Increased stem cells delivered using a silk gel/scaffold complex for enhanced bone regeneration

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The low in vivo survival rate of scaffold-seeded cells is still a challenge in stem cell-based bone regeneration. This study seeks to use a silk hydrogel to deliver more stem cells into a bone defect area and prolong the viability of these cells after implantation. Rat bone marrow stem cells were mingled with silk hydrogels at the concentrations of 1.0 × 10^5/mL, 1.0 × 10^6/mL and 1.0 × 10^7/mL before gelation, added dropwise to a silk scaffold and applied to a rat calvarial defect. A cell tracing experiment was included to observe the preservation of cell viability and function. The results show that the hydrogel with 1.0 × 10^7/mL stem cells exhibited the best osteogenic effect both in vitro and in vivo. The cell-tracing experiment shows that cells in the 1.0 × 10^7 group still survive and actively participate in new bone formation 8 weeks after implantation. The strategy of pre-mingling stem cells with the hydrogel had the effect of delivering more stem cells for bone engineering while preserving the viability and functions of these cells in vivo.

Stem-cell-based bone regeneration was recently considered to be a preferable method to reconstruct bone defects compared to autologous bone grafts or allogeneic bone grafts, which have obvious flaws such as requiring extra surgery or potentially producing a severe immune response1–4. Seeded stem cells initiate local bone formation with biomaterials as carriers5–7. The in vivo osteogenic differentiation potential of seeded bone mesenchymal stem cells (BMSCs) has been indicated by various studies8–10. However, along with the effort to enhance the osteoconductivity of the biomaterials, some researchers have shown that the seeded stem cells exhibit a low survival rate after implantation in vivo11–15. Meanwhile, the uncertainty of the in vivo cell fate and the failure of some clinical trials caused by cell deficiency remain unsolved questions regarding clinical approaches16–18. Strategies to improve this situation rely on exploiting innovative biomaterials that could amplify the initial seeding quantity of stem cells while possessing excellent cytocompatibility for maintaining the seeded cells’ viability.

Silk fibroin is a biodegradable material with strong mechanical properties, superior biocompatibility, a simple fabrication process and tunable processing parameters19–22; above all, it can be easily fabricated into various forms, such as sponges, films, fibers or gels23, 24. Our previous study successfully fabricated this material into a hydrogel and scaffold; both have exhibited excellent biocompatibility because they have successfully improved bone regeneration by delivering stem cells and growth factors to the defect area25, 26. Notably, the silk-fibroin-based hydrogel allows stem cells to be encapsulated before gelation, and, as a highly hydrated material, it can suspend cells homogeneously within the hydrogel network. This distinguished capacity of the silk hydrogel renders it as a possible carrier to deliver a large quantity of cells to the bone regeneration area27–30.

In the present study, a silk hydrogel was used to deliver more bone marrow stem cells (BMSCs) into the porous three-dimensional (3D) scaffolds to enhance bone regeneration, where the mass of stem cells carried by the silk hydrogel could grow inside the specifically shaped scaffold. In vitro, we tested the transportation rates of small...
molecules and large proteins into the gel, which might enhance the survival rate of inner seeded BMSCs. After osteogenic induction in vitro, the silk gel/scaffold complex containing $1.0 \times 10^7$/mL stem cells was implanted into critical-sized calvarial defects in rats to evaluate the bone regeneration effects. The schematic illustration of this strategy is shown in Fig. 1.

Results

Nutrient transportation performance of the silk gel/scaffold complex. This experiment was carried out after the cell-free silk hydrogel was added dropwise to the scaffold (the SEM images and FT-IR spectra of the silk scaffold can be found online as Supplementary Fig. S1 and Supplementary Fig. S2, respectively). The results showed that the alizarin red solution can consistently permeate through the silk gel/scaffold complex. At 1 min and 5 min, the alizarin red solution permeates gradually from every interface to the core of the complex. From gross observation, the alizarin red solution almost went through the complex in 20 min from both the sagittal and coronal angles (Fig. 2a). The silk gel/scaffold complex could also absorb and release large protein molecules, such as bovine serum albumin (BSA), in a short period of time. The OD value of the 1 min group was significantly higher that of the control group ($p < 0.01$), which indicates that the BSA was rapidly absorbed by the complex from the first minute. In addition, there were also significant differences between the 1 and 5 min groups and the 5 and 20 min groups ($p < 0.01$). These results indicate a consistent absorbance of BSA by the silk gel/scaffold complex during the first 20 min. Noticeably, there is no significant difference between the 20 min group and the pure BSA group, where we speculate that the complex could almost thoroughly absorb the BSA in 20 min (Fig. 2b and c).

Cell interactions within the hydrogel. Both microscopy and confocal laser scanning microscopy (CLSM) showed the intensiveness of the cell distribution in the $1.0 \times 10^7$ group compared to the $1.0 \times 10^5$ group or the $1.0 \times 10^6$ group (Fig. 3). More importantly, CLSM indicated that both the $1.0 \times 10^7$ and $1.0 \times 10^6$ groups had almost no cell interactions, that is, cells were independently dispersed within the hydrogel (Fig. 3a). However, for the $1.0 \times 10^7$ group, CLSM showed a high rate of cell–cell interaction, where most cells were connected to other cells and present as a network (Fig. 3b). In the $1.0 \times 10^7$ group, the entire silk gel was well distributed, with cells that could produce local calcium deposition and mineralization even in the very core of the hydrogel.

Cell proliferation. Both the $1.0 \times 10^5$ group and the $1.0 \times 10^6$ group underwent a stable increase in cell quantity from day 1 to day 10. Significant differences were detected between day 4 and day 10 for these two groups ($p < 0.01$). For the $1.0 \times 10^7$ group, cell quantity declined after initial seeding, with significant differences detected between day 1 and day 4 ($p < 0.01$). However, the cell quantity then consistently increased until day 10. Statistically significant differences that indicated this increase were also detected between day 4 and day 7 as well as day 4 and day 10 ($p < 0.01$). Finally, the cell quantities in the $1.0 \times 10^7$ group showed no significant differences between day 1 and day 10 (Fig. 4).

Osteogenic potential in vitro. Both the alkaline phosphatase (ALP) activity assay and calcium deposition assay showed significant differences among the three groups. In the ALP activity assay, the $1.0 \times 10^7$ group appeared to have the highest ALP expression, followed by the $1.0 \times 10^6$ group and the $1.0 \times 10^5$ group (Fig. 5a). The calcium deposition in the $1.0 \times 10^7$ group was $1.6458 \pm 1.1770$ mg/well, which was significantly higher than that in the $1.0 \times 10^5$ group ($0.2575 \pm 0.028$ mg/well) and the $1.0 \times 10^6$ group ($0.0143 \pm 0.002$ mg/well) ($p < 0.01$).
There was also a statistically significant difference between the $1.0 \times 10^5$ group and the $1.0 \times 10^6$ group ($p < 0.01$) (Fig. 5b). The results of the real-time quantitative polymerase chain-reaction (qPCR) assay are presented relative to the value for the $1.0 \times 10^5$ group. There were statistically significant differences in the expression of both ALP
and osteocalcin (OCN) genes between the $1.0 \times 10^5$ group and the $1.0 \times 10^6$ group ($p < 0.05$), and more significant differences between the $1.0 \times 10^6$ group and the $1.0 \times 10^7$ group ($p < 0.01$) (Fig. 5c and d).

**Micro-CT.** Figure 6a shows the reconstructed image of the newly formed bone in the rat calvarial defect area obtained using Micro-CT from both the apical and antapical views. In contrast to the $1.0 \times 10^5$ group and the $1.0 \times 10^6$ group, which exhibited few calcium nodules with a large amount of vacancy in the defect area, the $1.0 \times 10^7$ group showed significantly higher new bone volume ($6.767 \pm 0.481$ mm$^3$) and the highest trabecular numbers ($1.098 \pm 0.197$) ($p < 0.01$) after implantation in vivo for 8 weeks, where the newly formed calcium nodules grew evenly all over the defect area and connected to each other as a network (Fig. 6a, b and c). There were also statistically significant differences between the $1.0 \times 10^5$ group and the $1.0 \times 10^6$ group with respect to both new bone volume ($p < 0.01$) and trabecular number ($p < 0.05$).

**Histological analysis.** Figure 7 shows the histological sections stained with Van Gieson’s picro fuchsin staining. Consistent with the results of Micro-CT, the $1.0 \times 10^7$ group demonstrated the highest amount of new bone area ($44.45 \pm 2.461\%$), notably higher than the $1.0 \times 10^6$ group ($19.133 \pm 1.112\%$) ($p < 0.01$). Mostly occupied
by the remnant silk hydrogel, the $1.0 \times 10^5$ group presented a percentage of new bone area of approximately 6.236 ± 1.172%, which was significantly lower than that for the $1.0 \times 10^6$ group and the $1.0 \times 10^7$ group ($p < 0.01$).

**Cell tracing.** The CM-Dil labeled BMSCs were still surviving after 8 weeks of implantation in both ossification and non-ossification zones. In the ossification zone, the calcein labeling area (green) indicated the new bone

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**Figure 6.** Micro-CT. Micro-CT images of the rat calvarial defect area after 8 weeks of implantation. (a) Representative image of the newly formed bone at the defect area from both apical and antapical views. Green circles indicate the calvarial defect area. (b) Quantitative morphometric analysis of the newly formed trabecular number. (c) Quantitative morphometric analysis of the new bone volume. (*) Represents $p < 0.05$; (**) Represents $p < 0.01$.

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**Figure 7.** VG Staining. VG-stained histological sections. (a) The green dot line shows the calvarial defect area. The yellow frames show the representative area of the new bone formed by cell carrying silk gel. (b) Quantitative morphometric analysis of the new bone area. (**) Represents $p < 0.01$. 
that formed between 6 weeks and 8 weeks after implantation, where the labeled cells were actively participating in this procedure (Fig. 8).

Discussion

Stem-cell-based bone engineering is very promising and opens the possibility of developing cell-carrying materials with optimized abilities to carry as many cells as possible and prolonged lifespans for exerting certain functions in vivo. The conventional cell seeding process relies on the cell to adhere to the scaffold. In addition to the initial seeded cell quantity used in another study, which varied from $1.0 \times 10^5$ to $5.0 \times 10^5$, the seeding efficiency was only 50% to 70% because most of the stem cells adhered only at the scaffold's surfaces and easily fell off. Therefore, the actual seeding quantity in vivo is beyond prediction. We see this utterly inadequate and unstable cell seeding quantity as a huge challenge, combined with the more difficult problem of maintaining the cell quantity for a long period of time after implantation.

Based on the evidence that silk fibroin possesses excellent mechanical properties, fine biocompatibility and a controllable degradation rate, scientists have been incubating various cell lines with silk fibroin in the form of films, fibers or porous scaffolds for bone or soft tissue engineering. Among all of those forms made using silk fibroin, we found hydrogel to be an ideal cell carrier because of its high water content, adequate mechanical strength and easily controlled gelation process. More importantly, the manufacturing process for producing silk hydrogel allows the mingling of a determined quantity of cells before gelation, which could ensure the initial cell seeding number and allow for optimization. In this study, we quantitatively seeded selective densities of rat BMSCs into the silk hydrogels. The maintained cell viability in the three experiment groups (Fig. 4) demonstrated the marked cytocompatibility of the silk hydrogel.

We compared the osteogenic potential of these cell containing silk hydrogels and found that silk gels encapsulated with higher cell quantities tended to have better osteogenic potential both in vitro and in vivo. The $1.0 \times 10^7$ group exhibited the highest ALP activity, the most calcium deposition and the strongest osteogenic–related gene expression in vitro. Consistent with the in vitro experiment, the $1.0 \times 10^7$ group showed a clear increase in bone formation in vivo (Figs 6 and 7). Micro-CT showed that the $1.0 \times 10^7$ group had more and faster calcium deposition from the seeded BMSCs, which led to a prominent performance with respect to both new bone volume and trabecular number in the defect area compared to the other two groups (Fig. 6). The histological analysis also confirmed this result, as Van Gieson's staining for the $1.0 \times 10^7$ group showed the highest ratio of new bone area (Fig. 7). To further confirm that the rapid bone regeneration was produced by the encapsulated BMSCs, we conducted a cell tracing experiment that showed that the encapsulated stem cells maintained their viability and actively participated in the local bone formation process after implantation (Fig. 8).

This comparison of the three experimental groups verifies that the excellent bone formation was primarily a result of the larger quantity of encapsulated stem cells. Generally, the $1.0 \times 10^7$ group exhibited more mineralization locations in the defect area, providing more cores for calcium nodule formation. More importantly, the long-term in vivo preservation of the viability and function of the encapsulated stem cells in the $1.0 \times 10^7$ group guaranteed a continuous bone formation process. It is interesting that the expression of osteogenesis-related genes (ALP and OCN) in the $1.0 \times 10^7$ group was detected to be the strongest among the three groups (Fig. 5), which implies the better osteogenic differentiation potential of single cells when they are in a higher degree of encapsulation. We speculated that the result may be attributed to the enhanced cell–cell interactions in the $1.0 \times 10^7$ group (Fig. 3), where this optimization could increase the formation of gap junctions that directly transfer signaling molecules and metabolites between adjacent cells. In addition, the increased cell quantity may also lead to more cytokines and proteins being secreted into the microenvironment that stimulate cell behavior.

Conventional cell scaffolds rely on the seeded cells to grow into the center of the defect area, in which the long-term repair process and the insufficient oxygen and nutrients inside the scaffolds make it a challenge to preserve the survival of implanted cells. However, the strategy of mingling stem cells with a silk hydrogel before gelation ensures that the cells are homogenously suspended in the material, which leads to mineralization of the
entire defect area and reduction of the repair time. In addition, the silk hydrogel has a permeability that allows the transfer of both small molecules and proteins (Fig. 2) that could nourish the seeded cells to preserve their viability after implantation. This study emphasized the crucial role of stem cells in rapid bone regeneration while confirming the cell carrying properties and in vivo long-term cell viability supporting properties of the silk gel/scaffold complex.

The fast and efficient bone-forming property achieved by the stem-cell-carrying silk hydrogel could be applied to multiple shapes of small defects by simply changing the nature or material of the scaffolds. We have now applied this strategy only to small defect areas to confirm the encapsulated cell functions with respect to rapid bone regeneration; verification of its applicability to large defect repair is still needed. Notably, achievements in \textit{in vitro} osteogenic induction of hydrogel-enwrapped stem cells also hint at the possibility of pre-inducing the encapsulated stem cells into osteogenic progenitor cells to attain calcium deposition and local mineralization before implantation, which might further shorten the time needed for bone regeneration and reduce the risk of \textit{in vivo} cell necrosis.

**Methods**

**Animals.** The animals used in this study were all obtained from the Ninth People's Hospital Animal Center (Shanghai, China) for both the calvarial defect repair experiment and BMSC isolation and culture. All animal experiments were conducted in accordance with the regional Ethics Committee guidelines, with the protocols approved by the Animal Care and Experiment Committee of Ninth People's Hospital.

**Rat BMSC isolation and culture.** BMSCs were obtained and cultured from 4-week-old male F344 rats, as we previously published\cite{26, 49}. Briefly, after euthanizing the rats with an overdose of pentobarbital injected intraperitoneally, the femurs were separated with the epiphysis being cut off. The marrow was then quickly rinsed out using Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco, USA). The isolated BMSCs were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum. Cells were incubated at 37 °C in an environment containing 5% CO$_2$. Non-adherent cells were removed by changing the medium after 24 h. When the confluence reached 80–90%, the BMSCs were subcultured at a density of 1.0 × 10$^6$ cells/mL with trypsin-ethylenediamine tetra-acetic acid (EDTA, 0.25% w/v trypsin, 0.02% EDTA). Cells at passage 2–3 were collected and resuspended in DMEM for subsequent cell encapsulation.

**Preparation of the materials.** Purified silk fibroin stock solutions were prepared at 8.0 wt% with deionized water diluted to approximately 4.0 wt% and used in the subsequent studies, as previously described\cite{32, 36, 30, 51}. The sterilized silk fibroin solution sterile DMEM powder was blended and sonicated to initiate gelation; approximately 10 min was required for the solution to fully transform into a hydrogel. Before it turned into a gel, a certain volume of cell suspension was added into the silk solution to reach three different final concentrations of 1.0 × 10$^5$ cells/mL, 1.0 × 10$^6$ cells/mL and 1.0 × 10$^7$ cells/mL. To observe the cell conditions inside the silk gel and evaluate their proliferation and osteogenic differentiation abilities \textit{in vitro}, 20 μL of the mixed solutions was added dropwise to 96 well-plates and incubated at 37 °C for 10 min for gelation before conducting \textit{in vitro} experiments. In addition, 20 μL of the silk gel was added dropwise to a porous silk scaffold (pore sizes 350–420 μm, 5 mm in diameter and 2 mm in thickness)\cite{20} to evaluate the transfusion condition of this silk gel/scaffold complex. For the \textit{in vivo} rat calvarial repair experiment, different densities of cell-containing silk gels were added dropwise to the silk scaffold for gelation, and then they were incubated in osteogenesis-induced medium for 7 days before \textit{in vivo} implantation.

**Nutrient transportation performance of the silk gel/scaffold complex.** After the silk gels were fully gelled in the silk scaffolds, the gel/scaffold complexes were immersed in alizarin red solution and removed at selected time points (1 min, 5 min and 20 min) to observe the transfusion condition of the alizarin red solution from both the coronal and sagittal angles. To further evaluate the ability to transport large protein molecules, we placed the gel/scaffold complex in a 24 well plate containing 1 mL of distilled water and 20 μL of BSA (0.5 mg/mL) in each well, making the final BSA concentration of the immersed solution to be 10 μg/mL (the control group well contained only 20 μL of distilled water). After immersing the complex in the plate for different time periods (1 min, 5 min and 20 min), the gel/scaffold complex was removed and washed with PBS 3 times to remove the redundant BSA. Each gel/scaffold complex was then placed into 1 mL of distilled water at 4 °C overnight to release the encapsulated BSA (20 μL, 10 μg/mL BSA solution was added to 1 mL of distilled water to serve as positive control in this study). A mixture of bicinechonic acid and copper sulfate solution was added into each well as the BCA working solution (Beyotime, Shanghai, China). The plate was then incubated at 37 °C for 30 min. The quantitative measurement of released BSA was present as the optical density (OD) value of the solutions at a length of 630 nm by an ELX ultra microplate reader (BioTek, Winooski, VT). Both transfusion experiments included only cell-free silk gel/scaffold complexes.

**Analysis of BMSCs within the silk hydrogel \textit{in vitro}**

**Cell interactions.** To observe the cell interactions within the silk hydrogel, we added dropwise 20 μL of silk solutions from three groups (1.0 × 10$^5$ cells/mL, 1.0 × 10$^6$ cells/mL, 1.0 × 10$^7$ cells/mL) into a 96-well plate. The plate was incubated at 37 °C for 10 min for full gelation. Then, 200 μL of DMEM was added into each well and incubated at 37 °C for 24 h before observation with a microscope. For a more comprehensive observation, the encapsulated cells were stained with FITC-phalloidin and DAPI (Invitrogen) and further observed under confocal laser scanning microscopy (CLSM, Leica, Germany).

**Cell proliferation.** Cell proliferation activity was evaluated by the MTT cell metabolic assay (Sigma, St. Louis, USA). Silk hydrogels containing different densities of BMSCs were incubated in 96-well plates, as described...
The specimens were collected and fixed in 10% buffered formaldehyde solution. The specimens were imaged with CellTracker™ CM-Dil (Invitrogen, Carlsbad, CA, USA) and then encapsulated into the silk gel/scaffold for 4 h to form formazan. The formazan was then dissolved with dimethyl sulfoxide, and the optical density (OD) was measured at 490 nm.

**Osteogenic potential in vitro.** To detect the osteogenic differentiation potential of each study group, we performed an ALP activity assay, a calcium quantification experiment and a qPCR assay. All experiments were performed in triplicate.

For the ALP activity assay, different groups of silk hydrogels were incubated in osteogenic induced medium for 3 and 7 days. After being fixed in paraformaldehyde for 30 min, the hydrogel was stained with an ALP kit (Beyotime, Shanghai, China) to evaluate the osteogenic potential of the encapsulated cells. Hydrogels with no cells were set as the control in this study.

For the calcium deposition assay, hydrogels were first fixed in neutral formalin at day 21 and then treated with 0.6 N HCl and gently shaken to decalcify for 24 h. The cell lysates were collected and transferred into a 96-well plate, and the cell lysates were incubated with a chromogenic reagent and calcium assay buffer from the calcium assay kit (Sigma, St. Louis, USA) for 10 min away from light. The optical density of the mixed solutions was then measured at 575 nm. A standard curve was set up to define the calcium concentration of each experiment group.

For the real-time quantitative polymerase chain-reaction (qPCR) assay, the total RNA of the cells in silk gel was extracted with Trizol reagent (TaKaRa, Shiga, Japan) after 7 days of incubation and reverse transcribed into cDNA with a PrimeScript 1st strand cDNA synthesis kit (TaKaRa, Shiga, Japan). The qPCR results were measured using a real-time qPCR system (Bio-Rad, Hercules, CA) to evaluate the expression of ALP and OCN genes, with the housekeeping gene GAPDH set for normalization. The final result was calculated using the comparative delta Ct method. The primers used in this study were commercially synthesized (Sangon Biotech, Shanghai, China), and the sequences are listed in Table 1.

**Surgical procedure for the rat calvarial defect model.** A rat calvarial defect model was established, as previously described25. Briefly, eighteen F344 rats were anesthetized with pentobarbital through an intraperitoneal injection (3.5 mg/100 g). A 5 mm diameter full-thickness calvarial defect was then created on both sides of the rat’s skull. Different groups of silk gel/scaffold complexes were pre-incubated in osteogenic medium for 7 days. After being fixed in paraformaldehyde for 30 min, the hydrogel was stained with an ALP kit (Beyotime, Shanghai, China) to evaluate the osteogenic potential of the encapsulated cells. Hydrogels with no cells were set as the control in this study.

**Micro-CT.** After 8 weeks of implantation, the rats were sacrificed by injecting an overdose of pentobarbital. The specimens were collected and fixed in 10% buffered formaldehyde solution. The specimens were imaged with a desktop Micro-CT system (μCT-80, Scanco Medical, Switzerland) and scanned in high-resolution mode (pixel matrix, 1024 × 1024; voxel size, 20 μm; slice thickness, 20 μm). We used an image analysis software package (Scanco Medical, Switzerland) to reconstruct the 3D images and detect new bone formation. The new bone volume (BV) and trabecular number (Tb.N) were then analyzed, as previously described25.

**Histomorphometric observation.** After Micro-CT analysis, the specimens were dehydrated in gradient from 75% to absolute ethanol and embedded in polymethylmethacrylate (PMMA). The specimens were cut into 150 μm thick sections with a Leica SP1600 saw microtome (Leica, Germany) and further polished to a final thickness of 40 μm. The sections were stained with Van Gieson’s picro fuchsin and observed under a confocal laser scanning microscope (CLSM, Leica, Germany). The new bone area was calculated using the Image-Pro Plus™ software program.

**Fluorescence cell tracing experiment.** Another 3 rats were included to determine whether the encapsulated osteogenic cells within the silk hydrogel participated in the bone formation process. The cells were labeled with CellTracker™ CM-Dil (Invitrogen, Carlsbad, CA, USA) and then encapsulated into the silk gel/scaffold complex at a density of 1.0 × 10⁶ cells/mL. After incubation in osteogenic medium for 7 days, the complexes were implanted into rat calvarial bone defects. Six weeks after implantation, 20 mg/kg Calcein (Sigma, St. Louis, USA) was intraperitoneally injected into rats to detect new bone formation. At 8 weeks, all rats were euthanized with overdose pentobarbital, and the specimens were harvested. After dehydration, the specimens were embedded in PMMA and then cut and polished into 40 μm thick sections. The sections were further stained with DAPI and observed using a fluorescence stereomicroscope (Leica, Wetzlar, Germany).

| Gene | Prime sequence | Product size (bp) | Accession number |
|------|----------------|------------------|-----------------|
| GAPDH | F:GGCAAGTTCAACGCCACAGT  R:GGCCAGTAGACTCCAGCACAT | 76 | NM_017008.3 |
| OCN | F:GGCCCTGACTGCTTCCGGCCTCT  R:TCCACCCCTGACTCCGCTTG | 103 | NM_013414.1 |
| ALP | F:GTCCCCACAAAGACGCCCACAAT  R:CAACGCCAGAGCCAGGAAAT | 172 | NM_013059.1 |

Table 1. Primers for real-time and reverse transcriptase polymerase chain reaction. OCN, osteocalcin; ALP, alkaline phosphatase; F, Forward; R, Reverse.
Statistical analysis. The data are all presented as the mean ± standard deviation. ANOVA and SNK post hoc based on the normal distribution and equal variance assumption test were used to test for statistically significant differences (p < 0.05; p < 0.01) between the different groups in all studies. Statistical analyses were calculated with the SAS 8.2 statistical software package (Cary, USA).

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Author Contributions
Xun Ding wrote the main manuscript text. Guangzheng Yang and Wenjie Zhang were responsible for the in vitro experiments. Guanglong Li and Shuxian Lin contributed to the animal experiments and results. Prof David. L. Kaplan offered instructions about the silk protein. Prof Xinquan Jiang conceived and guided most of the experiments and article writing.

Additional Information
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