Fabrication and Characterization of Silk Fibroin Microfiber-Incorporated Bone Marrow Stem Cell Spheroids to Promote Cell–Cell Interaction and Osteogenesis

Wonchan Lee,*† Joo Hee Choi,*† Sumi Lee, Jeong Eun Song, and Gilson Khang*

ABSTRACT: In this study, silk fibroin microfiber (mSF) was applied to assist spheroid assemblies of rBMSCs (rabbit bone marrow stem cells) (S/B). Alkaline hydrolysis was induced with different times and conditions to manufacture the various sizes of mSF. The mSF was incorporated in the rBMSC with different amounts to optimize proper content for spheroid assembly. The formation of the S/B was confirmed under optical microscopy and SEM. Proliferation and viability were characterized by CCK-8 and live/dead staining. Osteogenesis was analyzed with ALP (alkaline phosphatase) activity studies and real-time polymerase chain reaction. The S/B was successfully produced and displayed uniformly distributed cells and mSF with the presence of a gap in the structure. Proliferation and viability of the S/B significantly increased when compared to rBMSC spheroids (B), which is potentially due to the enhanced transportation of oxygen and nutrients to the cells located in the core region. Additionally, ALP activity and osteogenic markers were significantly upregulated in the optimized S/B under osteogenic media conditions. Overall, a hybrid–spheroid system with a simple 3D cell culture platform provides a potential approach for engineering therapeutic stem cells.

1. INTRODUCTION

Mesenchymal stem cells (MSC) have been a promising material in tissue engineering (TE) due to their multiple translocation and self-renewal ability.1–3 Traditionally, culturing of MSCs was maintained and expanded based on tissue culture plates (TCPs), a two-dimensional (2D) environment. However, compared to real tissue, a 2D culture has difficulty producing cell–cell–matrix interaction, and thus it cannot be an optimal condition to mimic the microenvironment of natural tissue.

In TE, a three-dimensional (3D) culture of cells has been noted to overcome these limitations, and various approaches to designing a 3D environment have been performed and developed.4–9 Among them, a spheroid-based cell culture provides an environment in which cells can easily interact with each other and shows improved interaction and differentiation ability compared to the 2D culture. However, spheroids have a limit in long-term culture and for therapeutic application due to inadequate mass transport and development of a hypoxic core within the internal cells of the spheroid.9–12 As an alternative, various biomaterials such as hydroxyapatite microparticles and fragmented fibers have been introduced into culturing of spheroids.13–16 Microparticles have a high specific surface area, which can cause non-uniform dispersion in the spheroid and interfere with cell interaction, but fragmented fibers have uniform dispersion, which can improve the properties of spheroids.17

Based on these examples, silk fibroin microfiber (mSF) is introduced as an artificial ECM (extracellular matrix) into the spheroid to enhance the interaction of cell–cell–matrix. Silk fibroin (SF) is excellent in terms of biocompatibility, biodegradability, and cell adhesion and has been verified as a stem cell-based TE template in various studies.18–20 In addition, it has the advantage of being easy to apply because there are many cases of SF manufacturing methods and applications to other TE technologies.21,22

In this study, mSF was manufactured without using other types of equipment, and mSF-incorporated rabbit bone marrow stem cell (rBMSC) spheroids (S/B) were manufactured using a centrifuge. The construction of spheroids with various types of mSF was first investigated, and the viability and proliferation of the system were confirmed. Characterization was further carried out with the spheroids loaded with the optimized mSF. ECM formation and osteogenesis of the
assemblies were analyzed to confirm the differentiation effect of the system.

2. MATERIALS AND METHODS

2.1. Preparation of mSF. Silkworm cocoons were cut into pieces, and 10 g was added into boiling 0.02 M Na₂CO₃ (Showa Chemical, Japan) for 30 min to remove sericin. After 30 min, the degummed silk fibroin (dSF) was washed with distilled water (DW) and fully dried in an oven at 60 °C overnight. A total of 4.2 g of dSF and 21 g of sodium hydroxide (8.35 M, Sigma-Aldrich, USA) were mixed uniformly, and 60 mL of DW was added for an alkaline hydrolysis reaction. To obtain various lengths of mSFs, the hydrolysis reaction was carried out for 30 min (30-mSF) and 60 min (60-mSF). An intensive hydrolysis reaction was performed by adding boiled DW for 30 s (0.5-mSF). DW was added to quench the reaction, and the microfibers were washed repeatedly with DW and centrifuged under conditions of 3500 rpm, 3 min, and room temperature (RT). The pH of the prepared mSF was adjusted to 7 using 2 N HCl/NaOH. Dried mSFs were obtained through lyophilization and stored in a desiccator. The fabricated mSF was characterized by optical microscopy and Bio-LV scanning electron microscopy (S-3000N, Hitachi, Japan). The length of the mSF was analyzed with ImageJ software. The samples for SEM observation were obtained by gold-sputtering on the surface of the mSF.

2.2. Bone Marrow Stem Cell (rBMSC) Isolation from a Rabbit Model. All the animal experiments were performed following the guidelines and approval of Chonbuk National University Animal Care Committee, Jeonju, Republic of Korea (CBNU 2016-50). rBMSCs were cultured in an alpha modified Eagle’s medium (alpha MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (PS, Gibco, USA) under standard culture conditions (5% CO₂ and 37 °C). rBMSCs were isolated from New Zealand white rabbits (6 week old; female; Hanil, South Korea) following a previously reported study. All the animal experiments were performed following the guidelines and approval of Chonbuk National University Animal Care Committee, Jeonju, Republic of Korea (CBNU 2016-50). rBMSCs were cultured in an alpha modified Eagle’s medium (alpha MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (PS, Gibco, USA) under standard culture conditions (5% CO₂ and 37 °C). rBMSCs were isolated from New Zealand white rabbits (6 week old; female; Hanil, South Korea) following a previously reported study.

2.3. Preparation of mSF-Incorporated rBMSC Spheroids. Ten milligrams of mSF was first sterilized with 70% pure ethanol (EtOH, Samchun Chemical, South Korea) for 30 min under an ultraviolet (UV) lamp and washed three times with PBS to remove the residual EtOH. Ten milliliters of alpha MEM was added and incubated for 30 min. rBMSCs were trypsinized with 0.5% trypsin–EDTA (10×) (Gibco, USA), and 2 × 10⁵ cells were seeded with various amounts of mSF (0, 5, 10, and 20 μL) in PCR tubes (Thermo Scientific, USA), adding more media to make a total of 50 μL volume in each tube. The tubes were spin down for 30 s and were incubated for 24 h in a humidified cell culture incubator under 37 °C and 5% CO₂ conditions (Figure 1). 48-well plates were coated with 100 μL of sterilized agarose gel (1.5% (w/v)), and the fabricated spheroids were transferred into each well. The structured spheroids were observed under an optical microscope (ECLIPSE TE2000-U, Nikon, Japan).

2.4. Histology. Spheroids were first stained with 1% eosin for 10 min to make it easier to distinguish them during sectioning. Excess stain was washed with PBS, and spheroids were collected using a pipette. mSF-incorporated spheroids were placed on a CryoMatrix (Thermo Fisher Scientific, USA) and were frozen in a deep freezer (−70 °C). The specimens were cross-sectioned with a cryomicrotome (Thermo Fisher Scientific, USA) at a thickness of 10 μm and placed on microscope slides. Samples were fixed in 4% formaldehyde for 15 min, and hematoxylin and eosin (H&E) staining was carried out following the standard histological technique. Briefly,
samples were washed with PBS for 10 min and (Fisher Scientific, USA) was treated with hematoxylin for 5 min. The residual reagent was removed, and the sample (Sigma-Aldrich, USA) was treated with eosin for 30 s. The samples were washed with PBS for 10 min, and dehydration was carried out by sequential treatment of 70, 80, 90, and 100% EtOH.

2.5. Cell Viability and Proliferation Analysis. A CCK-8 (cell counting kit-8, Dojindo, Japan) assay was carried out for 1 and 7 days of culture. The spheroids were transferred to a 96-well plate coated with TCP, and fresh media were added. The samples were stimulated with constant agitation for 3 h. Treatment with the CCK-8 reagent was done by following the manufacturer’s protocol. The treated cells were incubated under standard conditions (37 °C and 5% CO2) for 1 h. The viability of the cells was calculated by following eq 1.

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\text{cell viability(\%)} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100 (\%)
\]

2.6. Morphological Analysis. Viability, cytotoxicity, and morphology were analyzed under a super-resolution confocal laser scanning microscope (LSM 880 with Airyscan, Carl Zeiss, Germany) in Z-stack mode and a Bio-LV SEM. A live/dead cell imaging kit (Invitrogen, USA) was applied following the manufacturer’s instruction. The spheroids were cultured for 7 days, transferred to a confocal dish (cover glass bottom dish, SPL, South Korea), and treated with the staining reagent. The spheroids were homogenized in an extraction solution (Sigma-Aldrich, USA) was treated with eosin for 30 min, and the images were observed. Live cells and dead cells were stained with calcine-AM (green) and an ethidium homodimer (red), respectively. Furthermore, for the SEM observation, the spheroids were cultured for 7 days and were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, USA) for 24 h at 4 °C. The cross-sectioned spheroids were gold-sputtered under a vacuum condition.

2.7. ALP Activity Study. An ALP assay kit (Takara Bio, Japan) was used to study the ALP activity of the fabricated spheroids. The spheroids were cultured for 7 days, and the samples were washed with physiological buffer three times. The spheroids were homogenized in an extraction solution following the manufacturer’s protocol. p-Nitrophenyl phosphate (pNPP) was added into each sample and incubated in a humidified incubator under 5% CO2 and 37 °C conditions for 1 h. The reaction was stopped with 0.9 N NaOH, and the reaction at an absorbance of 405 nm was measured with a microplate reader (Emax Molecular Devices, CA, USA).

2.8. Real-Time Polymerase Chain Reaction. The spheroids were cultured for 7 days and washed with PBS three times. Cell lysis was performed by homogenizing the cells in trizol (Takara Bio, Japan) and chloroform (Samchun Chemical, South Korea). The specimens were centrifuged at 12000g at 4 °C for 15 min. The supernatant was collected in a 1.5 mL Eppendorf tube (USA). cDNA was synthesized with TopscriptTM RT DryMIX (dT18 plus) (Enzynomics, South Korea), and a reverse transcription reaction was performed with a polymerase chain reaction (PCR) thermal cycler (Takara Bio, Japan). The real-time polymerase chain reaction (RT-PCR) was performed with an SYBR Green PCR Master Mix (Applied Biosystems, USA) and StepOnePlus real-time PCR system (Applied Biosystems, USA). ALP (alkaline phosphatase), Runx2 (runt-related transcription factor 2), COL1A1 (collagen type I α1 chain), and OCN (osteocalcin), which are osteogenesis-related markers, were used for this study.

3. RESULTS AND DISCUSSION

3.1. Characterization of mSF. mSF was prepared by utilizing the well-documented procedure of alkaline hydrolysis of proteins. This method is cost-effective, and the product can be obtained in a short time. Various lengths of mSF can be acquired from different treatment times or concentrations of alkaline ions (sodium hydroxide). The process occurs by the conversion of amide bonds to a carboxylic acid and an amine or ammonia. The length of the mSF was relatively broad and displayed a stepwise decrease: 324.74 ± 46.25 μm (30-mSF) and 241.02 ± 40.32 μm (60-mSF) (Figure 2A,B). The external heat induced faster hydrolysis with a shorter length of the mSF (138.27 ± 23.6 μm (0.5-mSF)). This is due to the fast cleaving of both less crystalline and crystalline regions in the SF. However, fast alkaline hydrolysis from external heat produced branches on the surfaces of the mSF (Figure 3). Therefore, based on the results of the subsequent experiments where heat-treated mSF did not display satisfying results, heat treatment is not recommended.

3.2. Preparation of mSF-Assisted rBMSC Spheroids. Spheroids assisted with different amounts (0, 5, 10, and 20 μL) of mSF were well fabricated. The size of the spheroids increased as the amount of mSF was higher (Figure 4). The B group displayed areas of 0.061 ± 0.005 mm² after 1 day of culture and 0.047 ± 0.006 mm² after 7 days of culture. The decrement in size may be due to the cell detachment of live and dead cells from the surface of the spheroids. The S, 10, and 20 μL 0.5-S/B groups showed areas of 0.228 ± 0.002, 0.363 ± 0.029, and 0.648 ± 0.126, respectively, after 1 day of culture. The spheroidal structure was well formed in 5 μL S/B, but the morphology collapsed as the content increased, and many cells were detached from the surface. After 7 days of culture, the areas of the S, 10, and 20 μL 0.5-S/B groups were 0.213 ± 0.012, 0.341 ± 0.027, and 0.603 ± 0.022 mm², respectively.
The detached cells were observed around the spheroids, and especially, 20 μL 0.5-S/B exhibited breakage of the spheroidal structure. The 5, 10, and 20 μL 30-S/B and 60-S/B groups had areas of 218 ± 0.014, 0.438 ± 0.069, and 0.457 ± 0.024 mm² and 0.206 ± 0.032, 0.352 ± 0.032, and 0.557 ± 0.03 mm², respectively. The spheroidal form was stable in all groups, but detachment of the cells occurred when 20 μL S/B was incorporated. The area of these groups decreased, or the 1 day spheroids has a similar size to those after 7 days of culture: 5 μL 30-S/B (0.198 ± 0.019 mm²); 10 μL 30-S/B (0.28 ± 0.023 mm²); 20 μL 30-S/B (0.433 ± 0.027 mm²); 5 μL 60-S/B (0.22 ± 0.011 mm²); 10 μL 60-S/B (0.322 ± 0.016 mm²); and 20 μL 60-S/B (0.538 ± 0.032 mm²). In particular, the spheroids treated with 30-mSF showed a significantly reduced area, and detachment of cells was not observed on day 1. After 7 days of culture, all the groups displayed some displacement of cells around the spheroids, which may be apoptosis due to aging or telomere erosion.26−30

3.3. Histological Analysis. The cross-sectioned spheroids were stained with H&E stain (Figure 5). The mSF-loaded spheroids exhibited evenly distributed cells and fibers. The 0.5-mSF-incorporated spheroids displayed breakage of the structure when a higher amount is added, which coincides with the optical microscope observation. Forming fibers by applying heat has the advantage of forming short fibers in a short time, but it is speculated that spheroid formation is disturbed from the deformed surface of the mSF. The staining showed well-fabricated spheroids that are loaded with 30-mSF and 60-mSF. The incorporation of mSF produced void spaces within the structure, which may contribute to transporting oxygen and nutrients to the cells that are in the center of the spheroids. However, the inclusion of 20 μL of mSF in the spheroids exhibited a gap between cells, which may hinder the formation of the spheroids and reduce the interaction between cells, leading to cell death and hindrance of cell growth.

Figure 3. Morphological analysis of mSF characterized by SEM (scale bar: 100 μm).

Figure 4. (A) Characterization of fabricated B and S/B cultured for 1 and 7 days (scale bar: 200 μm). (B) Analysis of area of B and S/B at different time points.
3.4. Viability and Cell Proliferation Assay. The CCK-8 assay was performed after 1 and 7 days of culture (Figure 6). The viability of the mSF-loaded spheroids had higher values compared to that of group B after 1 day of culture. The high viability of cells could be attributed to the restricted aggregation of cells that provides higher exposure to sufficient gases and nutrients. After 7 days of culture, the 30-mSF- and 60-mSF-treated spheroids exhibited an increase in viability, which may be due to the proliferation of the cells. Along with the full images of the spheroids, these results suggest that the inclusion of mSF improves the stability of the spheroid by enhancing the interaction among cells and the matrix and increases the viability and expansion of cells.

3.5. Morphological Analysis. The live/dead staining was carried out with the optimal content and treatment time (10 μL 30-S/B) (Figure 7A). During the incubation period, the B group showed a preference for TCP over the interaction between cells and exhibited detached cells around the spheroid. Also, dead cells were observed around and inside the spheroids. On the other hand, in the case of the spheroids containing mSF, although mSF was stained with an ethidium homodimer (red), dead cells did not appear. Also, cells did not fall on the periphery, which verifies the strong interaction among cells and mSF. SEM observation was also carried out after 7 days of culture (Figure 7B). The B group exhibited a compact arrangement of cells at the surface and inside the spheroids. On the other hand, the mSF-loaded spheroids exhibited interaction of cells and fibers. The secretion of ECM was captured on the surface of the spheroids, which confirms the strong cell–cell and cell–mSF interactions. The inner area of the mSF-loaded spheroids exhibited spaces among cells and fibers, which coincides with the staining result.

3.6. Evaluation of Osteogenic Differentiation. Alkaline phosphatase (ALP) activity was characterized and real-time polymerase chain reaction (RT-PCR) was performed after 7 days of culture to investigate osteogenic differentiation of the spheroids (Figure 8). ALP activity, an important indicator of bone formation differentiation, was significantly higher in spheroids containing mSF. This verifies that more inorganic phosphates forming an organic ECM were produced from mSF-loaded spheroids.31–33 The gene expression of ALP was also higher in the RT-PCR result, which showed to be 2.5-fold higher in the S/B group. This may be due to the strong interaction among cells and the great transportation of nutrients and oxygen in the center of the spheroids. The gene expression of osteogenic markers exhibited upregulation in the S/B group. RUNX2, which regulates osteogenic differentiation34 and proliferation of cells, showed to be 2.32-fold higher in the mSF-loaded spheroids. COL1, which is involved in ECM formation and is important for bone matrix formation and calcification, showed to be 3.67-fold higher in the S/B.35 The OCN, which is a noncollagenous protein secreted by osteoblasts, showed to be 3.48-fold higher in the mSF-loaded group. It is presumed that mSF provides relatively strong stiffness and a topologically stable structure that facilitates osteogenesis.36,37 The overall result confirms that the incorporation of mSF provides a suitable environment for osteogenic differentiation of rBMSCs and suggests that mSF

Figure 5. H&E staining of cross-sectioned B and S/B (scale bar: 200 μm).

Figure 6. Investigation of cell viability of B and S/B by CCK-8 assay.

Figure 7. (A) Live/dead staining and (B) SEM images characterized after 7 days of culture (scale bar: (A) 200, (B) 100, and 30 μm; magnified).
can be a promising material for enhancing the property of the spheroid.

4. CONCLUSIONS

In this study, mSF was integrated with rBMSCs to improve the cellular viability and function of the spheroid. Different sizes of the mSF were fabricated by different times of exposure and heat treatments. The formation of the spheroid was stable when the proper amount and size of the mSF were incorporated. The histology of the cross-sectioned spheroids showed evenly distributed cells and fibers and exhibited void spaces that allowed spheroids to overcome hypoxia and improve the delivery of nutrients. In S/B, an increase in viability, proliferation, and ECM expression was confirmed, and it can be inferred that S/B morphology improved cell–cell and cell–matrix interaction. The osteogenic differentiation was observed in both B and S/B, but S/B exhibited significantly higher levels of ALP activity and osteogenesis gene expression. Taken together, the developed system enhanced the viability, proliferation, and ECM expression was confirmed. The histology of the cross-sectioned spheroids showed evenly distributed cells and fibers and exhibited void spaces that allowed spheroids to overcome hypoxia and improve the delivery of nutrients.

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**Author Contributions**

W.L. and J.H.C. equally contributed the study. W.L. and J.H.C. designed and conceived the study. W.L. and J.H.C. synthesized and characterized mSF with the support of S.L. W.L. and J.H.C. wrote the manuscript. J.E.S. and G.K. contributed reagents and materials for this study.

**Notes**

The authors declare no competing financial interest.

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