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Silk Fibroin Microparticles as Carriers for Delivery of Human Recombinant Bone Morphogenetic Protein-2: *In Vitro* and *In Vivo* Bioactivity

Paulo César Bessa, Ph.D.,¹⁻⁴ Elizabeth R. Balmayor, Ph.D.,^{1,2} Joachim Hartinger,⁴ Gerald Zanoni, M.Sc.,⁴ Daniela Dopler, B.Sc.,⁴ Alexandra Meinel, Ph.D.,^{4,5} Asmita Banerjee, M.Sc.,⁴ Margarida Casal, Ph.D.,³ Heinz Redl, Ph.D.,⁴ Rui L. Reis, Ph.D.,^{1,2} and Martijn van Griensven, Ph.D.⁴

The *in vitro* and *in vivo* efficiency of fibroin microparticles as a delivery carrier for bone morphogenetic protein-2 (BMP-2) was evaluated. BMP-2 was encapsulated in silk fibroin particles that were produced by a simple and very mild processing method. The dose–response of BMP-2-loaded fibroin particles was examined in C2C12 cells, after 5 days of culture. The BMP-2 retained most of its activity as observed by the increase in alkaline phosphatase activity, which was much higher when BMP-2 was encapsulated into the particles rather than just surface-adsorbed. After 2 weeks of culture, increased mineralization was observed with BMP-2-loaded particles in comparison to soluble added growth factor. No significant cytotoxicity was detected. When implanted in a rat ectopic model, bone formation was observed by *in vivo* micro-computed tomography after 2 and 4 weeks post-implantation, with particles loaded with 5 or 12.5 μg BMP-2. An increase in bone density was observed over time. Histology revealed further evidence of ectopic bone formation, observed by strong alizarin red staining and osteocalcin immunostaining. Our findings show that fibroin microparticles may present an interesting option for future clinical applications in the bone tissue engineering field, and therefore, further studies have been planned.

Introduction

BONE MORPHOGENETIC PROTEINS (BMPs) are a group of cytokines from the transforming growth factor-beta (TGF- β) superfamily, which have been used as powerful osteoinductive components in several late-stage tissue engineering products for bone grafting.¹⁻³ Currently, two devices using BMPs have been approved by the Food and Drug Administration for human clinical application based on the use of collagen sponges.⁴ However, as collagen poses several drawbacks such as suboptimal handling conditions and the risk of disease transmission, there is still a need for an optimized BMP delivery system.

The search for efficient, simple, and cheap delivery systems for drug targeting has led to a great investment in the area of nano- and microparticles for drug delivery. In tissue engineering, these systems are excellent choices for growth factor delivery, because they can be easily prepared

and sterilized. They can be processed into injectable systems allowing an easy and noninvasive implantation in the patient.⁵

Diverse synthetic and natural origin polymers have been suggested as alternatives for the delivery of BMPs in bone tissue engineering.^{3,6} Silk fibroin offers a versatile option as a natural biocompatible and biodegradable protein polymer that has been shown to efficiently deliver BMPs in a variety of works.⁷⁻¹⁰ Recently, a simple method for producing fibroin microparticles was described,¹¹⁻¹³ and these microparticles were explored as a potential new carrier for the encapsulation and sustained release of different BMPs.¹⁴ In this study, we examined the activity of BMP-2 released from fibroin microparticles *in vitro* in C2C12 cells, by quantification of the alkaline phosphatase (ALP) activity and calcium mineralization, and in a rat ectopic bone formation model, using microcomputed tomography (μCT) and histological analysis.

¹B's Research Group—Biomaterials, Biodegradables, and Biomimetics, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, University of Minho, Guimarães, Portugal.

²PT Associated Laboratory, IBB—Institute for Biotechnology and Bioengineering, Guimarães, Portugal.

³CBMA—Centre of Molecular and Environment Biology, University of Minho, Braga, Portugal.

⁴Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center, Austrian Cluster for Tissue Regeneration, Vienna, Austria.

⁵Bernhard Gottlieb University School of Dentistry, Vienna, Austria.

Materials and Methods

Materials

Silk-containing *Bombyx mori* cocoons were purchased from Halcyon Yarn (Bath, ME). The silk fibroin was isolated from the cocoons, following an adapted degumming protocol.¹² Fibroin microparticles were prepared using the method of Cao and colleagues,¹³ with modifications. Briefly, a solution of 1% (w/v) fibroin in phosphate-buffered saline was prepared, to which 0.5 μg BMP-2 (Wyeth, Maidenhead, United Kingdom) was loaded, per mg of fibroin. This solution was incubated at room temperature with stirring (600 rpm), while absolute ethanol was added dropwise at a ratio of 1:2 to the initial volume of fibroin. The solution was incubated overnight at -20°C , washed with distilled water, collected by centrifugation, and freeze-dried. The characterization of the fibroin microparticles by scanning electron microscopy, dynamic light scattering, and swelling behavior and the release profile of BMP-2 have been described elsewhere.¹⁴ The resulting particles had an average diameter of $2.7 \pm 0.3 \mu\text{m}$. All other chemicals were of analytical grade.

Methods

Bioactivity in C2C12 cells. C2C12 cells have been used for screening the osteogenic activity of BMPs in a variety of works.^{15–18} These cells do not express significant amounts of endogenous BMPs, thus making them an effective model for testing the activity of the released BMP-2.¹⁹ C2C12 cells were seeded at 10^5 cells/mL per well in a 24-well plate, attached in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) fetal calf serum, at 37°C and 5% CO_2 , in a humidified environment. The cells were incubated with unloaded particles or with particles containing 0.1, 0.5, 1.0, or 2.5 μg encapsulated BMP-2 (Wyeth). BMP-2 was added as a positive control to the culture medium at 0.1, 0.5, 1.0, or 2.5 $\mu\text{g}/\text{mL}$. As another control, unloaded particles that were immersed for 30 min in 5 $\mu\text{g}/\text{mL}$ BMP-2 and washed twice were also added to the cells (adsorbed BMP control); these particles were added in the same volumes as the particles with encapsulated growth factor were added to the cultures. ALP enzymatic activity was measured according to standard procedures,²⁰ after 5 days in C2C12 cultures. The activity recovery of the encapsulated BMP-2 was estimated by comparing the ALP activity induced by a specific amount of growth factor released from the fibroin particles¹⁴ to the activity induced by the same amount of growth factor added to the culture medium as a positive control.

Alizarin red mineralization. For the alizarin red mineralization assay, C2C12 cells were seeded at 3×10^4 cells/mL per well and cultured in DMEM supplemented with 100 μM ascorbic acid and 10 mM β -glycerophosphate, in the presence of both unloaded and BMP-2-loaded (0.5 μg) microparticles. BMP-2 was also added as a control at 0.5 $\mu\text{g}/\text{mL}$ to the culture media. Media and particles were replaced every 5 days. After 14 days of culture, the cells were washed for three times with phosphate-buffered saline (without Ca^{2+} , Mg^{2+}) and fixed in 4% (v/v) formaldehyde for 30 min. After removal of the fixative, cells were washed twice with distilled water and covered with alizarin red 1% solution, followed by gentle agitation in an orbital shaker for 10 min. The solution

was then removed and the cells were washed three more times with distilled water. The cells were observed using a Axiovert10 (Zeiss, Jena, Germany) optical microscope and imaged with a coupled Coolpix950 (Nikon, Tokyo, Japan) camera. The mineralization was quantified by the method proposed by Gregory *et al.*²¹ Briefly, the calcium from cells was extracted by adding 800 μL acetic acid (10%, v/v) to each well and incubating for 30 min in an orbital shaker. Then the suspensions were transferred to a clean 1.5 mL Eppendorf tube and vortexed for 30 s; 500 μL of mineral oil was added and the suspensions were heated to 85°C for 10 min and then cooled in ice for 5 min. The suspensions were centrifuged (20 min, 10,000 g), the supernatant carefully removed, and 200 μL ammonium hydroxide (10%) was added (until pH ~ 4.1). The solutions were read for absorbance at 405 nm in a Spectra III spectrophotometer (SLT, Salzburg, Austria).

Cell viability assay. Human osteosarcoma cell line (SaOS-2 cell line; European collection of cell cultures (ECACC) was used for cell viability tests instead of murine C2C12 cell line, because it is a well-established human cell line of an osteoblast phenotype. Human osteosarcoma cells were seeded at 10^6 cells/mL in a 24-well plate, attached overnight in DMEM with 1% fetal calf serum and no antibiotics, at 37°C and 5% CO_2 , in a humidified environment, and cultured for 1, 3, and 5 days. Unloaded fibroin particles were added to the cell culture at 0.5 mg/mL. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) cell viability assay was determined after these time periods, using a standard procedure.²⁰ Latex rubber (Velos-Perforex, Manchester, United Kingdom) and standard culture medium were used as positive and negative controls, respectively. Latex rubber is known to have a strong cytotoxic effect leading to extensive cell death and lysis and is commonly used as a positive control for cell death. All the samples were tested in triplicate for at least two independent experiments with reproducible results. The results are expressed as a percentage of the control (scored as 100% viability) as mean \pm standard deviation.

Rat ectopic bone formation model. Male Sprague-Dawley rats (weighing 340–360 g) were purchased from the Institut für Labortiergenetik und -genetik (Himberg, Austria). The rats were housed in light- and temperature-controlled facilities and given food and water *ad libitum*. In three animals, surgery was performed after general anesthesia with an intramuscular injection of 60 mg/kg of body weight (BW) ketamine (Ketavet[®]; Pharmacia, Erlangen, Germany) and 7.5 mg/kg BW xylazine (Rompun[®]; Bayer, Leverkusen, Germany). After shaving and disinfecting the dorsum of the animals, four midsagittal incisions were made dorsally in the proximal area of each limb and subcutaneous pockets were created by blunt dissection. The fibroin particles were implanted subcutaneously into each pocket. Three groups were defined as follows: Group I (unloaded particles), Group II (particles + 5 μg BMP-2), and Group III (particles + 12.5 μg BMP-2). The animals were randomized and were implanted with four samples ($n = 4$) per each group. Only one group was assigned to each animal to avoid the potential interference of released BMP by blood circulation. After placement of the particles, the incisions were closed with sutures, and the wound was covered with vapor-

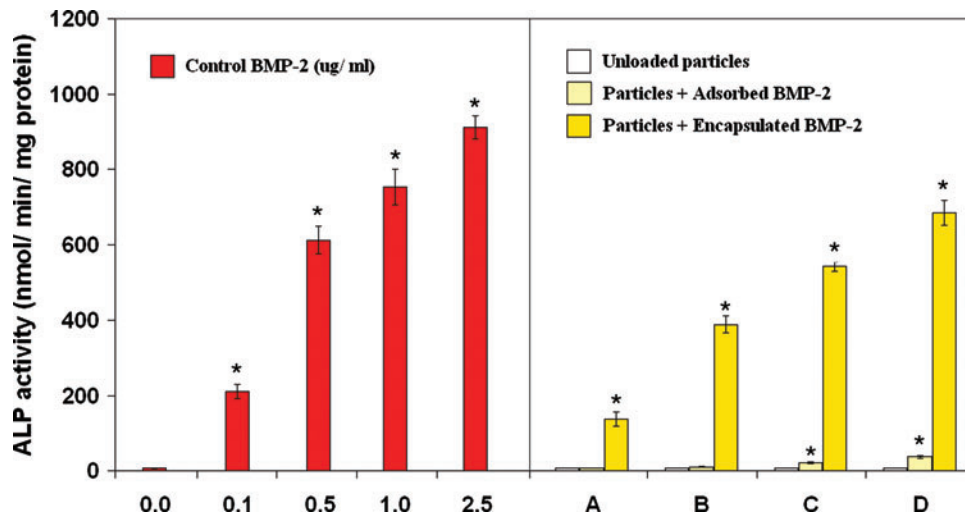


FIG. 1. Alkaline phosphatase (ALP) activity of C2C12 cells after 5 days of culture. A–D: Particles were added in similar amounts to the cell cultures containing encapsulated BMP-2 (0.1, 0.5, 1.0, or 2.5 μg , respectively), adsorbed BMP-2, or no growth factor (unloaded particles). Controls were obtained from cells incubated only with 0.0, 0.1, 0.5, 1.0, or 2.5 $\mu\text{g}/\text{mL}$ BMP-2. ALP activity is reported as nmol/min/mg of total protein (mean \pm standard deviation, $n=3$). Statistical differences indicated

(* $p < 0.01$) are relative to the negative control without growth factor or particles. BMP-2, bone morphogenetic protein-2. Color images available online at www.liebertonline.com/ten.

permeable spray dressing (Opsite®; Smith & Nephew, London, United Kingdom). Analgesia treatment was given postoperation, with 0.01 mg/kg BW Buprenorphin. At the end of each implantation period (4 weeks), the animals were sacrificed and samples from the tissues surrounding the implantation site were harvested. The animal protocol was approved by the authority of the city government of Vienna.

Microcomputed tomography. Live μCT (vivaCT 75; Scanco Medical AG, Brüttisellen, Switzerland) was used to examine ectopic bone formation in individual rats. μCT imaging was performed at weeks 2 and 4. The animals were anesthetized for 5 min with 2% isoflurane during measurement, and the dorsal regions in proximity to the limbs were examined. No X-ray contrast media were used in this study. Image reconstruction was performed using the built-in three-dimensional visualization software (Scanco Medical AG). The evaluation of newly formed bone and bone density was performed using IPL version v5.06b (Scanco Medical AG).

Histology. Harvested samples were fixed in neutral buffered 10% formalin and embedded in paraffin. Three-micrometer or 4-mm sections were cut and stained with

hematoxylin and eosin (H&E) or alizarin red S, respectively, for light microscopy observation. For a detailed analysis of tissue differentiation, immunohistochemical staining was performed in selected samples. Briefly, paraffin sections were dewaxed with xylene and rehydrated in a graded ethanol series. After enzymatic antigen retrieval with 0.1% proteinase (Sigma Aldrich, St. Louis, MO), sections were incubated with an osteocalcin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber at 4°C overnight. After rinsing, sections were incubated with the EnVision™ system (Dako, Glostrup, Denmark) at room temperature for 30 min and immunoreactivity was visualized with 3-amino-9-ethylcarbazole (AEC) chromogen solution (Thermo Fisher Scientific, Hudson, NH). Slides known to express osteocalcin were used as positive controls, and those without the primary antibody served as negative controls.

Statistical analysis. Experiments were performed in triplicate and expressed as means \pm standard deviation, unless stated otherwise. Student's *t*-test was used for statistical analysis using a two-tailed nonpaired test. Statistical significance was defined as $p < 0.05$ for a 95% confidence, and $p < 0.01$ for a 99% confidence.

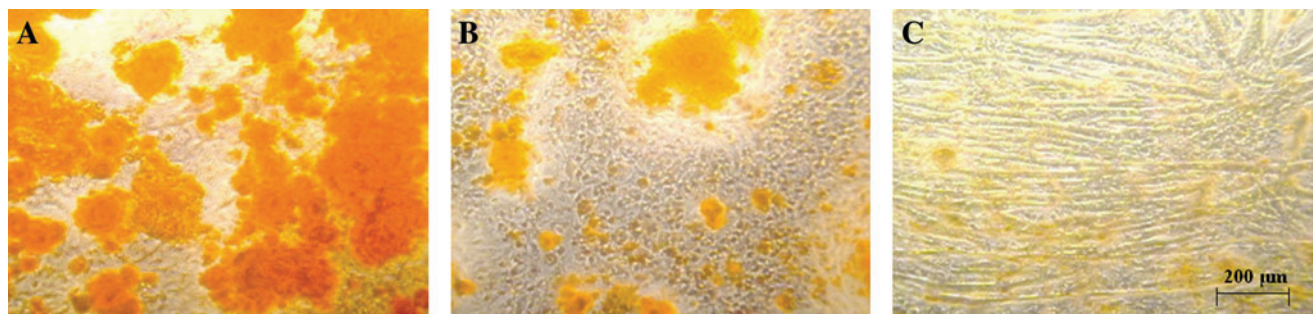


FIG. 2. Alizarin red mineralization staining showing sites of calcium phosphate deposits (orange color) in C2C12 cell line differentiated into osteoblast after 14 days of culture with particles loaded with 0.5 μg BMP-2 (A), and 0.5 $\mu\text{g}/\text{mL}$ BMP-2 added to culture medium (B). Unloaded fibroin particles did not show any mineralization (C). Color images available online at www.liebertonline.com/ten.

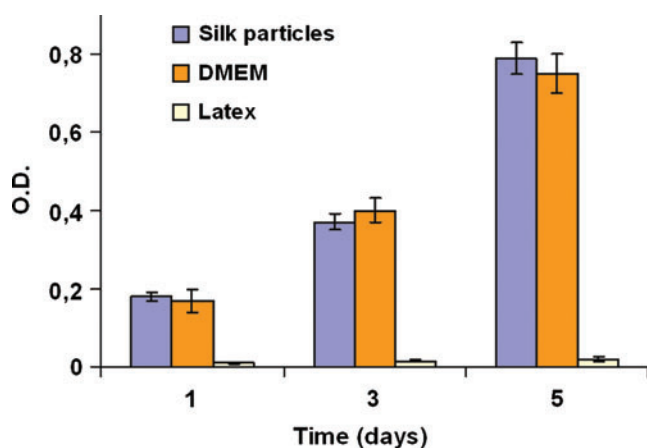


FIG. 3. Cell viability tetrazolium salt 3-(4,5)-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) test performed in SaOS-2 cells as a function of the time of cell culture with 0.5 mg/mL silk fibroin microparticles. Dulbecco's modified Eagle's medium (DMEM) and latex were used as a negative and a positive control for cell death, respectively (mean \pm standard deviation, $n=3$). Color images available online at www.liebertonline.com/ten.

Results

ALP and osteogenic mineralization in C2C12 cells

The bioactivity of BMP-2 released from the silk fibroin microparticles was studied by determining the ability to induce ALP enzymatic activity over basal levels in C2C12 cells (negative controls) after 5 days of culture, and by comparing the cell response to a BMP-2 dose-response effect directly added to the culture medium (positive control). The BMP-2 released from the fibroin particles was able to induce a significant increase in the ALP activity over basal levels after 5 days ($p < 0.01$; Fig. 1). The remaining activity of encapsulated and released BMP-2 was estimated as $88.9\% \pm 6.1\%$. At this period, $76.9\% \pm 6.4\%$ of loaded BMP-2 were released from the particles, as calculated elsewhere.¹⁴ Cells were observed to differentiate into osteoblast morphology (data not shown). The fibroin particles with only adsorbed BMP-2 were able to induce solely a small increase, but significant ($p < 0.01$), in ALP activity. Unloaded particles did not induce any increase in ALP activity or osteoblast morphology. After 2 weeks of cell culture, the BMP-2-loaded particles were able to induce mineralization as observed by alizarin red staining (Fig. 2). No mineralization was observed for cells cultured with unloaded particles. The amount of calcium was significantly higher ($p < 0.01$) for fibroin particles loaded with BMP-2 ($1897.7\% \pm 16.0\%$) compared with a

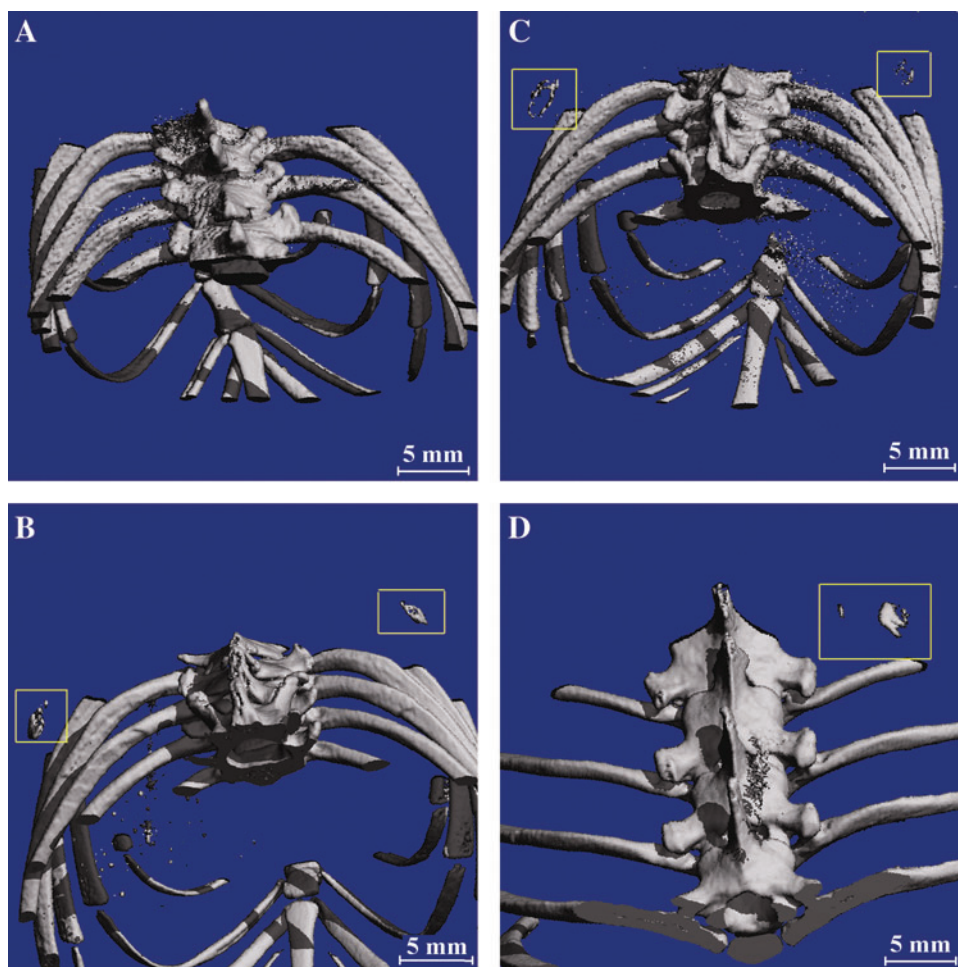


FIG. 4. μ CT images of ectopic bone formation (see insets) induced by unloaded fibroin particles after 4 weeks (A), fibroin particles + 5 μ g BMP-2 after 4 weeks (B), and fibroin particles + 12.5 μ g BMP-2 after 2 weeks (C) and after 4 weeks (D). Scale bars: 5 mm. μ CT, microcomputed tomography. Color images available online at www.liebertonline.com/ten.

corresponding dose of BMP-2 added to media as a standard positive control ($899.4\% \pm 11.3\%$).

Cell viability

The silk fibroin microparticles did not show any significant evidence of cytotoxicity in human osteosarcoma cells, after 1, 3, or 5 days of cell culture (Fig. 3). The cells were able to proliferate normally during this time period. MTS is a viability/proliferation test, and an inverse relationship of toxicity to cells can be assumed.²⁰ Data correlated with the morphological observations of the cells by optical microscopy (data not shown). Similar data were also obtained when particles were tested in primary cultures of human adult adipose-derived stem cells (data not shown).

In vivo μ CT

All animals showed no complications in wound healing, during the 4-week follow-up. After 2 and 4 weeks, all sites of fibroin particle implantation were easily identified and retrieved for analysis. The μ CT reconstructions (Figs. 4 and 5)

clearly showed ectopic calcifications in sites where BMP-2-loaded particles (Groups II and III) were implanted. In Group II (fibroin particles + $5\ \mu\text{g}$ BMP-2), no ectopic bone could be detected after 2 weeks, possibly because it was below the threshold of sensitivity of the μ CT equipment. However, at week 4, ectopic bone was clearly visible having a bone volume of $2.5 \pm 0.5\ \text{mm}^3$ and $288.2 \pm 20.8\ \text{mg}$ hydroxyapatite (HA)/ mm^3 (mineral density). In Group III (fibroin particles + $12.5\ \mu\text{g}$ BMP-2), ectopic bone formation was observed at both weeks 2 and 4 postimplantation. Comparing the μ CT reconstructions in this group, there was an increase in both the bone volume and bone density (1.9 ± 0.6 to $2.3 \pm 0.9\ \text{mm}^3$ and 178.3 ± 11.3 to $353.0 \pm 88.0\ \text{mg}$ HA/ mm^3 ; $p < 0.05$; Fig. 6). The dimensions of the newly formed bone were nearly similar between the two time periods, which varied from 1 to 3 mm in width. At week 2, the structure of bone was rather indistinct; however, at week 4, the bone formed as a solid shell on part of the surface and had a trabecular structure inside. In the control group (Group I, unloaded fibroin particles), no ectopic bone formation could be detected.

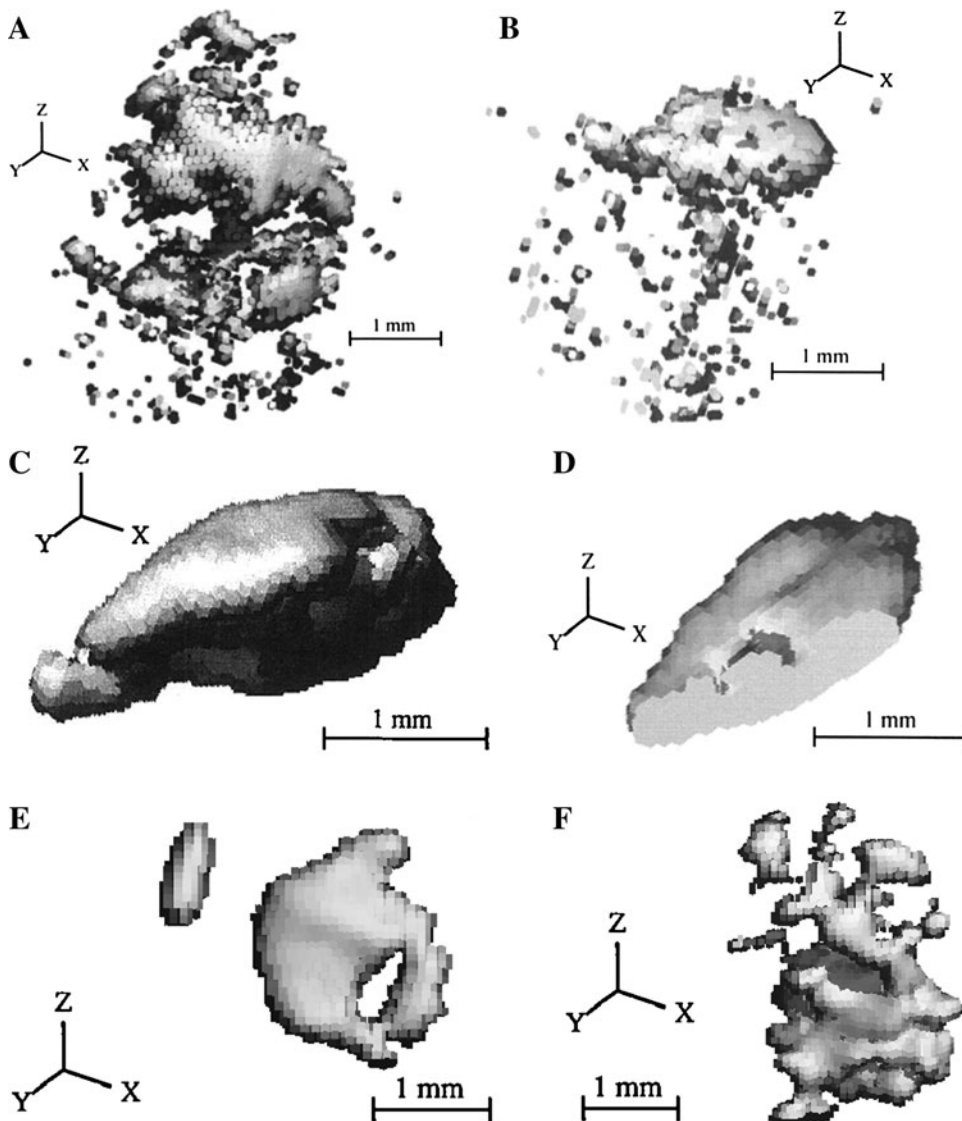


FIG. 5. Detailed μ CT images of ectopic bone formation induced by fibroin particles + $12.5\ \mu\text{g}$ BMP-2 after 2 weeks (A, B) and 4 weeks (E, F), and fibroin particles + $5\ \mu\text{g}$ BMP-2 after 4 weeks (C, D). Background signal in A and B is due to a lower threshold used for imaging. Scale bars: 1 mm.

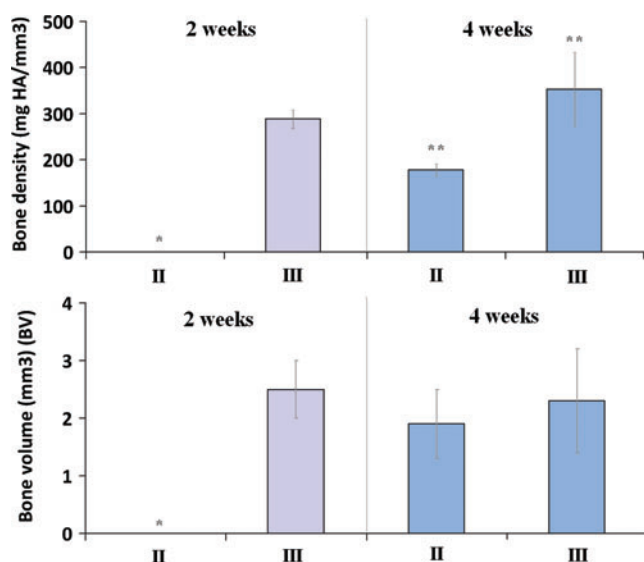


FIG. 6. Bone density (mg hydroxyapatite/mm³) and bone volume (mm³) of ectopic bone evaluated by μ CT. Fibroin particles + 5 μ g BMP-2 (II); fibroin particles + 12.5 μ g BMP-2 (III). Error bars reflect standard error. *No ectopic bone was detected. **Significant ($p < 0.05$) difference between each other. Color images available online at www.liebertonline.com/ten.

Histology

H&E staining revealed no signs of vascularization, but in Group II some parts showed calcified tissue. In this group, H&E staining revealed abundant presence of osteoblasts and osteocytes surrounded by an extracellular matrix (Fig. 7A). These tissues showed intense alizarin red staining (Fig. 7B). In Group III, no alizarin red-positive areas could be observed, despite the fact that in the μ CT, ectopic bone formation was observed. At the time of harvest it was not possible to localize where the new bone formation was occurring. No staining was detected in the control group as expected (data not shown). Immunohistological evaluation with an osteocalcin-specific antibody revealed moderate staining in sections of Group II (Fig. 7C), in the site where osteoblasts surrounding a mineralized matrix were detected. Staining was not detected in a negative control with no incubation with the osteocalcin antibody (data not shown).

Discussion

The need for an efficient delivery system for BMPs as a way of providing effective and sustained stimulation of bone formation, as shown in several experimental models,²² has been well recognized. The main role of a carrier is to retain these growth factors at the site of injury for a prolonged time frame, protecting the immobilized drugs from degradation and maintaining its bioactivity, while releasing the protein in a time- and site-controlled way to promote the formation of new bone at the treatment site.

Silk fibroin biomaterials have been explored as novel alternatives for drug delivery and in tissue engineering, as a result of their biocompatibility, biodegradability, mechanical strength, and versatility of formats into which the material could be processed.²³ In this study we examined the

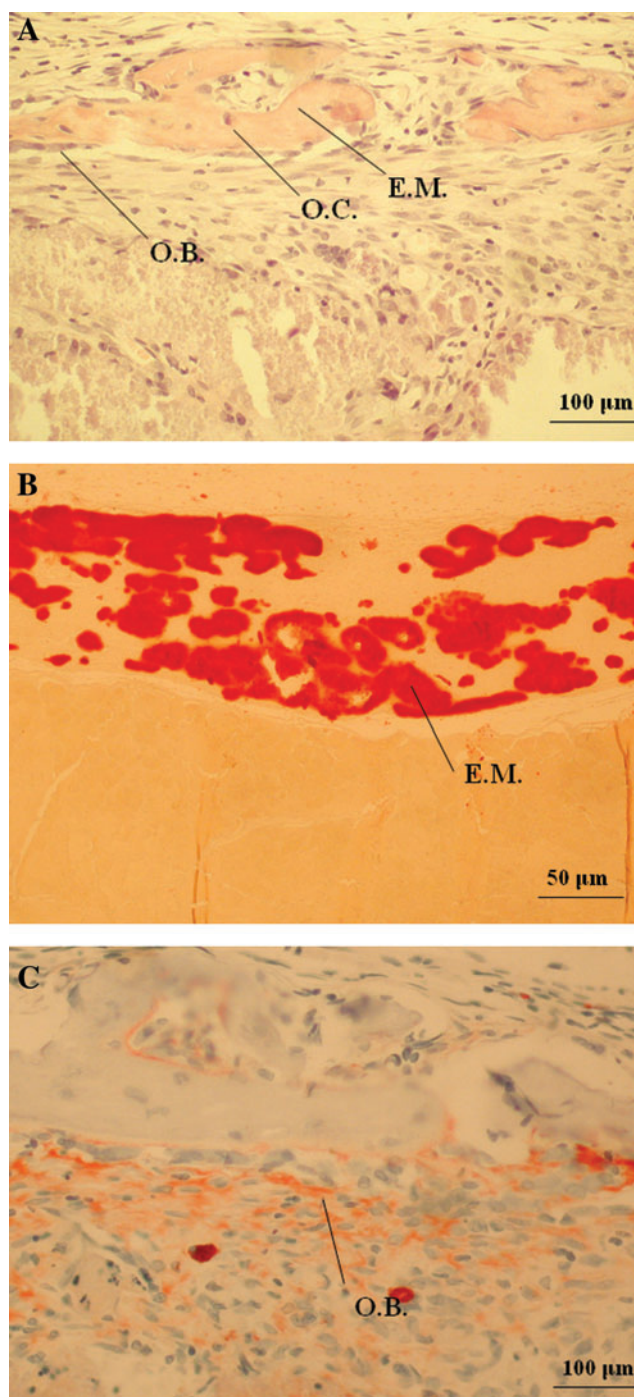


FIG. 7. Hematoxylin and eosin staining for animals implanted with fibroin particles + 5 μ g BMP-2 at 4 weeks after implantation (A). Newly formed bone was indicated by the presence of a mineralized extracellular matrix (E.M.) stained with alizarin red (B), osteocytes (O.C.), and osteoblasts (O.B.) stained by osteocalcin immunostaining (C). Color images available online at www.liebertonline.com/ten.

in vitro and *in vivo* delivery of BMP-2 from fibroin micro-particles.

The basis for evaluating the activity of the BMP released by the fibroin particles was to test initially the particles loaded with growth factor in C2C12 cell line and then using a

rat ectopic bone formation model. C2C12 murine premyoblast cells are well defined by their ability to rapidly differentiate into osteoblasts when cultured in the presence of BMPs.^{15,16,24} In these cells, fibroin particles loaded with BMP-2 were able to induce a significant increase in ALP activity (after 5 days of culture), osteoblast-like morphology, and the formation of a mineralized matrix (after 2 weeks of culture), thereby confirming that the growth factor was loaded into the particles, retaining its bioactive state. This adds value to the method formerly developed to produce fibroin particles by ethanol precipitation,¹¹⁻¹³ confirming that it retains the activity of encapsulated drugs. Similar cases were reported but using different methodologies. In one report, fibroin particles retained the activity of loaded horseradish peroxidase, using lipid vesicles as particle templates, followed by methanol treatment,²⁵ whereas in another work, silk spheres, produced by nozzle vibration and methanol treatment, retained the activity of insulin growth factor-1.²⁶ In contrast, the method herein described is more simple, and its milder processing steps may allow the encapsulation of growth factors while retaining most of their activity. In our study, the activity recovery was estimated as $88.9\% \pm 6.1\%$, whereas no such results were presented in the former works using silk particles as carriers for BMP-2 delivery.

As particles with surface-adsorbed BMP-2 induced only low levels of ALP, it is likely that most of the growth factor accountable for the significant increase in ALP levels is BMP-2 encapsulated into the particles during their fabrication. This adds support to our previous report that BMP-2 is released in a sustained way for a period of up to 2 weeks.¹⁴ This is also confirmed by the increased mineralization, over the same period, induced by particles containing the BMP-2, when compared with the corresponding doses of growth factor added to the culture media. No cytotoxicity was detected by the use of fibroin particles *in vitro*, as inferred by the MTS cell viability assay. In general, silk fibroin has been regarded as a good biocompatible material, effecting low immune responses *in vivo*, and with an historical use as sutures in clinical applications.²⁷⁻²⁹ This adds further evidence that these materials could be used as a viable option for future clinical applications.

Ectopic bone formation models in rodents have been used to test the activity of BMPs in a wide variety of studies.³⁰⁻³⁴ The microparticles could form a slurry, which was easily implanted ectopically in rats. After 2 and 4 weeks, bone formation was observed in all sites of implantation of BMP-2-loaded particles and the extent of ectopically formed bone was measured by μ CT. Mineralized areas of bone were found as early as after 2 weeks postimplantation and increased in both bone volume and HA density after 4 weeks. Live μ CT is a reliable and noninvasive method for scanning and evaluation along time of bone mass, structure, geometric, and density parameters.³⁵ The use of μ CT imaging maximizes the results from single animals, offering sequential data within the same animal, thus reducing this way the required number of animals per study.³⁶ Ectopic bone formation was also confirmed by histological analysis performed at 4 weeks postimplantation. New bone formation was detected by the presence of a calcified extracellular matrix, which was stained by alizarin red, revealing only surface mineralization without quantitative data, when compared with the μ CT data. In addition, osteocalcin, a calcium-binding protein and a marker of mature

bone formation,³⁷ was also detected in the cells surrounding the newly formed bone.

New bone formation occurs as released BMP-2 triggers the recruitment of stem cells that differentiate into osteoblasts and form a calcium phosphate matrix.³⁸ This can only occur if there is not only an initial stimulation of the site by BMP-2, but also a sustained release of the growth factor in the following weeks, allowing the formation of new bone. In the lack of a sustained release, the BMP-2 is usually cleared from the implant site within hours, supposedly terminating its local effect.³⁹ This is demonstrated by studies where surface-adsorbed BMP-2 was used in the particles that failed to induce new bone formation probably because of either loss of the BMP bioactivity or insufficient duration of stimulus.⁴⁰ In fact, we have observed the same lack of activity in our *in vitro* data for particles with adsorbed BMP. In one report, TGF- β 2, another member of the TGF- β superfamily, completely lost activity after 1 week of incubation at 37°C *in vitro*.⁴¹ A similar situation has also been reported with BMP-2-adsorbed fibroin scaffolds, hypothesized to degrade at a similar rate, in which the growth factor loaded inside the scaffold retained activity for a longer period.⁸ Thus, it has been suggested that materials exhibiting an higher retention of BMPs yield increased osteoinductive activity and are therefore more interesting for future clinical applications.^{42,43}

In our contribution, the amounts of BMP-2 loaded into the fibroin particles (5 and 12.5 μ g) were similar to the values reported in the literature to achieve ectopic bone formation in rats with BMP-2, using other delivery systems.^{40,44,45} Whether the particulate system could be used *in vivo* in combinations with hydrogels or scaffolds, as part of more complex three-dimensional tissue engineering constructs, remains a question to be explored in future studies. The feasibility of the use of fibroin to heal bone fractures has been demonstrated in previous works.^{8,10,46} One possibility could be the use of the fibroin microparticles as part of injectable systems or loaded into scaffolds to achieve a delayed release of BMP-2, as was explored for other types of materials.⁴⁷

The fibroin particles have clearly shown to be able to deliver BMP-2 in a sustained way, allowing the formation of new bone in rodents, and may thus constitute a promising delivery system of BMPs for future bone tissue engineering clinical trials.

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Disclosure Statement

No competing financial interests exist.

References

- Reddi, A.H. BMPs: from bone morphogenetic proteins to body morphogenetic proteins. *Cytokine Growth Factor Rev* **16**, 249, 2005.
- Bessa, P.C., Casal, M., and Reis, R.L. Bone morphogenetic proteins in tissue engineering: the road from laboratory to the clinic, part I (basic concepts). *J Tissue Eng Regen Med* **2**, 1, 2008.
- Bessa, P.C., Casal, M., and Reis, R.L. Bone morphogenetic proteins in tissue engineering: the road from laboratory to the clinic, part II (BMP delivery). *J Tissue Eng Regen Med* **2**, 81, 2008.
- Gautschi, O.P., Frey, S.P., and Zellweger, R. Bone morphogenetic proteins in clinical applications. *ANZ J Surg* **77**, 626, 2007.
- Kim, K., and Fisher, J.P. Nanoparticle technology in bone tissue engineering. *J Drug Target* **15**, 241, 2007.
- Mano, J.F., Silva, G.A., Azevedo, H.S., Malafaya, P.B., Sousa, R.A., Silva, S.S., Boesel, L.F., Oliveira, J.M., Santos, T.C., Marques, A.P., Neves, N.M., and Reis, R.L. Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. *J Royal Soc Interface* **4**, 999, 2007.
- Karageorgiou, V., Meinel, L., Hofmann, S., Malhotra, A., Volloch, V., and Kaplan, D. Bone morphogenetic protein-2 decorated silk fibroin films induce osteogenic differentiation of human bone marrow stromal cells. *J Biomed Mater Res A* **71**, 528, 2004.
- Karageorgiou, V., Tomkins, M., Fajardo, R., Meinel, L., Snyder, B., Wade, K., Chen, J., Vunjak-Novakovic, G., and Kaplan, D.L. Porous silk fibroin 3D scaffolds for delivery of bone morphogenetic protein-2 *in vitro* and *in vivo*. *J Biomed Mater Res A* **78**, 324, 2006.
- Li, C., Vepari, C., Jin, H.J., Kim, H.J., and Kaplan, D.L. Electrospun silk-BMP-2 scaffolds for bone tissue engineering. *Biomaterials* **27**, 3115, 2006.
- Kirker-Head, C., Karageorgiou, V., Hofmann, S., Fajardo, R., Betz, O., Merkle, H.P., Hilbe, M., von Rechenberg, B., McCool, J., Abrahamsen, L., Nazarian, A., Cory, E., Curtis, M., Kaplan, D., and Meinel, L. BMP-silk composite matrices heal critically sized femoral defects. *Bone* **41**, 247, 2007.
- Nam, J., and Park, Y.H. Morphology of regenerated silk fibroin: effects of freezing temperature, alcohol addition, and molecular weight. *J Appl Polym Sci* **81**, 3008, 2001.
- Zhang, Y.Q., Shen, W.D., Xiang, R.L., Zhuge, L.J., Gao, W.J., and Wang, W.B. Formation of silk fibroin nanoparticles in water-miscible organic solvent and their characterization. *J Nanoparticle Res* **9**, 885, 2007.
- Cao, Z., Chen, X., Yao, J., Huang, L., and Shao, Z. The preparation of regenerated silk fibroin microspheres. *Soft Matter* **3**, 910, 2007.
- Bessa, P.C., Balmayor, E.R., Azevedo, H.S., Nürberger, S., Casal, M., van Griensven, M., Reis, R.L., and Redl, H. Silk fibroin microparticles as carriers for delivery of human recombinant BMPs. Physical characterization and drug release. *J Tissue Eng Regen Med* 2009 (In press).
- Klößch, B., Fürst, W., Kneidinger, R., Schuller, M., Rupp, B., Banerjee, A., and Redl, H. Expression and purification of biologically active rat bone morphogenetic protein-4 produced as inclusion bodies in recombinant *Escherichia coli*. *Biotechnol Lett* **27**, 1559, 2005.
- Long, S., Truong, L., Bennett, K., Phillips, A., Wong-Stall, F., and Ma, H. Expression, purification, and renaturation of bone morphogenetic protein-2 from *Escherichia coli*. *Protein Expr Purif* **46**, 374, 2006.
- Bessa, P.C., Pedro, A.J., Klosch, B., Nobre, A., van Griensven, M., Reis, R.L., and Casal, M. Osteoinduction in human fat-derived stem cells by recombinant human bone morphogenetic protein-2 produced in *Escherichia coli*. *Biotechnol Lett* **30**, 15, 2008.
- Bessa, P.C., Cerqueira, M.T., Rada, T., Gomes, M.E., Neves, N.M., Nobre, A., Reis, R.L., and Casal, M. Expression, purification and osteogenic bioactivity of recombinant human BMP-4, -9, -10, -11 and -14. *Protein Expr Purif* **63**, 89, 2009.
- Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Roscn, V., Wozney, J.M., Fujisawa-Sehara, A., and Suda, T. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteogenic lineage. *J Cell Biol* **127**, 1755, 1994.
- Salgado, A.J., Gomes, M.E., Chou, A., Coutinho, O.P., Reis, R.L., and Hutmacher, D.W. Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds. *Mater Sci Eng* **20**, 27, 2002.
- Gregory, C.A., Gunn, W.G., Peister, A., and Prockop, D.J. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* **329**, 77, 2004.
- Seeherman, H., and Wozney, J.M. Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. *Cytokine Growth Factor Rev* **16**, 329, 2005.
- Lammel, A., Schwab, M., Slotta, U., Winter, G., and Scheibel, T. Processing conditions for the formation of spider silk microspheres. *ChemSusChem* **1**, 413, 2008.
- Vallejo, L.F., Brokelmann, M., Marten, S., Trappe, S., Cabrera-Crespo, J., Hoffmann, A., Gross, G., Weich, H.A., and Rinas, U. Renaturation and purification of bone morphogenetic protein-2 produced as inclusion bodies in high-cell-density cultures of recombinant *Escherichia coli*. *J Biotechnol* **94**, 185, 2002.
- Wang, X., Wenk, E., Matsumoto, A., Meinel, L., Li, C., and Kaplan, D.L. Silk microspheres for encapsulation and controlled release. *J Control Release* **117**, 360, 2007.
- Wenk, E., Wandrey, A.J., Merkle, H.P., and Meinel, L. Silk fibroin spheres as a platform for controlled drug delivery. *J Control Release* **132**, 26, 2008.
- Altman, G.H., Diaz, F., Jakuba, C., Calabro, T., Horan, R.L., Chen, J., Lu, H., Richmond, J., and Kaplan, D.L. Silk-based biomaterials. *Biomaterials* **24**, 401, 2003.
- Meinel, L., Hofmann, S., Karageorgiou, V., Kirker-Head, C., McCool, J., Gronowicz, G., Zichner, L., Langer, R., Vunjak-Novakovic, G., and Kaplan, D.L. The inflammatory responses to silk films *in vitro* and *in vivo*. *Biomaterials* **26**, 147, 2005.
- Wang, Y., Rudym, D.D., Walsh, A., Abrahamsen, L., Kim, H.J., Kim, H.S., Kirker-Head, C., and Kaplan, D.L. *In vivo* degradation of three-dimensional silk fibroin scaffolds. *Biomaterials* **29**, 3415, 2008.
- Bulpitt, P., and Aeschlimann, D. New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. *J Biomed Mater Res* **47**, 152, 1999.
- Maire, M., Chaubet, F., Mary, P., Blanchat, C., Meunier, A., and Logeart-Avramoglou, D. Bovine BMP osteoinductive potential enhanced by functionalized dextran-derived hydrogels. *Biomaterials* **26**, 5085, 2005.
- Zhang, C., Hu, Y., Xiong, Z., Zhang, S., Yan, Y., and Cui, F. [Tissue engineered bone regeneration of periosteal cells us-

- ing recombinant human bone morphogenetic protein 2 induce]. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **19**, 100, 2005.
33. Kato, M., Namikawa, T., Terai, H., Hoshino, M., Miyamoto, S., and Takaoka, K. Ectopic bone formation in mice associated with a lactic acid/dioxanone/ethylene glycol copolymer-tricalcium phosphate composite with added recombinant human bone morphogenetic protein-2. *Biomaterials* **27**, 3927, 2006.
 34. Jeon, O., Song, S.J., Kang, S.W., Putnam, A.J., and Kim, B.S. Enhancement of ectopic bone formation by bone morphogenetic protein-2 released from a heparin-conjugated poly (L-lactic-co-glycolic acid) scaffold. *Biomaterials* **28**, 2763, 2007.
 35. Hangartner, T.N. Thresholding technique for accurate analysis of density and geometry in QCT, pQCT and microCT images. *J Musculoskelet Neuronal Interact* **7**, 9, 2007.
 36. Cowan, C., Aghaloo, T., Chou, Y.-F., Walder, B., Zhang, X., Soo, C., Ting, K., and Wu, B. MicroCT evaluation of three-dimensional mineralization in response to BMP-2 doses *in vitro* and in critical size rat calvarial defects. *Tissue Eng* **13**, 501, 2007.
 37. Price, P.A. Gla-containing proteins of bone. *Connect Tissue Res* **21**, 51, 1989.
 38. Cunningham, N.S., Paralkar, V., and Reddi, A.H. Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor beta 1 mRNA expression. *Proc Natl Acad Sci USA* **89**, 11740, 1992.
 39. Louis-Ugbo, J., Kim, H.S., Boden, S.D., Mary, M.T., Li, R.C., Seeherman, H., D'Augusta, D., Blake, C., Jiao, A., and Peckham, S. Retention of 125I-labeled recombinant human bone morphogenetic protein-2 by biphasic calcium phosphate or a composite sponge in a rabbit posterolateral spine arthrodesis model. *J Orthop Res* **20**, 1050, 2002.
 40. Wei, G., Jin, Q., Giannobile, W.V., and Ma, P.X. The enhancement of osteogenesis by nanofibrous scaffolds incorporating rhBMP-7 nanospheres. *Biomaterials* **28**, 2087, 2007.
 41. Bentz, H., Schroeder, J.A., and Estridge, T.D. Improved local delivery of TGF- β 2 by binding to injectable fibrillar collagen via difunctional polyethylene glycol. *J Biomed Mater Res* **39**, 539, 1998.
 42. Uludag, H., D'Augusta, D., Palmer, R., Timony, G., and Wozney, J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. *J Biomed Mater Res* **46**, 193, 1999.
 43. Uludag, H., D'Augusta, D., Golden, J., Li, J., Timony, G., Riedel, G., and Wozney, J.M. Implantation of recombinant human bone morphogenetic proteins with biomaterial carriers: a correlation between protein pharmacokinetics and osteoinduction in the rat ectopic model. *J Biomed Mater Res* **50**, 227, 2000.
 44. Kenley, R., Marden, L., Turek, T., Jin, L., Ron, E., and Hollinger, J.O. Osseous regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenetic protein-2 (rhBMP-2). *J Biomed Mater Res* **28**, 1139, 1994.
 45. Hosseinkhani, H., Hosseinkhani, M., Khademhosseini, A., and Kobayashi, H. Bone regeneration through controlled release of bone morphogenetic protein-2 from 3-D tissue engineered nano-scaffold. *J Control Release* **117**, 380, 2007.
 46. Meinel, L., Betz, O., and Fajardo, R. Silk based biomaterials to heal critical sized femur defects. *Bone* **39**, 922, 2006.
 47. Yilgor, P., Tuzlakoglu, K., Reis, R.L., Hasirci, N., and Hasirci, V. Incorporation of a sequential BMP-2/BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering. *Biomaterials* **30**, 3551, 2009.

Address correspondence to:

Paulo César Bessa, Ph.D.

Ludwig Boltzmann Institute for Experimental

and Clinical Traumatology

AUVA Research Center

Austrian Cluster for Tissue Regeneration

Donauerschlingenstrasse 13

1200 Vienna

Austria

E-mail: paulo.bessa@dep.uminho.pt

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