**In vitro** generation of a multilayered osteochondral construct with an osteochondral interface using rabbit bone marrow stromal cells and a silk peptide-based scaffold

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**Abstract**

Tissue engineering of a biological osteochondral multilayered construct with a cartilage-interface subchondral bone layer is a key challenge. This study presented a rabbit bone marrow stromal cell (BMSC)/silk fibroin scaffold-based co-culture approach to generate tissue-engineered osteochondral grafts with an interface. BMSC-seeded scaffolds were first cultured separately in osteogenic and chondrogenic stimulation media. The two differentiated pieces were then combined using an RADA self-assembling peptide and subsequently co-cultured. Gene expression, histological and biochemical analyses were used to evaluate the multilayered structure of the osteochondral graft. A complete osteochondral construct with a cartilage-subchondral bone interface was regenerated and BMSCs were used as the only cell source for the osteochondral construct and interface regeneration. Furthermore, in the intermediate region of co-cultured samples, hypertrophic chondrogenic gene markers type X collagen and MMP-13 were found on both chondrogenic and osteogenic section edges after co-culture. However, significant differences gene expression profile were found in distinct zones of the construct during co-culture and the section in the intermediate region had significantly higher hypertrophic chondrocyte gene expression. Biochemical analyses and histology results further supported this observation. This study showed that specific stimulation from osteogenic and chondrogenic BMSCs affected each other in this co-culture system and induced the formation of an osteochondral interface. Moreover, this system provided a possible approach for generating multilayered osteochondral constructs. Copyright © 2013 John Wiley & Sons, Ltd.

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**Keywords** multilayer; osteochondral; BMSCs; co-culture

**1. Introduction**

Osteochondral tissue consists of multiple tissue layers with different structures, cell types and functions (Martin et al., 2007; Yang and Temenoff, 2009). However, its self-regeneration ability is limited (Hunziker, 2002). During occurrence of osteochondral defects such as traumatic injuries, osteochondritis dissecans and chondromalacia, this multilayered tissue was unable to recover and instead filled up with fibrous tissues resulting in mechanical instability (Bhosale and Richardson, 2008; Cain and Clancy, 2001; Martin et al., 2007). Osteochondral defects are occasionally derived from superficial cartilaginous layer defects. Such damages of the cartilage surface are often the precursor of degeneration of the complete osteochondral tissue and degeneration can progress to the subchondral region (Mano and Reis, 2007; Martin et al., 2007). Current surgical treatments such as microfracture and autografts improve regeneration of osteochondral defects. However, certain limitations such as donor scarcity and unwanted fibrocartilage regeneration continue to hamper
surgical outcomes (Kellett et al., 2006; Lewis et al., 2006). As a result, an osteochondral engineered graft generated using tissue-engineering methods has the potential to resolve this problem.

Several studies have reported positive results of isolated cartilage constructs without a mineralization zone or subchondral bone region (Allan et al., 2007; Boskey, 1992) by developing various scaffolds (Cao et al., 2003; Khanarian et al., 2012; Malafaya and Reis, 2009) and by using different cells sources, cytokines and bioreactors (Csaki et al., 2008; Mahmoudifar and Doran, 2005; Mohan et al., 2011). However, an ideal method for regenerating a complete osteochondral graft containing an osteochondral interface remains to be explored. Recently, a multilayered scaffold strategy was used to treat patients and results showed that both cartilage and subchondral bone could be regenerated (Kon et al., 2010). This raises the possibility that regeneration of a complete osteochondral graft comprised of the cartilage layer, subchondral bone and an osteochondral interface required a multilayered design for scaffolds and different cell arrangements.

Among current strategies in use, co-culture could be an effective method. The current study was based on cell-cell interactions between chondrogenic and osteogenic BMSCs. Some studies have shown that under regulatory effects of osteoblasts, chondrocytes were able to differentiate into hypertrophic chondrocytes (Jiang et al., 2005). Conversely, BMSCs could differentiate into osteoblasts and chondrocytes after specific stimulations (Liu et al., 2006; Miao et al., 2009; Wu et al., 2007; Zou et al., 2008), thereby supporting the possibility that BMSCs can be applied as the only cell source to generate the complete osteochondral tissue. As a result, by co-culturing chondrogenic and osteogenic differentiated progenies, a complete osteochondral construct with osteochondral interface could be regenerated, as reported in this article.

In the current study, two pieces of silk sponge scaffolds were seeded with BMSCs and then glued together with RADA self-assembling peptides with the aim of engineering a complete osteochondral construct. A silk fibroin sponge was used to provide a 3D culture environment for rabbit BMSCs. Silk fibroin has been shown to be a biocompatible material that allows good cell attachment. Moreover, this sponge scaffold can provide a 3D porous structure and the necessary mechanical support for bone and cartilage tissue generation (Vepari and Kaplan, 2007; Wang et al., 2005, 2006). Self-assembling peptides have been recently used to fabricate nanofibrous scaffolds in tissue-engineering research. These self-assembling nanofibrous scaffolds contain more than 99% water and possess excellent biocompatibility (Zhang et al., 2005). The generated nanofibers mimic natural ECM and enhance attachment, growth and differentiation of a variety of cells, including chondrocytes (Gelain et al., 2007; Kisiday et al., 2002). The schematic approach used is shown in Figure 1. After BMSCs were seeded onto two separate scaffolds, they were first cultured in chondrogenic and osteogenic media separately for two weeks. Finally, the two pieces were connected by RADA self-assembling peptides to achieve the complete osteochondral graft and then investigated. As described in our previous study, by co-culturing with osteoblasts, chondrogenic BMSCs received different influences depending on the distances from osteoblasts (Chen et al., 2012). As a result, another objective of the current study was to analyze the layered differentiation in both pieces of scaffolds and formation of the interface.

2. Materials and methods

2.1. Scaffold preparation

Fabrication of scaffolds was described in our previous study (Chen et al., 2012). Briefly, Bombyx mori raw silk fibres were immersed in a degumming solution of 0.25% (w/v) Na2CO3 and 0.25% (w/v) sodium dodecyl Sulfate (SDS) between 98°C and 100°C until the sericin was removed. The degummed silk was rinsed with distilled water for 1 h to remove any residual degumming solution and then dried. It was then dissolved in a mixture of calcium chloride, ethanol and water (CaCl2–CH3OH–H2O) (3:1:2:8) at 65°C under continuous stirring. Next, the silk solution was dialyzed against distilled water using SnakeSkin™ pleated dialysis tubing (MWCO 3500; Pierce, Thermo Scientific, Singapore). The concentration (w/v) of the dialyzed solution was determined and adjusted to 3% w/v using distilled water. The silk solution was transferred into Petri dishes and frozen at -20°C followed by freeze-drying for 24 h. Freeze-dried silk sponge scaffolds were treated with 90% methanol for 10 min and then rinsed with distilled water. Scaffold dimensions were 5-mm diameter and 2-mm thick.

2.2. Cell culture and in vitro 3D co-culture model

Rabbit bone marrow-derived stromal cells (rBMSCs) were obtained from New Zealand White Rabbits using a previously described protocol approved by the NUS Institutional Animal Care and Use Committee, National University of Singapore, Singapore (Fan et al., 2008; Liu et al., 2008). Passage 3 rabbit BMSCs were used in all tests. 0.5 million BMSCs were mixed with 50 μl of 0.5% RADA peptide (Sigma-Aldrich Pte. Ltd., Singapore) solution before being seeded on each silk scaffold. They were then cultured for two weeks in chondrogenic medium consisting of: high glucose DMEM supplemented with 10^{-3} M dexamethasone, 1% ITS + premix, 50 mg/ml ascorbic acid, 1 mM sodium pyruvate and 4 mM proline. Chondrogenic differentiation was induced in the presence of 10 ng/ml transforming growth factor β3 (TGF-β3) (R&D Systems, Minneapolis, MN, USA) and osteogenic medium consisting of: high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 10^{-7} M dexamethasone, 50 mg/ml ascorbic acid and 10nM β-glycerophosphate (Sigma). After two weeks of culture, scaffolds in the two different media were combined by using RADA self-assembling peptide and co-cultured for another two
weeks using cocktail culture medium consisting of high glucose DMEM supplemented with 2% FBS, $10^{-7}$ M dexamethasone, 1% ITS + premix, 50 mg/ml ascorbic acid, 1 mM sodium pyruvate, 4 mM proline and 10 nM β-glycerophosphate (Sigma). Culture medium was changed three times a week. Two control groups were designed in this study: 1) two combined osteogenic pieces (OS-control) and two combined chondrogenic pieces group (CH-control). All samples were cultured in cocktail culture medium. All tests were performed after co-culture and time points were recorded after co-culture.

2.3. Total RNA extraction, cDNA synthesis and real-time PCR analysis

Three gene expression tests were performed. In the first, the effect of co-culture was investigated. After one and two weeks of co-culture, samples from the co-culture and the two control groups ($n = 3$ for each group) were harvested. In the second gene expression test, the formation of osteochondral zones was analyzed in co-culture samples alone. Samples from the co-culture group at week 2 were harvested and split into two parts: a top and bottom chondrogenic layer to be evaluated and compared. In the final test, the location of hypertrophic chondrogenic cells was also investigated in the co-culture group. The complete osteochondral construct was divided into four layers: CH-TOP, CH-MID, OS-MID and OS-TOP, and then tested. All samples for gene expression were rinsed in phosphate buffered saline (PBS) and total RNA was extracted using an RNeasy Mini Kit (QIAGEN Singapore Pte. Ltd., Singapore) following manufacturer protocols. The final eluted RNA was stored at $-20^\circ$C and reverse transcribed to cDNA using iScript™ cDNA synthesis kit (Bio-Rad Laboratories Pte. Ltd., Singapore). Real-time RT-PCR was performed in an iQ5 multicolour real-time PCR detection system (Bio-Rad Laboratories Pte. Ltd., Singapore) using an iQ™ Green RT-PCR Kit (Qiagen). Assays were run in triplicates for each sample. The primer sequences of selected genes for real-time PCR are summarized in Table 1 (Hollister, 2005; Sahoo et al., 2011). GAPDH was used as the housekeeping gene and relative expression levels for each gene of interest were determined. Amplification was performed in triplicate and data were analyzed for relative expression using the $\Delta\Delta$Ct method.

2.4. GAG assays

The total insoluble sulfated glycosaminoglycans (GAG) produced on scaffolds was tested in Week 2 in three groups. To further analyze the influence from co-culture, co-cultured samples were separated into a chondrogenic (CH-PART) and osteogenic parts (OS-PART) and compared with one piece from the osteogenic (OS-control) and chondrogenic (CH-control) control groups. Samples ($n = 3$ samples/group) were harvested and washed three times with PBS. Five-hundred uL of 0.25 mg/ml pepsin solution with 0.5% Triton X 100 and 0.25 M HCl was added to each sample in a 2-mL centrifuge tube and samples were shaken at $37^\circ$C for 2 h. The digested solutions were harvested and neutralized by NaOH then analyzed for soluble sulfated GAG using a Blyscan™ Assay (Biocolor, Newtownabbey, Northern Ireland). The procedure followed vendor protocols. Absorbance was measured by a Sunrise™ remote 96-well microplate reader (Tecan Asia Pte Ltd., Singapore) to calculate the percentage reduction in dye at 656 nm wavelength with 550 nm as the reference wavelength. GAG content was obtained by comparison with the standard calibration supplied in the assay kit.

2.5. Morphological characterization

Cellular morphology in the cell-seeded silk scaffolds was also characterized using a Joel TSM-5000 scanning electron microscope JEOL ASIA PTE. Ltd., Singapore. Samples from the co-culture group were harvested after two weeks then fixed with formaldehyde, freeze-dried for 24 h, sputter-coated with gold and observed by SEM.

2.6. Histology

Histological examination of cell-seeded scaffolds after two weeks of co-culture was performed. Scaffolds were harvested and fixed with 3.7% formaldehyde, dehydrated, embedded in paraffin with 10-μm sections cut from each

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**Figure 1. Osteochondral co-culture system**
sample and stained with Hematoxylin and eosin (H&E) for histological observation. To investigate the mineralization of extra cellular matrix (ECM), Alizarin red staining was used. To investigate the GAG in ECM, Alcian blue staining was used.

To investigate ECM proteins, Type I, II and X collagen produced by BMSCs, an immunohistology staining kit, and a Leica CM3050S cryomicrotome with a labeled streptavidin-biotin immunoenzymatic antigen detection system (UltraVision Detection System Anti-Mouse, HRP/streptavidin-biotin) were used. Brieﬂy, sections were digested enzymatically with 1 mg/mL pepsin for 30 min at RT and then incubated with a mouse anti-pig monoclonal antibody (Sigma). Next, horseradish peroxidase (HRP) labeled secondary antibody was used. Finally, all sections were covered by coverslips using permanent mounting medium. Images were subsequently analyzed with Adobe Photoshop CS5 (Mountain View, CA, USA). The area for the pixels within the threshold set for the chromogen was divided by the total pixel area of the region to obtain the percentage stained (Tan et al., 2011); four regions from each sample were analyzed and compared.

2.7. Statistical analysis

All data were expressed as means ± standard deviation. Multiple comparisons were performed using one-way ANOVA and post hoc Tukey’s tests for pairwise comparisons (SPSS 13.0 software package). \( p < 0.05 \) was accepted as statistically significant.

3. Results and Discussion

3.1. Osteochondral co-culture construct

A BMSCs based co-culture method was used to regenerate a complete osteochondral graft comprised of the cartilage layer, the subchondral bone and an osteochondral interface. To achieve this, the ﬁrst step was to design a multilayered co-culture construct. As shown in Figures 1 and 2A, the osteogenic/chondrogenic co-culture constructs were developed with a combined 3D culture consisting of chondrogenic and osteogenic BMSCs in two pieces of scaffolds. Cells were observed to be well-distributed within the whole constructs and were surrounded by higher amounts of GAG in the chondrogenic layer (Figure 2B) and mineralized ECM in the osteogenic region (Figure 2C), supported by H&E staining (Figure 2). The interface gap (Figure 3A, arrows) was partly covered by tissues (Figures 2E, 3A, 3D), which was intended to integrate the layers. A few spherical shape cells were observed in the intermediate region; however, the gap between the two layers remained.

3.2. Osteogenic/chondrogenic BMSC co-culture system induced hypertrophic chondrogenic differentiation

GAG is one of the most important ECM constituents in cartilage (Harley and Gibson, 2008; Moroni et al., 2008). Corresponding to the results of the current study, the production of GAG (Figure 4) after two weeks of co-culture was signiﬁcantly less in the co-culture group than that of the chondrogenic control group but still greater than the osteogenic control. Co-culture samples were glued by osteogenic and chondrogenic pieces. As a result, we further separated the two pieces and further investigated. Results showed that after co-culture, the production of GAG in the chondrogenic part was down-regulated compared to the piece from the chondrogenic control group, which showed similar results to those of Jiang et al., (2005). They reported that when co-cultured with osteoblasts, the production of GAG from chondrocytes decreased. In contrast, the amount of GAG production in the osteogenic part from the co-culture group was greater than from the osteogenic control group but still greater than the osteogenic control. Co-culture samples were glued by osteogenic and chondrogenic pieces. As a result, we further separated the two pieces and further investigated. Results showed that after co-culture, the production of GAG in the chondrogenic part was down-regulated compared to the piece from the chondrogenic control group, which showed similar results to those of Jiang et al., (2005). They reported that when co-cultured with osteoblasts, the production of GAG from chondrocytes decreased. In contrast, the amount of GAG production in the osteogenic part from the co-culture group was greater than from the osteogenic control group; however, the difference was not signiﬁcant (*, \( p < 0.05 \)).

Hypertrophic chondrocytes are the main cell type in the osteochondral interface region (Keeney and Pandit, 2009; Yang and Temenoff, 2009). Co-culture has been reported by several studies as a possible method for introducing hypertrophic chondrocyte differentiation (Jiang et al., 2005; Sanchez et al., 2005). Osteoblasts have been thought to have the ability to stimulate chondrocytes into hypertrophic differentiation (Jiang et al., 2005). In addition, Cheng et al. (2011) reported that osteogenic/chondrogenic BMSCs could induce BMSCs to differentiate into hypertrophic chondrocytes in vitro. In the current study, quantitative real time-PCR was used to investigate hypertrophic differentiation by regulation of the co-culture. Results

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**Table 1. Real-time RT-PCR primer sequences**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer sequences</th>
<th>Reverse primer sequences</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>GCCATCAAAAGGTGTGTTAGTGTGACAGC</td>
<td>CTTCAACAAAGGCTGATGAGG</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>GGTAAGGGTGTGTTGTTCCACTCT</td>
<td>TGGGGAATCTGACAGCCTGAGT</td>
</tr>
<tr>
<td>Collagen II</td>
<td>AAAGGGGTAGACTAGTTTAGAT</td>
<td>TGCTGTCTACATAGCTGAGA</td>
</tr>
<tr>
<td>Collagen I</td>
<td>GCATGTCGTTAGGAAAGCTTACC</td>
<td>ATGATGCAATGCTGTTCTGC</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>GAAGTGAGGAAGGAGGAGA</td>
<td>GCCAGGAGGAGGAGGAGAAGAGAG</td>
</tr>
<tr>
<td>Runx-2</td>
<td>CTTTCACCTTCAGTAAAGAAGA</td>
<td>TAAGTAAAGGTTGCTGATAGT</td>
</tr>
<tr>
<td>Collagen X</td>
<td>CCCCACCCCAAGACAGACAGT</td>
<td>ATCACCTTTGAGTGGCCTGCT</td>
</tr>
<tr>
<td>MMP-13</td>
<td>TCCGCTTAGGAGGTGACAGG</td>
<td>ACTCCTGCGGTTGAGGTGTTG</td>
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showed that this co-culture system could also successfully guide the formation of hypertrophic chondrocytes from osteogenic and chondrogenic BMSCs by co-culture.

In the first gene expression study, samples from the co-culture group and two control groups were harvested during weeks 1 and 2. Results (Figure 5) showed that Type II Collagen in the co-cultured group decreased compared to the chondrogenic control (CH-control). Similarly, expression of Aggrecan decreased significantly at week 2. It is possible that hypertrophic differentiation could have downregulated chondrogenic markers. GAG content results provided similar results. However, the expression of Type II Collagen and Aggrecan genes was still 2x greater than the osteogenic control (OS-control). In contrast, Type I Collagen and Osteonectin were upregulated by co-culture compared to chondrogenic control but were still lower than the OS-control. Osteonectin is one of main molecules involved in tissue mineralization (Keeney and Pandit, 2009; Nanba et al., 1997), as a result, the upregulation expression of Osteonectin could be favourable to the formation of osteochondral calcified ECM. Moreover, the significant upregulation of Runx-2 in week 1 showed

Figure 2. (A) Macroscopic image of the construct and histology analysis of the two layered osteochondral constructs co-cultured after 2 weeks. B) Alcian blue staining. C) Alizarin red staining. Interface region was indicated by arrows. D-F) H&E staining, chondrogenic region; D) interface region; E) osteogenic region F). Cells indicated by arrows (B, C; scale bar = 500 μm; D-F, scale bar = 100 μm)

Figure 3. SEM photomicrographs showing two layers fused together after 2 weeks of co-culture (A). ECM in the osteogenic part covered almost all scaffold pores and star-shaped osteoblast-like cell morphology was found in this region (indicated by arrows; B). Chondrocyte-like cells were found the in chondrogenic part (indicated by arrows; C). Some parts between two layers were covered by chondrocyte like cells with ECM (D)
that co-culture influenced chondrogenic BMSC maturation and expressed hypertrophic gene markers on the scaffold (Kim et al., 2008). Type X Collagen has been considered a main hypertrophic marker (Boskey, 1992; Cheng et al., 2011; Lefebvre et al., 1995; Sanchez et al., 2005). In the current study, Type X Collagen appeared in week 1 in the co-culture group, which was significantly greater than that of the CH-control and there was no expression in OS-control at both time points. All gene expression results supported the concept that this co-culture system had the potential to induce hypertrophic differentiation.

3.3. Generation of multilayered osteochondral constructs with the interface

The main aim in osteochondral tissue engineering is to engineer a complete osteochondral tissue with cartilage, intermediate calcified cartilage and subchondral bone layers (Martin et al., 2007; O'Shea and Miao, 2008). Chondrocytes and osteoblasts are two main cell sources for osteochondral tissue generation (Keeney and Pandit, 2009). However, harvesting of chondrocytes can cause secondary injury to patients (Ho et al., 2009). Researchers
have attempted to generate an osteochondral multilayer in vitro using only BMSCs (Cheng et al., 2011). The results of the current study provided another possible method by using BMSCs as an only cell source and engineering a multilayered osteochondral construct.

The morphology of cells and composition of ECM was visibly different between layers (Figure 2). Alizarin Red staining results (Figure 2C) showed that the highest amount of calcium was deposited in the osteogenic portion in the interface region (Figure 2C, arrow); that fewer calcium deposits were detected; and that no calcium was detected in the chondrogenic layer. Furthermore, Alcian blue staining showed that in the chondrogenic and intermediate regions, that there was a greater amount of GAG distributed in ECM compared to the osteogenic region. Cells maintained a spherical morphology embedded in GAG (Figure 2B) and collagen II (Figure 7B) enriched ECM. On the contrary, osteoblast-like cells were embedded in a mineralized ECM with higher Collagen I content in the chondrogenic layer.

Results from the second set of gene expression analyses confirmed the formation of multilayered structures. Co-cultured samples were harvested at week 2 and separated into the CH and OS parts to analyse gene expressions. Figure 6A shows that in this co-culture model, both CH- and OS-parts demonstrated gene expressions had the tendency to become chondrocyte and osteoblast lineages (Karlsson et al., 2007; Mackay et al., 1998; Mori et al., 2011). The difference in Type II collagen expression between CH- and OS-parts was not significant. In addition, Aggrecan in CH-part was 5x greater than that in OS-part. On the other hand, the expression of Type I Collagen and Osteonectin in OS-part was 2x greater than that of CH. Both Collagen X and MMP-13 expression in CH-part were significantly greater than in the OS-part.

To locate the hypertrophic differentiation zones in the whole multilayered construct, both CH- and OS-parts were further separated into another two layers (Figure 6B): CH-TOP, CH-MID, OS-MID and OS-TOP and analyzed for hypertrophic chondrocyte gene markers Collagen X, MMP-13 and chondrogenic markers Aggrecan, Collagen II and two osteogenic markers, Osteonectin and Collagen I. A difference between layers was clearly observed. Hypertrophic gene markers were statistically greater in the two middle layers; especially expression of Type X Collagen in the CH-MID layer, which was 2-3x greater than that of other layers, thereby supporting the notion that hypertrophic chondrogenic differentiation was induced by attaching scaffolds and co-culturing. Furthermore, following interaction between the two pre-differentiated BMSC scaffolds, Aggrecan and Collagen II expressions were evidently downregulated in the CH-MID part compared with CH-TOP. Meanwhile, Osteonectin and Collagen I expression in the OS-MID part was also downregulated compared with OS-TOP. Results showed that chondrogenic BMSCs in direct contact with the osteochondral part had the greatest expression of Collagen X and MMP-13 (Sanchez et al., 2005; Wei et al., 2010); this result was also confirmed by Collagen staining analysis (Figure 7). Percentages stained for Collagen I from

Figure 6. (A) Gene expression analysis 2: normalized expression levels of chondrogenic, osteogenic and hypertrophy-related genes in the chondrogenic and osteogenic parts from the co-culture group after 2 weeks of co-culture (*p < 0.05). B) Gene expression analysis 3: normalized expression levels of chlorogenic, osteogenic and hypertrophic-related genes in the chondrogenic top part, Chondrogenic middle part: osteogenic middle and top parts from the co-culture group after 2 weeks of co-culture (*p < 0.05)
CH-TOP to OS-TOP were 33.9, 30.3, 52.6 and 59.5%. On the other hand, percentages stained for Collagen II from CH-TOP to OS-TOP were 67.7, 21.2, 21.1 and 14.5%. For Collagen X staining, the percentages stained were 11.7, 23.7, 12.2 and 7.7%. Collagen I had the greatest amount in OS-TOP and Collagen II had the greatest amount in CH-TOP. Moreover, the middle region of the whole construct had the greatest amount of Collagen X. In addition, GAG and calcium staining and SEM all showed that interface-like structures were found in the intermediate regions between two layers and chondrocyte like cells were found and their ECM contained both GAG and calcium. Results showed that the interaction between chondrogenic and osteogenic BMSCs was dependent on their distance from each other; cells located close to the contacted region received more stimulation (Chen et al., 2012).

In the current study, the potential of using co-cultured chondrogenic and osteogenic BMSCs for multilayered osteochondral construct was investigated. However, some limitations remain. Some studies have shown that chondrogenic BMSCs and chondrocytes have the possibility to redifferentiate into osteoblasts in long term culture in vitro (Galotto et al., 1995). In the current study, some growth factor such as VEGF were synthesized by hypertrophic chondrocytes and osteogenic BMSCs to induce redifferentiation (Carlevaro et al., 2000). In addition, the cocktail medium included FBS and β-glycerophosphate, which can also improve differentiation (Cheng et al., 2011). Thus, the whole structure had the possibility to be mineralized in long-term culture. To prevent such redifferentiation, methods such as providing TGF-β, bone morphogenetic proteins-4 and Fibroblast growth factor-2 can be used (Mandl et al., 2004; Pick et al., 2007). To better generate a multilayered construct, a precise controllable system should be used to supply stimulation factors to different regions of the constructs. Secondly, we also found that expression of Osteonectin, Runx-2 and Collagen Type X were all increased in the chondrogenic control. The proposed cause could be due to the β-glycerophosphate in the cocktail co-culture medium. The presence of β-glycerophosphate can induce hypertrophy and result in related gene expressions (Mackay et al., 1998). Therefore, efforts to isolate chondrogenic BMSCs from osteogenic constituents in future studies could enable a better understanding of the effects of osteogenic-chondrogenic cell interactions on chondrocyte hypertrophy. Moreover, the RADA peptide gel was used to glue the chondrogenic and osteogenic layers and provided an ECM-like environment for cell migration and attachment (Chau et al., 2008; Gelain et al., 2007). However, a tiny gap between these two layers was observed. To further fuse the two layers, samples could be cultured for longer periods and extra BMSCs could be seeded between layers. To further investigate the regeneration ability of the co-culture engineering constructs, in vivo tests will be performed. The engineered osteochondral plugs will be implanted into the rabbit knee to test its ability to regenerate the osteochondral defects.

In addition to osteochondral regeneration, the co-culture approach and layered analysis methods used in the current study could also be used in the study of other complex multilayer tissue generation. Interface regeneration is the basis of complex tissue engineering (Lu and Jiang, 2006; Spalazzi et al., 2006; Yang and Temenoff, 2009). Different cells can be layered in co-cultures to investigate cell-cell interactions and generate the interface.

### 4. Conclusions

This study reported that a pre-chondrogenic/pre-osteogenic BMSC co-culture system had the potential to induce chondrogenic hypertrophy. Furthermore, the system provided a new method of fabricating a multilayered osteochondral construct using only rabbit bone marrow stromal stem cells and a silk fibroin/peptide scaffold. By using this method, a multilayered construct with different cells and ECM was concurrently generated. The study showed that this co-culture method had the potential to engineer an osteochondral multilayered construct with interface in vitro, which also provided a possible method for the regeneration of other complex tissues.

### Conflict of interest

The authors have declared that there is no conflict of interest.
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