period, peaked before mineralization, and then decreased [34,35]. ALP results of this study in Figure 3(B) showed that MSCs (in the OS0± groups) encapsulated in the osteogenic layer followed the characteristic trend for ALP activity, indicative of their osteogenic differentiation. Although the OS0+ group (with TGF-β3 in the top layer) had higher ALP levels than the OS0− group (without TGF-β3 in the top layer) at days 7, 14 and 28, the ALP peak in the OS0+ group was seen to shift to a later time point (day 14) compared to the ALP peak of the OSO− group at day 7. The results suggest that TGF-β3 may prolong the immature stage of MSCs and delay the osteogenic process.

ALP activity of osteogenic cells was higher than that of plain MSCs after the preculture at day 0. Additionally, there was a trend that longer exposure to the osteogenic medium resulted in higher ALP values (although the differences were not significant). During the subsequent culture in the hydrogel composites, ALP levels of the osteogenic cells slight increased or remained unchanged. This indicates that the peaks of their ALP values may have occurred during the osteogenic preculture in tissue culture flasks. However, the cells were able to maintain their osteoblastic phenotype in the hydrogels.

Calcium deposition is a marker for late-stage osteogenic differentiation [34,35]. The results from the calcium assay on the osteogenic layer of the hydrogels did not reveal significant differences in calcium content among the groups, however significant increases in calcium content compared to the day 0 values were observed in some samples containing osteogenically precultured cells at later time points [Figure 3(C)]. Von Kossa staining of the cross sections of the hydrogel composites at day 28 confirmed the deposition of mineralized matrix in the samples containing precultured osteogenic cells in the bottom (osteogenic) layer and blank MPs in the top (chondrogenic) layer [the OS3−, OS6− and OS12− groups in Figure 6(E, G, I)]. In those samples, a darker staining of calcium deposition was observed in the middle area of the hydrogel composites, some even spread to the top layer, as shown in Figure 6(G, I). More calcium deposition in the middle area of the hydrogels could be due to a faster degradation rate in the center of the hydrogels (seen from cross sections of the hydrogel composites without cells, data not shown), giving the cells more space to lay down matrix. In contrast to the blank MP treatments, no staining of calcium was observed in either layer of the TGF-β3 treatments [Figure 6(D, F, H, J)]. This finding, along with the results from the ALP assay showing the delay of peak ALP activity in the presence of TGF-β3, suggest that TGF-β3, or chondrocyte-like cells induced by TGF-β3 had an inhibitory effect on the maturation and mineralization of osteoblastic cells encapsulated in the bottom layer. In fact, other studies investigating the effect of TGF-β3 on osteogenic differentiation of MSCs, MSC-derived osteoblasts and osteoblast-enriched bone cells, have also shown that TGF-β3 suppressed osteogenic differentiation and matrix synthesis of the cells [32,33].

Conclusions

In the current work, we fabricated a bilayered osteochondral construct with good integration between layers, using OPF hydrogel composites containing gelatin MPs and MSCs. A live/dead assay revealed that cell viability was maintained in both layers during the coculture, and the cellularity of each layer was influenced by the osteogenic preculture period of the cells encapsulated in the osteogenic layer. TGF-β3-loaded MPs significantly stimulated chondrogenic differentiation of MSCs in the chondrogenic layer. Additionally, cells of various osteogenic preculture periods in the osteogenic layer, along with TGF-β3, enhanced chondrogenic gene expression of the MSCs in the chondrogenic layer to different extents. In the osteogenic layer, osteogenically precultured cells maintained their osteoblastic phenotype, as evidenced by a sustained ALP activity and some calcium deposition. However, TGF-β3 showed an inhibitory effect on cell mineralization by Von Kossa staining. The results demonstrated the fabrication of bilayered osteochondral constructs using biodegradable hydrogel composites and suggested their application for the co-delivery of growth factors and MSCs for cartilage tissue engineering, while illustrating that encapsulated cells of different degrees of osteogenic differentiation can significantly influence the chondrogenic differentiation of co-cultured progenitor cells in both the presence and absence of chondrogenic growth factors.

Acknowledgments

This work was supported by the National Institute of Health (R01 AR48756).

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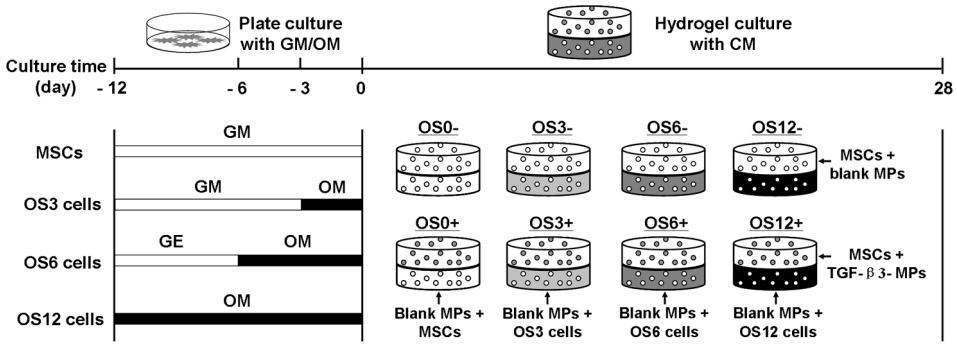

Figure 1.

A schematic representation of the overall experimental design. During the plate preculture, rabbit marrow mesenchymal stem cells (MSCs) were cultured with either general medium (GM) or osteogenic medium (OM) or both for a total of 12 days. However, cells were exposed to OM for different time periods: 0, 3, 6 and 12 days, and were named MSCs, OS3 cells, OS6 cells, OS12 cells, respectively. After cell encapsulation at day 0, bilayered hydrogel composites were cultured in chondrogenic medium (CM). Eight groups were included, namely the OS0±, OS3±, OS6± and OS12± groups. The top layer of the hydrogels encapsulated MSCs with blank microparticles (MPs) (indicated with −) or with transforming growth factor-β3 (TGF-β3)-loaded-MPs (indicated with +). The bottom layer of the hydrogel composites contained blank MPs and cells of different preculture periods: MSCs in the OS0± groups, OS3 cells in the OS3 ± groups, OS6 cells in the OS6± groups, and OS12 cells in the OS12± groups, respectively.

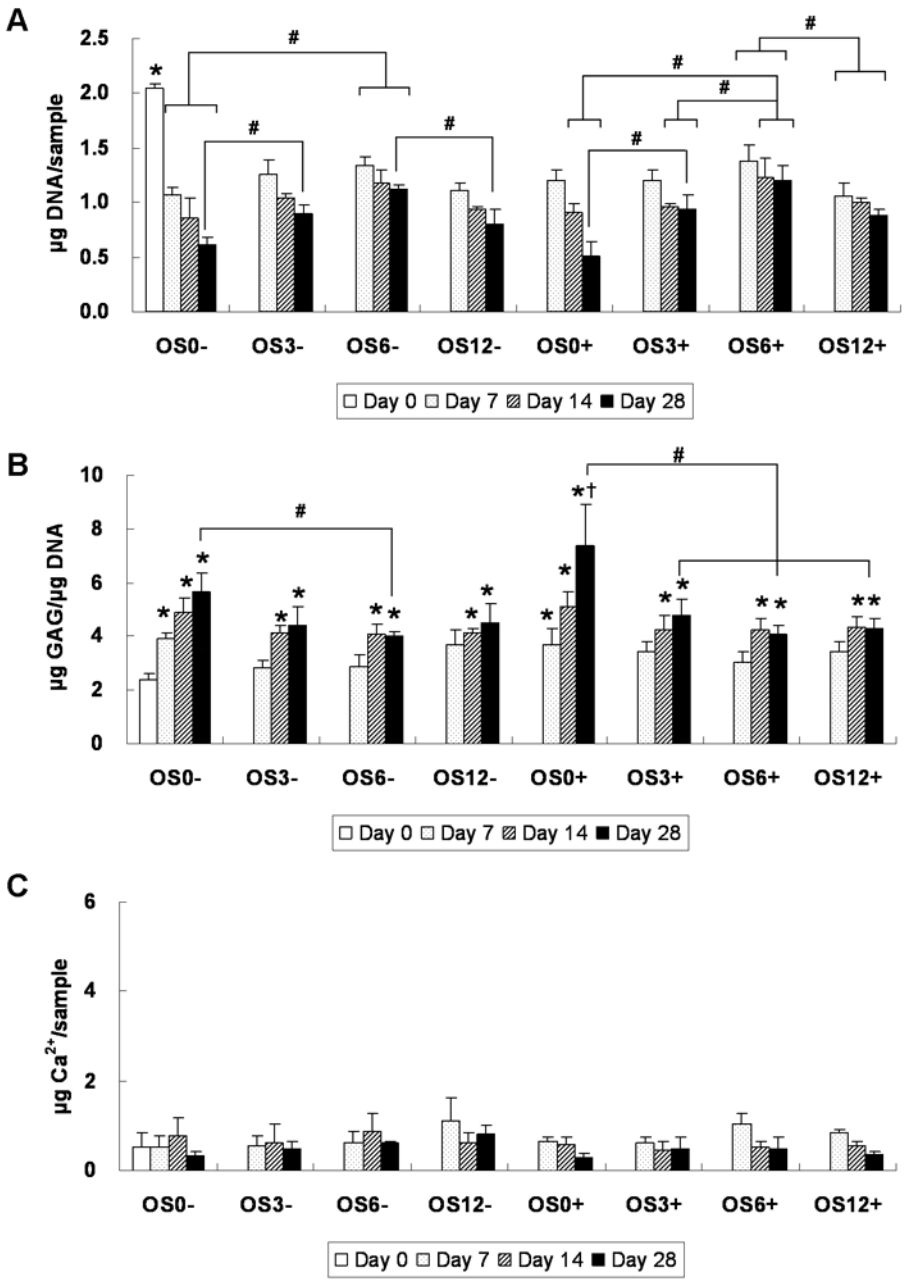

Figure 2.

DNA content (A), GAG content normalized to μg DNA (B) and calcium content (C) for the top layer of the bilayered hydrogel composites. Error bars represent means ± standard deviation for n = 4. At day 0, all groups shared top-layer samples for the biochemical assays. Statistical significance symbols are defined in Table 1.

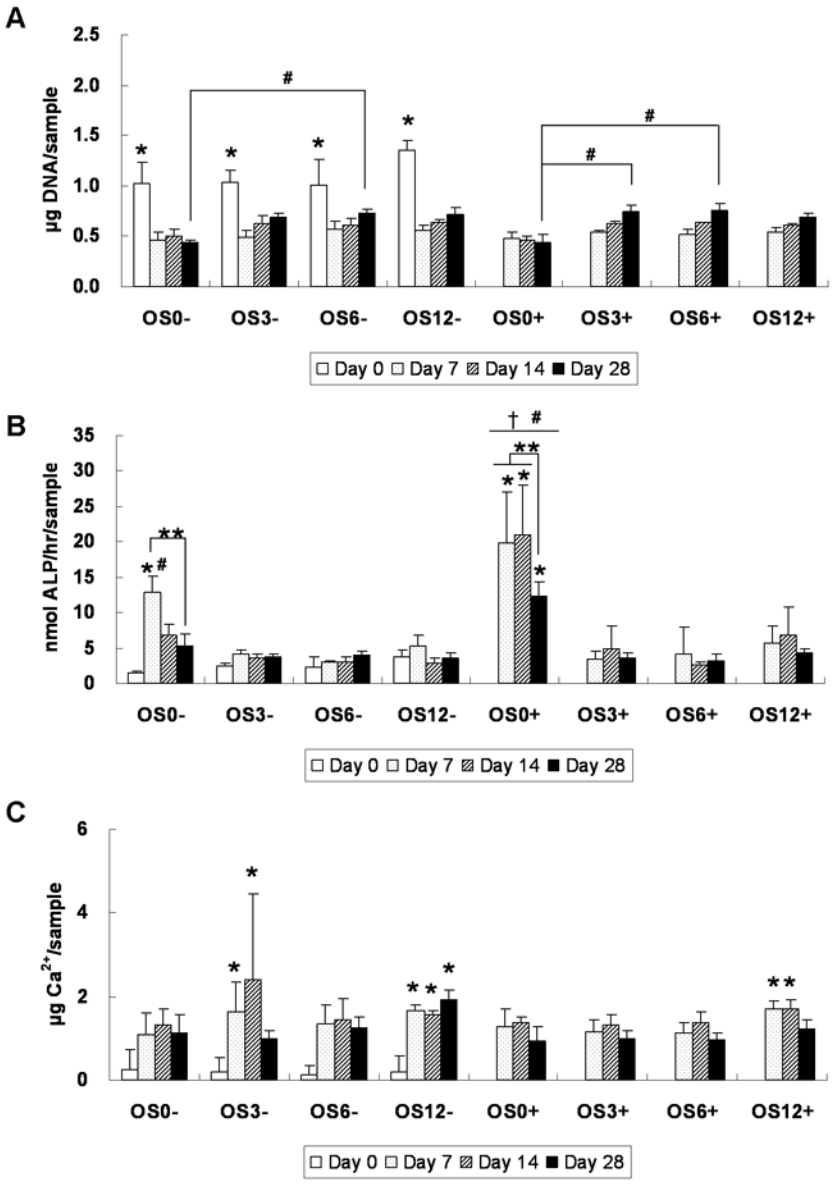

Figure 3.

DNA content (A), ALP enzyme activity (B) and calcium content (C) for bottom layer of the bilayered hydrogel composites. Error bars represent means ± standard deviation for n = 4. At day 0, the OS0± groups shared bottom-layer samples for all biochemical assays; and similarly the OS3± groups, OS6± groups, and OS12± groups shared samples respectively. Statistical significance symbols are defined in Table 1.

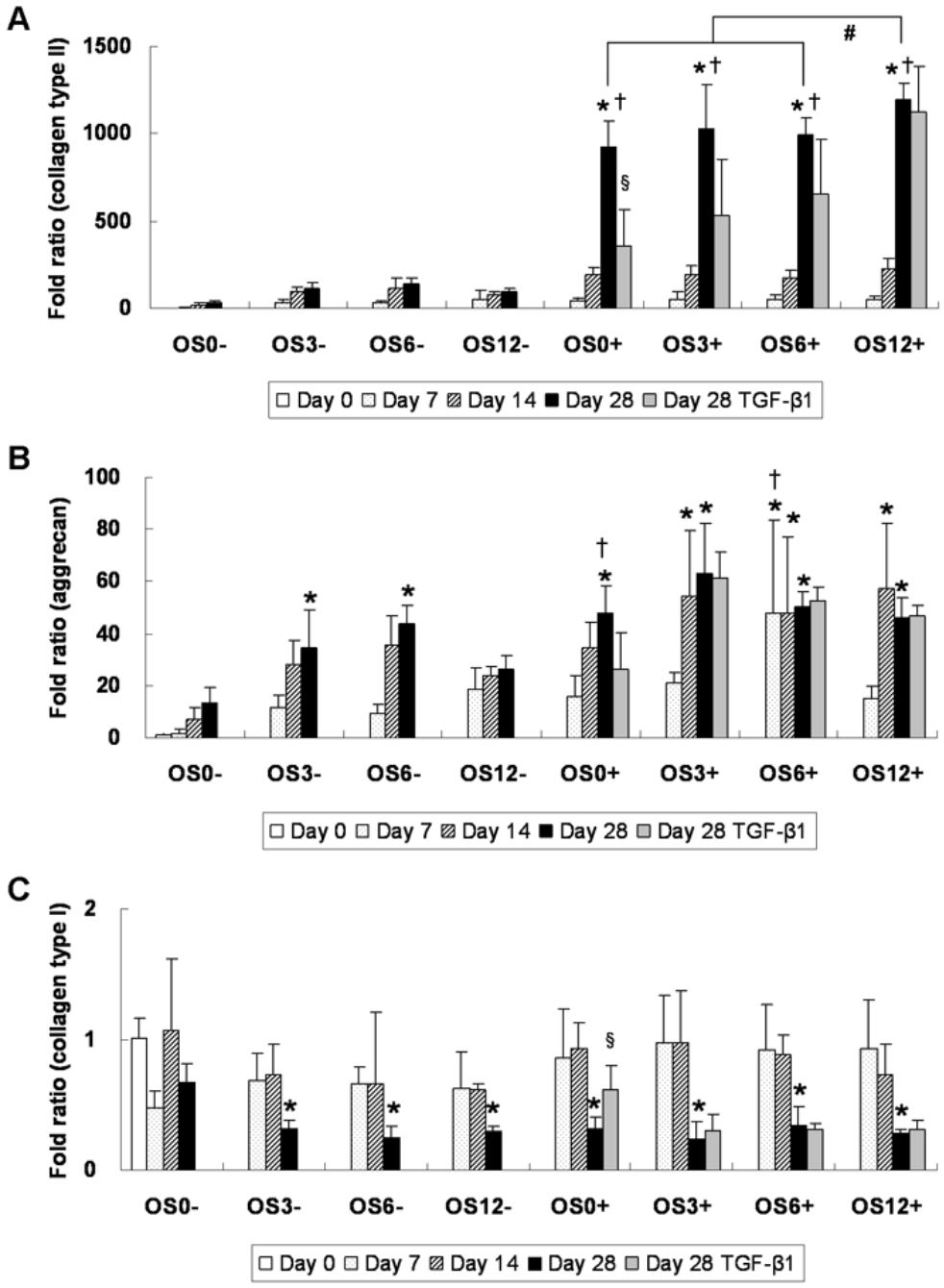

Figure 4.

Quantitative gene expression of collagen type II (A), aggrecan (B), and collagen type I (C) for the top layer of the bilayered OPF hydrogel composites. At day 28, gene expression levels were compared between top layer hydrogels encapsulating TGF-β3 and those encapsulating TGF-β1. Error bars represent means ± standard deviation for n = 4. Statistical significance symbols are defined in Table 1.

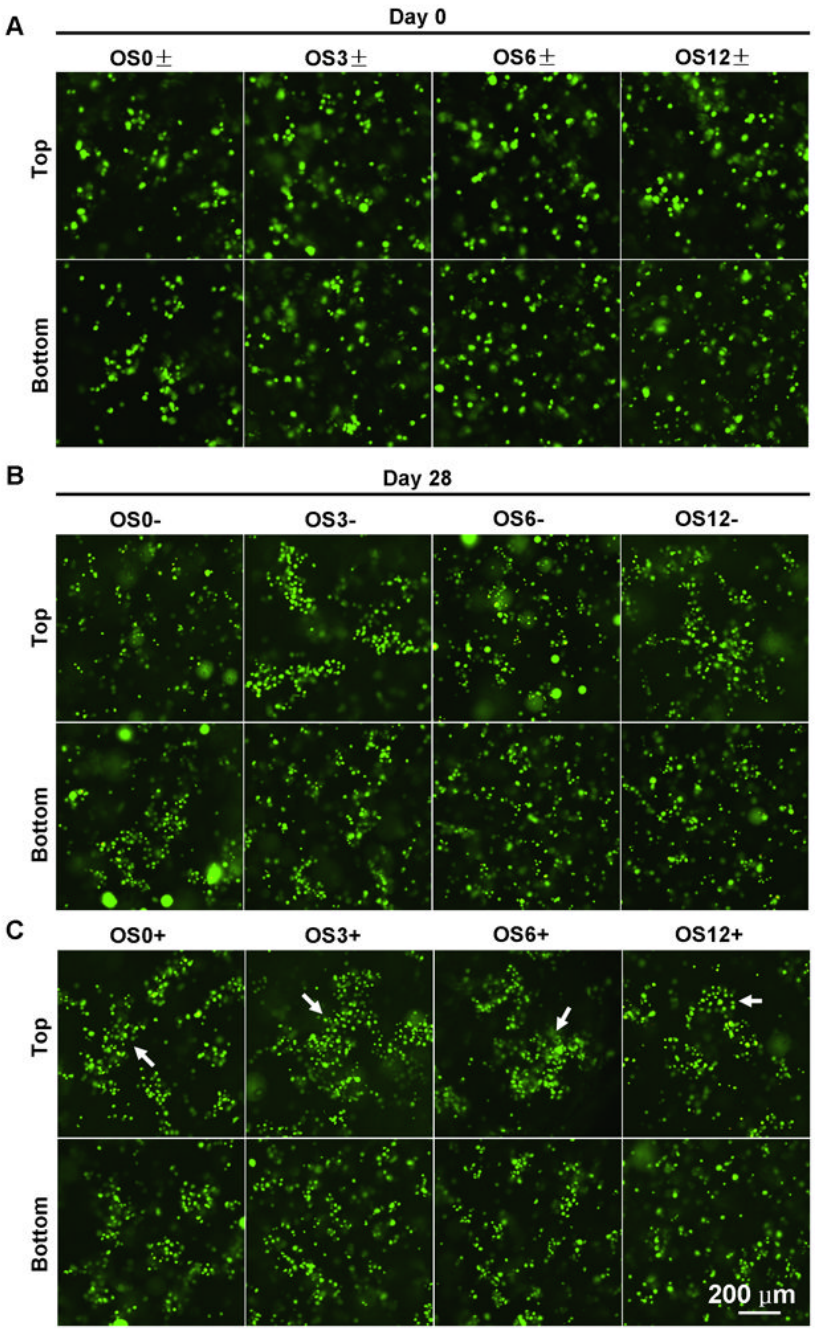

Figure 5.

Confocal fluorescence microscopy images of cells encapsulated in top and bottom layers of the bilayered composites after encapsulation (A) and after 28 days of culture (B–C) with live/dead reagents. A green fluorescence (calcein AM) designates live cells, whereas a red fluorescence (EthD-1) indicates dead cells. Arrows indicate aggregated rabbit marrow MSCs in OPF hydrogel composites. Scale bar represents 100 μm.

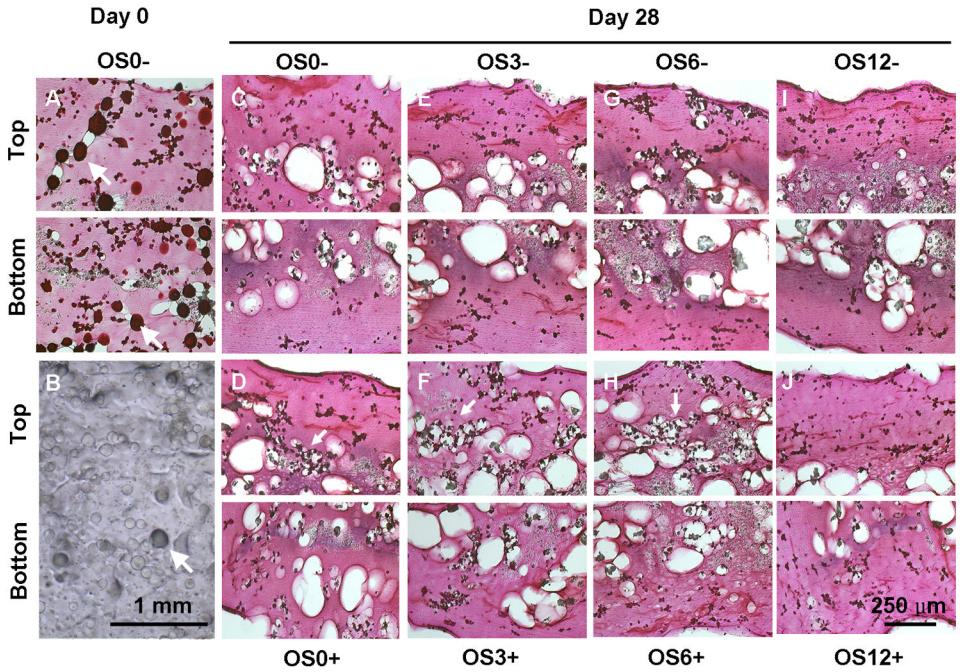

Figure 6.

Histological sections stained with Von Kossa and Safranin O stainings illustrating cells encapsulated in top and bottom layers of the bilayered composites after encapsulation (A) and after 28 days of culture (C–J). (B) shows a light microscopy image of a cross section of a bilayered hydrogel encapsulating MSCs and blank MPs in each layer at day 0. Small arrows indicate aggregated cells in OPF hydrogel composites. Big arrows indicate MPs. Scale bar represents 1mm in (B), and 250 μm in the histological images (A, C–J).

Table 1

Definition of Statistical Significance Symbols in Figures 2, 3 and 4

Symbols	Statistical Significance ($p<0.05$)
*	A significant difference between the values at a later time point and day 0 within a given group.
**	A significant decrease compared to a previous time point within a given group
†	A significant difference between a TGF-β3-loaded MP treatment and its blank MP control at the same time point
#	A significant difference compared to another group within the TGF-β3-treated groups or within blank MP groups at the same time point
§	A significant difference between the TGF-β3 and TGF-β1 groups, of the same bottom layer formulation at the same time point