The effects of pore architecture in silk fibroin scaffolds on the growth and differentiation of BMP7-expressing mesenchymal stem cells

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Abstract

Pore architecture of scaffolds is known to play a critical role in tissue engineering as it provides the vital framework for the seeded cells to organize into a functioning tissue. In this report, we investigated the effects of different concentrations of silk fibroin protein on 3 dimensional (3D) scaffold pore microstructure. Four pore size ranges of silk fibroin scaffolds were made by freeze-dry technique, with the pore sizes ranging from 50 to 300 μm. The pore size of the scaffold decreases as the concentration of fibroin protein increases. Human bone marrow mesenchymal stromal cells (BMSCs) transfected with BMP7 gene were cultured in these scaffolds. Cell viability Colorimetric assay (MTS), alkaline phosphatase （ALP）assay and reverse transcription-polymerase chain reaction (RT-PCR) were performed to analyze the effect of the pore size on cell growth, the secretion of extracellular matrix (ECM), and osteogenic differentiation. Cell migration in 3D scaffolds was confirmed by confocal microscopy. Calvarial defects in SCID mice were used to determine the bone forming ability of the silk fibroin scaffolds incorporated with BMP7-expressing BMSCs. The results showed that BMP7 expressing BMSCs preferred a pore size between 100 and 300 μm of silk fibroin protein fabricated scaffolds, with better cell proliferation and ECM production. Furthermore, *in vivo* transplantation of the silk fibroin scaffolds combined with BMP7-expressing BMSCs induced new bone formation. This study identified that optimized pore architecture of silk fibroin scaffolds could modulate the bioactivities of BMP7 transfected BMSCs in bone formation.

Key words: bone marrow mesenchymal stromal cell; bone morphogenetic protein; silk fibroin scaffold; bone

1. Introduction

Tissue engineering is a promising technology that uses cells and biomaterials to replace damaged tissues or organs[1,2]. A cell-based tissue engineering technique has proved to be one of the most promising alternative therapies for bone defects. This approach consists of an interactive triad of responsive cells, a supportive matrix, and bioactive molecules promoting differentiation and regeneration.

Bone marrow mesenchymal stromal cells (BMSCs) are a prospective cell source for the engineering of bone tissue. They are relatively easy to obtain from a small aspirate of bone marrow and have multiple potentials to differentiate into different cell lineages such as osteoblasts, chondrocytes, adipose cells, ligament cells, and neural cells [3]. In addition, they are relatively easy to expand in culture under conditions in which they retain some of their potential to differentiate into multiple cell lineages [4].

Use of bone morphogenetic proteins (BMPs) for bone regeneration represents one of the most promising emerging therapies for bone regeneration and is a viable alternative to autologous and homologous bone grafts. BMP-7 appears to be one of the most potent osteogenic factors for bone regeneration[5]. However, the physiological effects of BMPs may not be maximized by direct delivery of recombinant proteins because of their rapid *in vivo* diffusion and relatively short-term bioactivity. Local gene therapy of BMPs has the potential to induce sustained and relatively high level of BMP production at sites requiring bone formation.

A three-dimensional microenvironment is necessary to promote the differentiation of BMSCs into osteoblasts. The silk fibroin scaffolds have high mechanical strength, are easy to handle, are conducive for cell proliferation and have already been used for bone tissue engineering[6-9]. When used for cell culture a number of scaffold design parameters influence the transport of nutrients and metabolic waste products, such as the macroscopic shape of the scaffold, pore size, porosity, pore interconnectivity, pore orientation, and surface chemistry. These parameters therefore need to be optimized to promote tissue formation in 3D constructs[10,11].

Previously we have shown that adenovirus vector encoding BMP7 (AdBMP7) can enhance bone regeneration around dental implant[12]. In the present study, we investigated whether osteoblastic differentiation of the BMSCs expressing BMP7 influenced by different pore microstructure of silk fibroin scaffolds *in vitro.*

2. Materials and methods

2.1.Materials

MTS was purchased from Promege Chemical Com. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were from Gibco Company (Gibco BRL/Life Technologies, Victoria, Australia). All other reagents were of analytical grade.

Silk protein fibroin was extracted from mulberry silk cocoons following a standard extraction procedures[13]. Briefly, cocoons were cut into small pieces of approximately 1 cm × 1 cm dimensions. They were boiled in an aqueous solution of 0.02 M Na2CO3 for half an hour (around 10% w/v of silk cocoons and Na2CO3) to remove all traces of

sericin for complete degumming of the shells. The cocoon pieces were subsequently washed thoroughly in distilled water and dried briefly for 1 h at 50°C. Degummed shell pieces were dissolved in 9.3 M lithium bromide for 4 h at 60 °C and then centrifuged to remove the insoluble material. The supernatant was dialyzed extensively for 3 days against deionized water with frequent changes. The protein concentration was then measured using BCA protein assay (Progen, Australia) procedure[14].

Recombinant adenoviruses were created using the Ad-Easy system (Stratagene, La Jolla, CA). Production and purification of the recombinant viruses (Ad-BMP7) were performed as described in detail by He et al[15]. Viral titers were estimated by optical density and the standard plaque assay. Using these methods preparations of 2×10^{10} particles/ml were obtained.

2.2. 3D silk fibroin protein scaffold fabrication

Porous 3D silk fibroin scaffolds were prepared by pouring regenerated viscous silk solutions (1–6 wt%) into plastic petri dishes, which were first cooled to 4ºC for half an hour, and then immediately transferred to a freezer at -35ºC overnight to solidify the solvent and induce solid–liquid phase separation. The solidified mixture was maintained at -80ºC for 2 hr and was transferred into a freeze-drying vessel (OHRIST BETA 1-15, Germany) for 48 hr until dry, after which the dried samples were treated with 70% (v/v) ethanol for 12 hr to induce crystallization and sterilization. The resulting silk scaffolds were washed repeatedly with sterile PBS in a biosafety hood to remove any residual alcohol before cell culture.

Scaffolds were divided into four groups according to silk content: 1 wt% silk fibroin scaffolds (1 wt%); 2 wt% silk fibroin scaffolds (2 wt%); 3.5 wt% silk fibroin scaffolds (3.5 wt%); and 5 wt% (5 wt%) silk fibroin scaffolds.

2.3. Porosity measurements

The porosity values of the scaffolds were measured by liquid displacement, similar to a previously described method[16]. Briefly, scaffolds were cut into 5×5×1 mm pieces, and then were placed in a 10 mL cylinder containing a defined volume of ethanol (V1) and then pressed to force air out of the scaffold until all trapped air had been evacuated. The total volume of ethanol and the ethanol impregnated scaffold was recorded as V2. The ethanol impregnated scaffold was removed from the cylinder and the residual ethanol volume was recorded as V3. The porosity of the scaffold was expressed as:

$$\text{Porosity} = (V1 - V3) / (V2 - V3)$$

2.4 Human bone marrow stem cells (BMSC) culture and Ad-BMP7 infection

BMSCs were isolated and cultured as previously described [17]. Human BMSCs (passage 4) were cultured in cell culture flasks in DMEM supplemented with 10% FBS at 37 °C with 5% CO_2. For infection, cells were seeded at 20,000 cells/cm^2 and allowed to adhere overnight. Then cells were infected at specified multiplicity (MOI=100) in serum free medium by the recombinant viruses (Ad-BMP7). After 6 hr the medium was changed to BMSC culture medium mentioned above and cells were kept overnight. The

expression of BMP7 in infected BMSCs was confirmed by the green fluorescent protein (GFP) expression monitored by fluorescent microscope and western blot [12].

2.5. Cell proliferation on silk fibroin 3D scaffolds

Scaffolds were cut into 5×5×1 mm pieces, transferred into 96-well plastic culture and incubated in serum free DMEM overnight, then 50 μL cell suspensions with a total of $1x10^5$ BMSCs were placed onto each scaffold. The cells were allowed to adhere to the scaffolds for 3 hr, and then the cell–scaffold complexes were covered with 150 μL of culture medium (DMEM + 10%FBS). The cells were incubated for 3 and 7 days and then the medium was replaced with 100μL PBS and 20μL of CellTiter 96 Aqueous One Solution Reagent (Promega, Genesearch. QLD, Australia). Then the cell viability was determined by measuring the absorbance at 490nm using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK) by MTS method. The 7 day incubated scaffolds were also fixed in 2.5% glutaraldehyde for confocal microscopy. Scaffolds sections with cells were made at a distance of 100 μm from the scaffold surface and scanned by taking 30 Z sections of 10 μm each. The Z sections were clubbed together to form a composite image for analysis.

2.6 Scanning electron microscopy (SEM) examination

The porous structures of the scaffolds were studied by scanning electron microscopy (SEM, Hitachi X-650). Human BMSCs expressing BMP7 were cultured for 1 and 7 days

on the scaffolds, then fixed on with 2.5% glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.3) for 3 hr at room temperature, rinsed three times with PBS, and dehydrated in a grade ethanol series. Samples were then critically point dried, coated with gold, and observed by SEM.

2.7. Alkaline phosphatase (ALP) activity of BMSCs

Osteogenic differentiation was assessed by measuring a time course of alkaline phosphatase (ALP) activity of the BMSCs grown on the various scaffold types. Scaffolds were cut into 5×5×1 mm pieces and transferred into 96-well plastic culture plates and a total of $1x10^5$ BMP7 expressing BMSCs were placed onto each scaffold. After 1 day the medium was changed to osteogenic differentiation medium containing high glucose DMEM containing 10% FBS, 50 μM ascorbic acid 2-phosphate, 10 mM β-glycerol phosphate and 100 nM dexamethasone (Sigma Aldrich, Castle Hill, NSW, Australia), which was changed every 4 days. On days 7 and 14 samples were removed and ALP activity was measured. The scaffold were irrigated with PBS three times to remove as much residual serum as possible and then 1 mL of 0.02% Triton® X-100 were placed on the scaffold sample to dissolve the cells. The solution was transferred into a 1.5 mL tube, and then sonicated. The samples were centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant transferred to fresh1.5 mL tubes to which 100 μL 1 mol/L Tris-HCl, 20 μL 5 mmol/L MgCl2, and 20 μL 5 mmol/L p-nitrophenyl phosphate was added. After 30 min incubation at 37°C the reaction was stopped by the addition of 50 μL of 1N NaOH. Using p-nitrophenol as a standard, we measured the optical density at 410 nm with a

spectrophotometer. The ALP activity was expressed as the value of p-nitrophenol production quantity divided by the reaction time and the protein synthesis quantity, as measured by the BCA Protein assay kit (Thermo Scientific, Melbourne, Australia). The ALP activity in BMSCs without transfection of BMP7 in undifferentiated culture was used as blank control. ALP activity in BMSCs without transfection of BMP7 seeded on 3.5wt% silk scaffold in differentiated culture was used as BMP negative control and named as BMP(-).

2.8. Reserve transcription and real-time quantitative RT-PCR analysis

The osteoblastic differentiation was further assessed by real-time quantitative RT-PCR (RT-qPCR) measuring the expression of ALP, type I collagen (COL1) and osteocalcin (OCN) in all treatment groups. Scaffolds were cut into 8×8×2 mm pieces and transferred into 24-well plastic culture plates and a total of 10^6 BMP7 expressing MSCs were placed onto each scaffold. The medium were changed after 24 hours to osteogenic differentiation medium which was changed every 4 days. On day 14 the samples were removed and total RNA isolated using Tri-Reagent® (Sigma Aldrich) according to the manufacturer's protocol. Complementary DNA was synthesized from 1 μg of total RNA using SuperScript III (Invitrogen) following the manufacturer's protocol. RT-qPCR was performed in 25 μl reactions containing 12.5 μl SYBR green Master Mix (Applied Biosystems), 2.5 μl (10 μM) of each forward and reverse primer for each gene of interest for a final concentration of 20 pmol, 2.5 μl of cDNA template and RNA free water. Reactions were performed in triplicates to determine the expression of genes using

primers (Table I) for ALP, COL1, and OCN. The house keeping gene, 18S rRNA was used as a control. The reaction was carried out using ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the PCR amplification followed 1 cycle of 10 min at 95°C, 40 cycles of 15 s at 95 and 60°C for 1 min. Melting curve analysis was performed to validate specific amplicon amplification without genomic DNA contamination. Relative expression levels for each gene were normalized by the Ct value of the house keeping gene 18S rRNA and determined by using the $\triangle$Ct method The relative expression of each gene was analysed by one-way ANOVA and Student–Newman–Keuls (SNK) q-test. The significant difference was considered at $P<0.05$. The mRNA expression in BMSCs without transfection of BMP7 in undifferentiated culture was used as blank control. The mRNA expression in BMSCs without transfection of BMP7 seeded on 3.5wt% silk scaffold in differentiation culture was used as BMP negative control and named as BMP(-) group.

2.9. Transplantation into calvarial defects

The bone forming ability of the silk fibroin scaffolds carrying BMP7-expressing MSCs was assessed in a calvarial defect model in severe combined immunodeficient (SCID) mice[18]. 3.5wt% scaffolds were chosen for this purpose due to the improved compression strength compared to the 1%wt scaffolds (results shown in the supplemented data). The scaffolds were cut into 3×3×1 mm pieces, transferred into 24-well plastic culture plates and a total of $1x10^5$ BMSCs expressing BMP7 was placed on each scaffold. After 24 hr incubation the medium were changed to osteogenic induction medium which was changed every 4 days. On days 14 the complexes were

implanted into the induced bone defects of the mice.

The surgeries were carried out according to the guidelines of the Animal Research and Care Committee of the Herston Medical Centre and Queensland University of Technology. The surgical procedures were performed in aseptic conditions under general inhalation anesthesia. Briefly, a linear incision (1 cm long) was made in the left skull to reveal the bone surface. The periosteum was dissected from the bone surface and a full-thickness calvarial bone defect, 3 mm in diameter, was created with a trephine bur in a slow-speed dental drill. To prevent heating injuries to the animals, 0.9% physiological saline was dropped onto the contact point between the bur and bone and care was taken to avoid injury to the dura in all animals. The implant was trimmed to fit the defect and placed precisely into the defects, and soft tissue above the defect was closed with skin staples. Control sites included calvarial defects that did not receive any filling material, sites filled with silk fibroin scaffold alone, and sites filled with fibronin scaffold seeded with BMSCs without transfection of BMP7.

Animals were euthanized 4 weeks after surgery and the defect areas were collected. After the samples were fixed in 4% paraformaldehyde for 12 hr at room temperature and then the tissues were decalcified in 10% EDTA, which was changed twice weekly, for 2 to 3 weeks, and then embedded in paraffin. Serial sections of 5 μm were cut and mounted on polylysine-coated slides. All sections were stained with hematoxylin and eosin for general assessment of the tissue and wound healing. New bone formation was confirmed by immunohistochemical staining using monoclonal anti-human type I collagen (COL1) antibody (Sigma-Aldrich, St. Louis, MO) [18]. Specimens were examined with light microscope.

2.10. Statistical analysis

All experiments were carried out in triplicate, with each treatment also conducted in triplicate. Means and standard deviations (SD) were calculated, and the statistical significance of differences among each group was examined by one-way ANOVA and a Post hoc t-test. The significance was set at $p<0.05$ level.

3. Results

3.1 Scaffold morphology

High porosity silk scaffolds were prepared by solid–liquid phase separation. Fig. 1 shows the SEM examinations of the scaffolds and clearly demonstrated that the pore size decreases with increasing silk concentration. The range of pore size estimated from the SEM measurement is presented in Table 2. The porosity and average pore diameter decreased with the increase of silk fibroin solution concentration,

After 1 day of seeding, SEM images showed that cells had a uniform distribution in the scaffolds. Although cell density was low and the cells appeared to be flat. After one week's culture the cell density increased obviously in all four types of scaffolds compared to that at Day 1. (Fig. 1).

3.2 MTS assay

MTS assay was adopted to evaluate the cytotoxicity of the tissue-engineering materials. The BMSCs attached uniformly to all scaffolds with different pore size after one day of culture and the cell density increased proportionally with time in culture (Fig. 2). The data indicated that the percent of viable cells on 5wt% scaffold were significantly lower than that on other scaffolds in the same culture period ($P<0.05$), suggesting that MSCs proliferation decreased on the 5wt% scaffold compared with the other three types of scaffolds.

Three days after infection with AdEasy-1 gene at a MOI of 100, GFP expression showed around 80% bMSCs were infected. The cell migration in silk scaffolds was investigated by confocal laser microscopy (CLM). It was noted that BMSCs attached and migrated into the pore areas of all types of scaffolds. Inside the scaffold (100 μm from the surface) large amount of cells were detected with more cells in 3.5wt% scaffold and less cells in 5wt% scaffold. GFP expression revealed that the cells grew into the pores of scaffolds. Cells adhered to the pore walls and spread well (Fig.3).

3.3 ALP assay

To determine osteogenic differentiation of the BMSCs on the various scaffolds, endogenous alkaline phosphatase (ALP) were evaluated on days 7 and 14. All groups showed a continuous increase of ALP activity over the *in vitro* culture period. 3.5wt% scaffold, in particular, exhibited significantly higher ALP activity ($P<0.05$) after 7 days of culture compared with 5wt% scaffold. Compared with undifferentiated BMSCs, osteogenic differentiation culture resulted in significant upregulation of ALP activity. The ALP activity was also significantly higher in BMSCs transfected with BMP7 compared with BMSCs without Ad-BMP7 infection cultured in silk scaffold. On day 14, ALP level was slightly lower in 5wt% scaffold compared with the other three groups. There were, however, no significant differences between 1wt%, 2wt% and 3.5wt% scaffolds (Fig. 4).

3.4 Real time quantitative RT-PCR

The osteoblastic differentiation was further assessed by measuring the mRNA expression of alkaline phosphatase (ALP), type I collagen (COL1) and osteocalcin (OCN) by RT-qPCR (Fig. **5**). After 2 weeks osteogenic culture all three osteogenic markers were significantly upregulated in BMSCs expressing BMP7 on all four types of silk scaffolds compared to the controls of either undifferentiated BMSCs or differentiated BMSCs without BMP7 infection on silk scaffolds. Variations in the mRNA expression level of these markers was detected in each individual sample; on 5wt% scaffold, for example, had less mRNA expression of ALP, COL1 and OCN than the cells on other type of scaffolds. However, with the exception of 3.5wt% scaffold compared with 5wt% scaffold, there was no statistically significant difference in the gene expression between groups after 14 days of osteogenic induction, when the samples (n=4) were averaged and normalized against the house keeping gene ($p>0.05$) (Fig. **5**).

3.5 *In vivo* study

There was no histological evidence of an inflammatory reaction in any of the treatment groups four weeks after implantation (Fig. 6). Unfilled calvarial defects were covered with a thin fibrous connective tissue sheet with no evidence of new bone formation. Bone defects filled with silk fibroin scaffold alone showed increased amounts of cells and fibrous tissue within the defects and no detectable new bone formation. In the group of defect filled with silk scaffold with BMSCs without BMP infection, some new bone formation was detected close to the edge of bone defect, but no new bone formation was detected in the central of the defects. New bone formation was found in defects filled

with silk fibroin scaffolds seeded with BMP7 expressing BMSCs. Some bone islands formed inside silk fibroin scaffolds, not only close to the bone defects, but also in the central of the defect. In addition, mineralized bony trabeculae were found and these were surrounded by osteoblasts. The levels of COL1 expression in silk fibroin scaffolds were determined by immunohistochemical methods (Fig. 5). COL1 expression was located to bone matrix and newly formed osteoid, osteoblasts and their matrices, as well as around the silk fibroin fibers (Fig. 5).

Discussion

Pore architecture of scaffolds is known to play a critical role in tissue engineering as it provides the vital framework for the seeded cells to organize into a functioning tissue. This study demonstrated that BMSCs expressing BMP7 gene were able to proliferate and differentiate when seeded and cultured within various pore architectures of silk fibroin scaffolds and also that BMSCs-BMP7/scaffold complexes, implanted into critical-size defects in SCID mouse calvaria, were capable of inducing new bone formation.

In this study we controlled concentration of silk fibroin protein to fabricate different 3D scaffold pore microstructure. Four pore size ranges of silk fibroin scaffolds were made by a freeze-dry technique, with pore sizes ranging from 50 to 300 μm; the pore sizes decreasing as the silk concentration increased. To evaluate what effects the pore architecture of silk fibroin scaffolds had on BMSC attachment and proliferation, cells were seeded on the silk fibroin scaffolds. Localization and viability of the cells were

analyzed by SEM and confocal microscopy. Cells attached to the surface of all four porosity groups and proliferated over 14 days in culture. The cell proliferation and distribution 1wt%, 2wt%, and 3.5wt% scaffolds were equal, whereas proliferation of cells seeded on 5wt% scaffold was reduced. The cell morphology, proliferation and migration on the silk scaffolds indicated all surfaces were cytocompatible.

There is no general consensus as to the optimal pore size for cell growth and tissue formation. An effective pore size range of 200–400 μm has been reported for bone formation [19], while others found no selectivity of osteoblasts for pore sizes in the range of 150–710 μm[20]. Hulbert et al. found that bone regeneration in scaffolds with pore sizes smaller than 350 μm is variable, pore sizes between 100 and 200 μm resulted in bone in-growth, while pore sizes between 10 and 100 μm resulted in fibrous tissue or unmineralized osteoid in-growth[15]. Zeltinger et al. found that vascular smooth muscle cells showed equal cell proliferation and extracellular matrix formation in pores ranging in size from 38 to 150 μm [21], whereas Min et al found that scaffold pore size rangeing from 50 to 200 μm did not significantly affect smooth muscle cells growth[22]. These findings are consistent with our observations for pore sizes ranging from 100 to 300 μm, in which we found that BMSCs proliferation and ECM production were unaffected. The cell proliferation and ECM production decreased however when the pore size was in the 50-100 μm range, which may be the result of less cells initially attaching to these finer pore structures.

The effect of pore architecture of silk fibroin scaffolds on MSC differentiation was

assessed by several assays, including alkaline phosphatase activity, which is an indicator of osteogenic differentiation, bone formation, and matrix mineralization. Previous studies have demonstrated that scaffold pore architecture affects ALP activity [23]. Our results showed that ALP activity increased on all four surface structures compared with control, suggesting that differentiation and maturation of BMSCs to the osteoblastic phenotype had indeed taken place (Fig. 4). There was no significant difference in ALP activity between groups with the exception of 5wt% scaffold, which was lower compared to the other groups.

Real time quantitative PCR of osteoblast differentiation marker genes confirmed such phenotype change of the BMSCs with BMP expression on the scaffolds (Fig. 5). It was clearly seen that the expressions of all three osteogenic genes increased significantly in all silk scaffold groups compared with the controls of either undifferentiated BMSCs or BMScs without BMP7 transfection on the 3.5wt% scaffold. These results suggest that porous silk fibroin scaffolds are capable of providing an environment suitable for osteogenesis of human mesenchymal stem cells expressing BMP7 gene.

In the present study, critical-size defects were created in mouse calvaria. This model of bone repair has been used to study new bone formation in many studies [18,24]. An implant must contain a relatively high number of progenitor or differentiated cells, as well as bioactive factors to specifically attract reparative cells to the injury sites [25]. We identified new bone formation in the defect site and the formation of isolated bone islands in the silk fibroin scaffold, whereas little or no bone formation was found in the negative

controls. These findings are consistent with other studies, which showed that implants containing differentiated MSCs and developed matrices are involved in direct bone formation, and also appear to be osteoconductive during the repair of the critical-size bone defects [26,27].

5. Conclusions

In this study we showed that porous architecture of silk fibroin scaffolds in optimized porosity can support BMP7 expressing BMSCs delivery, facilitate ostegenic differentiation, and induce new bone formation.

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Figure legend：

Fig. 1 shows SEM micrographs of the scaffolds. (A, E, I) 1 wt% silk fibroin scaffolds; (B, F, J) 2 wt% silk fibroin scaffolds; (C, G, K) 3.5 wt% silk fibroin scaffolds; (D, H, L) 5 wt% silk fibroin scaffolds; (A, B, C, D) represents the scaffolds without cell seeding. At day 1 after seeding cells were evenly distributed inside the scaffolds, although cell numbers were low and the cells appeared to have a flat morphology (E, F, G, H). No differences were noted among the four scaffold types; One week after seeding the number of cells had significantly increased in all four scaffold types compared to day 1 after cell seeding (I, J, K, L).

FIG 2. The proliferation of human MSCs on the four scaffolds porosity groups was determined by an MTS assay. The viable cell percentage on 3.5 wt% silk fibroin scaffolds was significantly higher than those on 1 wt% silk fibroin scaffolds. In 5 wt% silk fibroin scaffolds, cells showed the lowest proliferation rate, which was statistically significant compared to other groups. (*$P<0.05$).

FIG 3. The 7 day incubated scaffolds with BMP7 expressing BMSCs. Confocal laser microscopy revealed that GFP-positive cells were adhered and spread in the middle of the scaffolds (Bar: 150 μm). (A) 1wt% silk fibroin scaffolds; (B) 2 wt% silk fibroin scaffolds; (C) 3.5 wt% silk fibroin scaffolds; (D) 5 wt% silk fibroin scaffolds.

FIG 4. All groups showed a continuous increase of *in vitro* ALP activity over the culture period. 3.5 wt% silk fibroin scaffolds had significantly higher ALP activity after one week of osteogenic culture compared with the other scaffolds. On day 14, minimal ALP was present within 5 wt% silk fibroin scaffolds; there was, however, no significant differences

between other groups (*P<0.05). Blank: BMSCs in undifferentiation culture. BMP(-): BMSCs without BMP7 infection in 3.5wt% silk scaffold.

FIG. 5. The osteoblastic phenotype was assessed by measuring the expression of alkaline phosphatase (ALP), type I collagen (COL1) and osteocalcin (OCN) by real-time quantitative PCR in all groups. The mRNA expression of ALP, COL1, and OCN was normalized against 18s. There was a significant upregulation of ALP, COL1, and OCN mRNAs in MSCs cultured in three-dimensional silk fibroin scaffolds in mineralized culture medium. Blank: BMSCs in undifferentiation culture. BMP(-): BMSCs without BMP7 infection in 3.5wt% silk scaffold.

FIG. 6. There was no histological evidence of an inflammatory reaction in any of the treatment groups 4 weeks after implantation in an induced calvarial defect in SCID mice. (A) hematoxylin and eosin image of control, scale bar : 100μm; (B) Sections were stained with monoclonal anti-human type I collagen antibody scale bar: 100μm; (C) Sections were stained with monoclonal anti-human type I collagen antibody scale bar : 20μm; (D) hematoxylin and eosin image of 3.5 wt% silk fibroin scaffolds without BMSCs, scale bar: 100μm; (E) Sections were stained with monoclonal anti-human type I collagen antibody scale bar : 100μm; (F) Sections were stained with monoclonal anti-human type I collagen antibody scale bar : 20μm; Arrowheads show fibrils of silk fibroin scaffolds. (G) hematoxylin and eosin image of 3.5 wt% silk fibroin scaffolds with BMP7 expressing BMSCs, scale bar: 100μm; (H) Sections were stained with monoclonal anti-human type I collagen antibody scale bar : 100μm; (I) Sections were stained with monoclonal

anti-human type I collagen antibody scale bar : 20μm. Arrowheads show fibrils of silk fibroin scaffolds and NB show the new bone. (J) hematoxylin and eosin image of 3.5 wt% silk fibroin scaffolds with BMSCs without BMP7 infection, scale bar: 100μm; (K) Sections were stained with monoclonal anti-human type I collagen antibody scale bar : 100μm; (L) Sections were stained with monoclonal anti-human type I collagen antibody scale bar : 20μm. Arrowheads show fibrils of silk fibroin scaffolds and NB show the new bone.