Biochemical and Biophysical Research Communications 433 (2013) 595–601

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Engineering tubular bone using mesenchymal stem cell sheets and coral particles

Wenxin Geng [a,1], Dongyang Ma [b,1], Xingrong Yan [a], Liangqi Liu [a], Jihong Cui [a], Xin Xie [a], Hongmin Li [a], Fulin Chen [a,*]

[a] Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University, No.229 North Taibai Road, Xi'an 710069, PR China
[b] Department of Oral and Maxillofacial Surgery, Lanzhou General Hospital, Lanzhou Command of PLA, BinHe 333 South Road, Lanzhou 730052, China

ARTICLE INFO

Article history:
Received 13 March 2013
Available online 21 March 2013

Keywords:
Bone
Tissue engineering
Mesenchymal stem cells
Cell sheets
Coral particles

ABSTRACT

The development of bone tissue engineering has provided new solutions for bone defects. However, the cell-scaffold-based approaches currently in use have several limitations, including low cell seeding rates and poor bone formation capacity. In the present study, we developed a novel strategy to engineer bone grafts using mesenchymal stem cell sheets and coral particles. Rabbit bone marrow mesenchymal stem cells were continuously cultured to form a cell sheet with osteogenic potential and coral particles were integrated into the sheet. The composite sheet was then wrapped around a cylindrical mandrel to fabricate a tubular construct. The resultant tubular construct was cultured in a spinner-flask bioreactor and subsequently implanted into a subcutaneous pocket in a nude mouse for assessment of its histological characteristics, radiological density and mechanical property. A similar construct assembled from a cell sheet alone acted as a control. *In vitro* observations demonstrated that the composite construct maintained its tubular shape, and exhibited higher radiological density, compressive strength and greater extracellular matrix deposition than did the control construct. *In vivo* experiments further revealed that new bone formed ectopically on the composite constructs, so that the 8-week explants of the composite sheets displayed radiological density similar to that of native bone. These results indicate that the strategy of using a combination of a cell sheet and coral particles has great potential for bone tissue engineering and repairing bone defects.

1. Introduction

Repairing large bone defects caused by tumor resection, trauma, infection or abnormal skeletal development remains a significant clinical problem despite advances in autologous bone grafts and surgical techniques [1]. The development of tissue engineering has provided promising alternatives for the repair of bone defects and has attracted more attention in recent years [2,3]. In the tissue engineering approach, isolated cell suspensions are usually seeded into block scaffolds to fabricate tissue substitutes. However, the cell-scaffold-based approaches often encounter problems in achieving high cell density and efficient cell delivery [4,5]. In addition, isolated cell suspensions are routinely harvested from culture dishes with proteolytic enzymes. This process may destroy extracellular matrix (ECM) components, cell–ECM interactions and cell–cell connections that are critical for cell growth, survival, differentiation and tissue development [6–8].

The cell sheet technique, in which high-density cells can be harvested together with their endogenous deposited ECM and intact cell–cell contacts, is a valuable extension of current initiatives in tissue engineering. Okano's group has developed a temperature-sensitive culture dish to harvest intact sheets non-invasively [9,10]. Transplantation of a cell monolayer or layered cell sheets has provided encouraging results in engineering functional myocardial patches and corneas [6,7]. Pouliot et al. [8] used dermal fibroblast sheets for skin, and L'Heureux et al. reported engineering human blood vessels with sheets of endothelial and smooth muscle cells [11].

For functional bone tissue engineering, the engineered graft should not only possess proper histological architecture and three-dimensional shape, but also possess adequate mechanical strength to bear loads. Hutmacher's group first reported fabricating tubular bone grafts with a combination of a biodegradable polymer mesh and sheets of marrow mesenchymal stem cells (MSCs) [4]. Histological evaluation showed that fibrous tissue formed around the scaffold, most likely because of non-specific inflammation caused by cytotoxic polymer degradation products and the low pH value in the implantation area, which is detrimental for new bone formation. Gao et al. wrapped a cell sheet around a tubular

* Corresponding author. Fax: +86 29 88302634.
E-mail address: chenfl@nwu.edu.cn (F. Chen).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.034

coral scaffold to create a tubular bone graft [12]. However, only a shell of ossification was observed at the surface of the engineered bone, indicating that the cells in the sheet could not migrate into the central zone of the block scaffold. Recently, we successfully fabricated bone grafts using osteogenic cell sheets alone, without alien biomaterials. However, we found that the cell sheet construct was flexible and deformable after detachment from the culture dish, making it difficult to manipulate while maintaining its initial three-dimensional shape; moreover, this fabricated construct also lacked the integral strength to withstand the handling necessary to implant it [13].

To address these issues, we proposed a new strategy for engineering tubular bone grafts using MSC sheets and coral particles, which were used to improve the handling properties of the cell sheet and serve as a volume extender. We first cultured the engineered composite constructs in a spinner bioreactor and investigated their *in vitro* characteristics. Then we evaluated the *in vivo* osteogenic potential of the engineered graft constructs *via* ectopic transplantation in nude mice.

2. Materials and methods

2.1. Culture medium

Two kinds of medium were used in the study. Medium I was Dulbecco's Modified Eagle Medium (Gibco Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 0.272 g/L L-glutamine (Sigma–Aldrich, St. Louis, MO, USA). Medium II was Medium I supplemented with 50 mg/mL of L-ascorbic acid 2-phosphate (Sigma–Aldrich, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma–Aldrich, St. Louis, MO, USA) and 100 nM dexamethasone (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Preparation of coral particles

Natural coral block (RegeMed Inc., batch number BS05321, China) was ground and sieved to obtain small particles of 0.1–0.2-mm diameter. The coral particles were immersed in 5% sodium hypochlorite for three days to remove foreign protein and were then washed with distilled water and sterilized by autoclave before use.

2.3. Cell culture and expansion

This study was approved by the Animal Care and Use Committee of Northwest University (Xi'an, China). Rabbit MSCs were isolated and cultured as previously described [13]. Briefly, rabbit ilia were split and the bone marrow was flushed out with Medium I. The marrow suspension was plated in 100-mm petri dishes (Nunc, Danmark) containing 10 mL of Medium I at 37 °C with a 5% CO_2 atmosphere. The non-adherent cells were removed by repeatedly washing with phosphate-buffered saline three days after plating. When the cells were approximately 80% confluent, the adherent cells were trypsinized and subcultured for further experiments.

2.4. Preparation of cell sheets and fabrication of constructs

The subcultured MSCs were plated at a density of 5×10^4 cells/cm^2 and cultured in 100-mm petri dishes in Medium II for 1 week without passage. For the composite cell sheets, 30 mg of coral particles were seeded on the surface of the confluent cells and the culture was grown for another week. For the cell sheets without coral particles, the MSCs were continuously cultured in Medium II for 2 weeks. When a cell sheet had formed, it was lifted from the culture dish with a cell scraper. Small pieces of the cell sheets were processed for scanning electron microscopy (SEM).

To test the interaction between MSCs and coral particles, 1 mL of Medium II containing 1×10^5 MSCs was seeded into each well of a 24-well culture plate and cultured according to the aforementioned method. For the composite group, 3 mg of coral particles were added to each well. After 1, 4 or 7 days of culture, the calcium ion (Ca^{2+}) concentration in the cell culture medium of each well was determined using an Atomic Absorption Spectrometer (SavantAA, GBC, Australia). The leach liquor of coral particles into the culture medium and the medium of cells sheet cultured without coral particles acted as the controls. The experiment was performed with six replicates.

To fabricate a tubular bone graft, a composite cell sheet was folded to form an oblong-shaped flap and then rolled around a 5-mm diameter mandrel. The composite construct (5-mm diameter × 20-mm long) was incubated in medium II for 3 days in a static condition to avoid displacement and to ensure fusion among the layers of the cell sheet. A similar construct assembled from a cell sheet alone acted as a control.

2.5. Engineering the tubular bone constructs in vitro

The fabricated constructs were transferred into spinner flasks (Bellco, USA) (rotating at a speed of 10 rpm) for dynamic culturing in Medium II; the medium was changed every 3 days. The mandrel was removed after 2 weeks and the tube-shaped grafts were cultured continuously for another 2 weeks. Following harvesting, the specimens were grossly inspected and processed for CT scans. The radiological density of the constructs was evaluated by measuring the Hounsfield density units (HU). The radiological density was measured at three longitudinal points (the two ends and the midpoint) of each construct and the mean value was calculated. The CT images were reconstructed using the 2004 GE Medical System to obtain the three-dimensional structure of the constructs. Then the specimens were divided into two parts. One part was fixed in 10% phosphate-buffered formalin and decalcified in 5% formic acid for histological examination. The samples were sectioned in the axial plane and stained with hematoxylin-and-eosin or Masson's trichrome stain. The other part was processed for a mechanical compression test that was performed with four replicates. Briefly, the compressive strength of the specimens was measured with an Instron 3365 Material Testing Machine (Instron Co., Canton, MA, USA) to 50% deformation with a flat-ended 10-cm^2 probe at a constant speed of 1 mm/min.

2.6. In vivo implantation

Using inhalation anesthesia and aseptic techniques, constructs fabricated from the composite MSC sheet or the MSC sheet alone were implanted into subcutaneous pockets on the backs of nude mice. At 4 weeks and 8 weeks after transplantation, four constructs of each group were harvested for macroscopic observation, CT scanning, radiological density measurement, histological examination and mechanical compression testing using the methods mentioned above.

2.7. Statistical analysis

All of the values were expressed as the mean ± standard deviation (SD). The data were analyzed using the paired Student's *t*-test after an analysis of variance (ANOVA) was performed, and $P < 0.05$ was considered significant.

3. Results

3.1. Characterization of the cell sheets

The MSCs proliferated rapidly and formed a thick cell sheet in 2 weeks in both groups (Fig. 1A and B). The cell sheet without coral particles had a smooth surface, whereas the composite cell sheet presented a rough surface. SEM analysis showed that both the spindle-shaped MSCs and coral particles were embedded in the ECM (Fig. 1C and D). Although the cell sheets in both groups could be lifted conveniently with a cell scraper and handled with forceps, the composite cell sheet was stronger and had better handling properties than did the counterpart. In addition, the composite cell sheet was easier to fabricate into a tubular construct with a rolling technique.

3.2. Interaction between cells and coral particles

The Ca^{2+} concentration in the medium of each group is listed in Table 1.The Ca^{2+} concentration in the medium of the cell sheet cultures remained stable during the experiment. On the first day after the coral particles were added, the Ca^{2+} concentration was not significantly altered. With coral particles on a cell sheet for 4 or 7 days, the Ca^{2+} concentration in the medium increased continuously and was significantly higher than in the other two groups ($P < 0.05$). The Ca^{2+} concentration in the coral leach liquor increased slightly but was not significantly different from that of culture medium without coral particles during the experimental period.

3.3. In vitro characterization of the tissue-engineered constructs

Both constructs maintained a cylindrical shape and their initial length after 4-weeks of dynamic culturing. The surface of the composite construct was rougher and more rigid than that of the control construct (Fig. 2A and B). CT images revealed evidence of hyper-dense structures in both constructs (Fig. 2C–F). The radiological density was measured to determine the extent of mineralization in the newly formed tubular grafts. The mean values of radiological density for the composite and control constructs were 19.4 ± 2.7 HU and 5.9 ± 1.2 HU ($n = 4$ each), respectively (Table 2). There was a significant difference between them ($P < 0.05$). As shown in Table 2, after 4 weeks of *in vitro* culture, the compressive strength of the constructs in the composite sheet group reached 0.55 ± 0.14N, which was significantly higher than that of the cell sheet group.

To examine the morphological characteristics and the ECM deposition in the tissue constructs, histology was performed. ECM deposition was observed in the constructs of both groups, particularly on the surfaces (Fig. 2H). The composite sheet constructs exhibited thick cartilage-like tissue formation and structural integration of the adjacent cell sheet layers, and the void area that was occupied by coral particles was clearly visible. Osteoid tissue was observed around the coral particles. Its control counterpart remained a distinctly multilayered structure with space between the cell sheets. The composite constructs showed more intense ECM deposition than did the control constructs (Fig. 2G–J).

Table 1
Concentration of calcium ions in each group at different time points (mM/L).

Group	1 day	4 days	7 days
Leach liquor	1.82 ± 0.04	1.85 ± 0.06*	1.86 ± 0.07*
Cell sheet	1.71 ± 0.09	1.70 ± 0.11*	1.68 ± 0.12*
Cell sheet + coral particles	1.90 ± 0.13	2.14 ± 0.15	2.33 ± 0.16

Results are shown as mean values ± SD ($n = 6$). *$P < 0.05$ compared with cell sheet + coral particles group at the same time point.

3.4. In vivo characterization of the engineered constructs

Both types of exgrafts were tightly connected to the surrounding host tissues. The surfaces of the exgrafts were covered with smooth hard tissue, and they maintained their initial tubular shape (Fig. 3A–D). The composite sheet constructs had larger volumes than did their counterparts. CT scanning revealed that ectopic

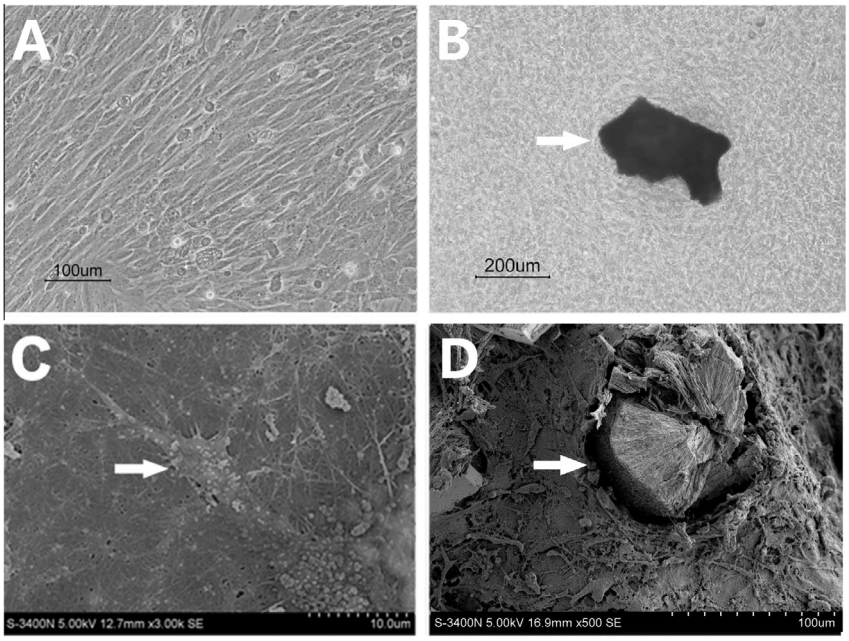

Fig. 1. Characteristics of the composite of cell sheets and coral particles. (A) MSCs grown to confluence to form a sheet. (B) Coral particles (arrow) were seeded on the surface of the confluent cells. (C) SEM image showing a spindle-shaped MSC (arrow) embedded in the ECM. (D) SEM image showing a coral particle (arrow) surrounded by ECM.

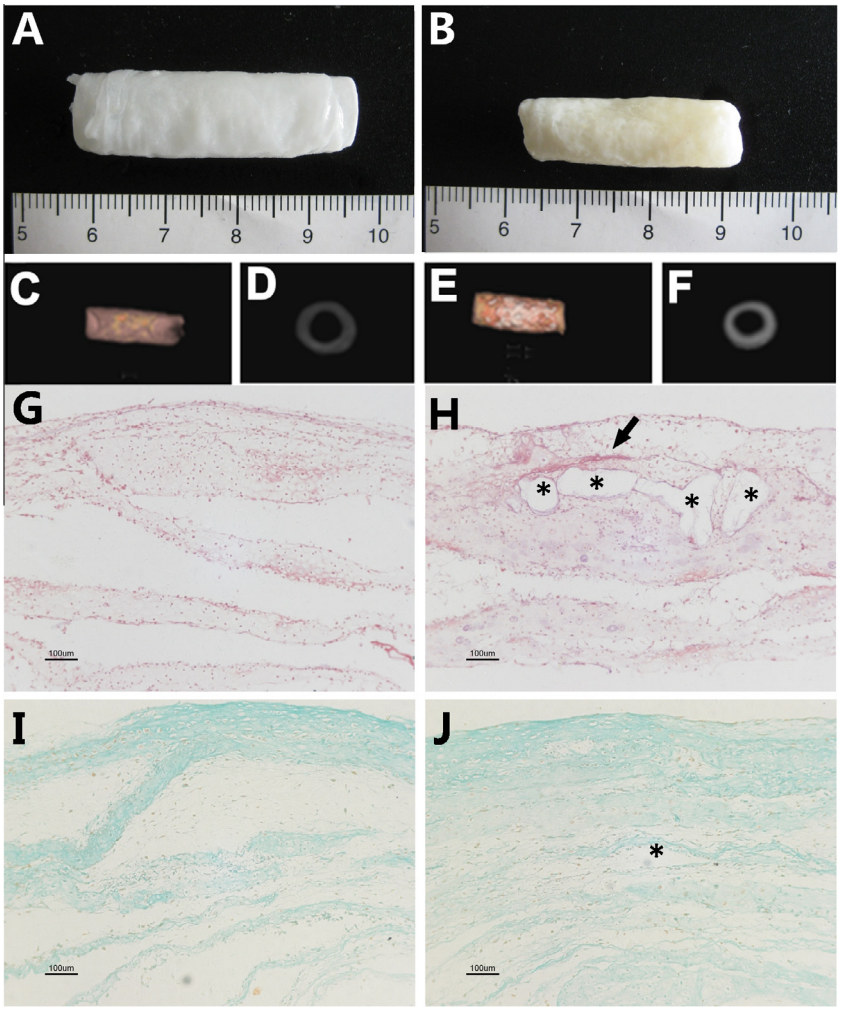

Fig. 2. Representative gross appearance, CT scans and histological examination of the constructs cultured *in vitro*. (A) A cell sheet construct. (B) A composite construct. (C) Three-dimensional CT image of a cell sheet construct. (D) Coronal CT image of a cell sheet construct. (E) Three-dimensional CT images of a composite construct. (F) Coronal CT image of a composite construct. (G) Histological examination (hematoxylin-and-eosin staining) of representative slices of a cell sheet construct. (H) Histological examination of a composite construct. The asterisks indicate coral particles and the arrow indicates osteoid tissue formation. (I) Masson's trichrome staining of a cell sheet construct. (J) Masson's trichrome staining of a composite construct. The asterisk indicates a coral particle.

Table 2
Analysis of the compressive strength and radiological density of the constructs.

Variable	Cell sheet			Cell sheet + coral particles		
	In vitro	4 weeks	8 weeks	*In vitro*	4 weeks	8 weeks
Compressive strength (N)	0.24 ± 0.09	4.63 ± 0.67	7.75 ± 0.92	0.55 ± 0.14*	8.42 ± 1.16*	10.57 ± 1.59*
Radiologic density(HU)	5.9 ± 1.2	37.9 ± 5.7	75.2 ± 6.9	19.4 ± 2.7*	87.2 ± 6.1*	125.5 ± 8.9*

Results are shown as mean values ± SD ($n = 4$). *$P < 0.05$ compared with the cell sheet group at the same time point.

mineralization had occurred in both groups by each time point (Fig. 3E and F). As shown in Table 2, the extent of mineralization in the tissue formations of both groups was low at 4 weeks and significantly increased by 8 weeks. At both time points, the average radiological density for the constructs of the composite sheet group was higher than that for the control constructs. In addition, the compressive strength of the composite sheet constructs was significantly higher ($P < 0.05$) than that of the control constructs. Importantly, the average radiological density of the 8-week ectopic grafts in the composite sheet group reached 125.5 ± 8.9 HU, similar to that of the native bone in the spines of the mice (129.3 ± 14.1 HU).

Representative histological images of the samples from the two groups are presented in Fig. 4. Bone formation mainly followed endochondral ossification, and trabecular bone was observed in the constructs in both implantation groups. Most of the coral particles had been absorbed by 4 weeks after implantation in the composite sheet constructs and were completely absorbed 4 weeks later (Fig. 4B–D). Consistent with the results for radiological density and mechanical strength, histological observation revealed more trabecular bone formation and ECM deposition in the composite sheet constructs than in the cell sheet constructs.

4. Discussion

The principle advantage of a cell sheet is that it is an entirely natural tissue assembled by cells, with a mature ECM, can be harvested by physical means. However, the poor mechanical proper-

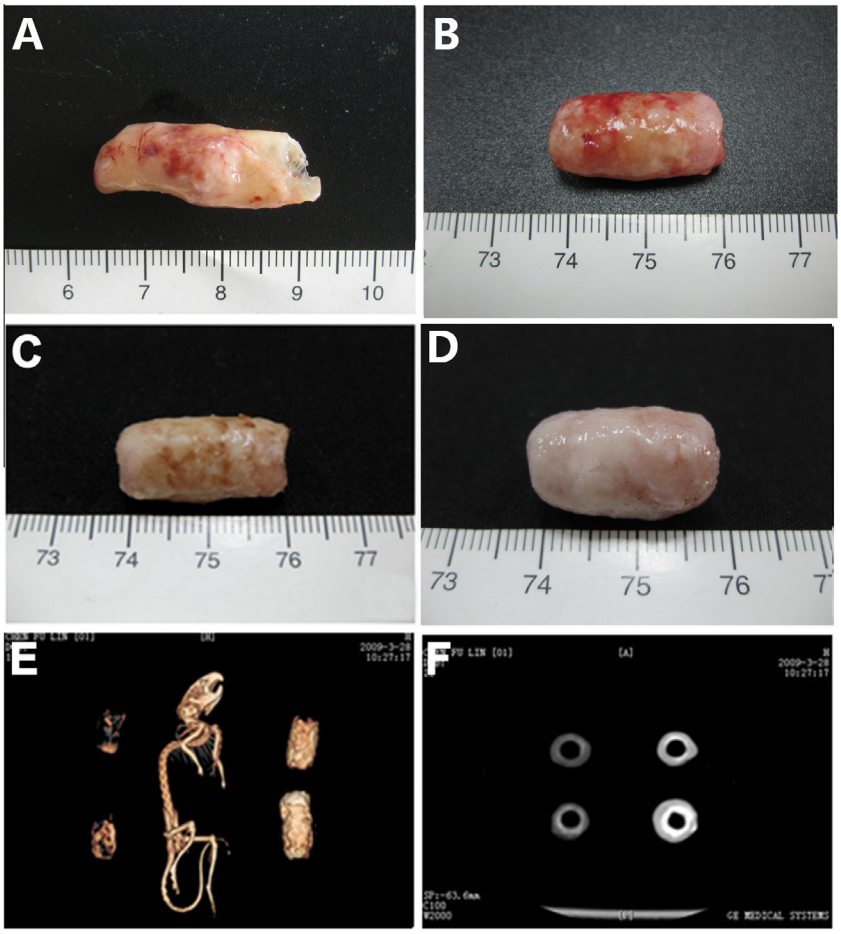

Fig. 3. Representative gross appearance and CT scans of the constructs after *in vivo* implantation. (A) Gross appearance of a harvested sample in the cell sheet group at 4 weeks post-implantation. (B) Sample from the composite group at 4 weeks post-implantation. (C) Sample from the cell sheet group at 8 weeks post-implantation. (D) Sample from the composite group at 8 weeks post-implantation. (E) Three-dimensional CT images of the constructs: Samples from the cell sheet group at 4 weeks (above left) and 8 weeks (below left) post-implantation, and a sample from the composite group at 4 weeks (above right) and 8 weeks (below right) post-implantation. (F) Coronal CT images of the constructs: Samples from the cell sheet group at 4 weeks (above left) and 8 weeks (below left) post-implantation, and a sample from the composite group at 4 weeks (above right) and 8 weeks (below right) post-implantation.

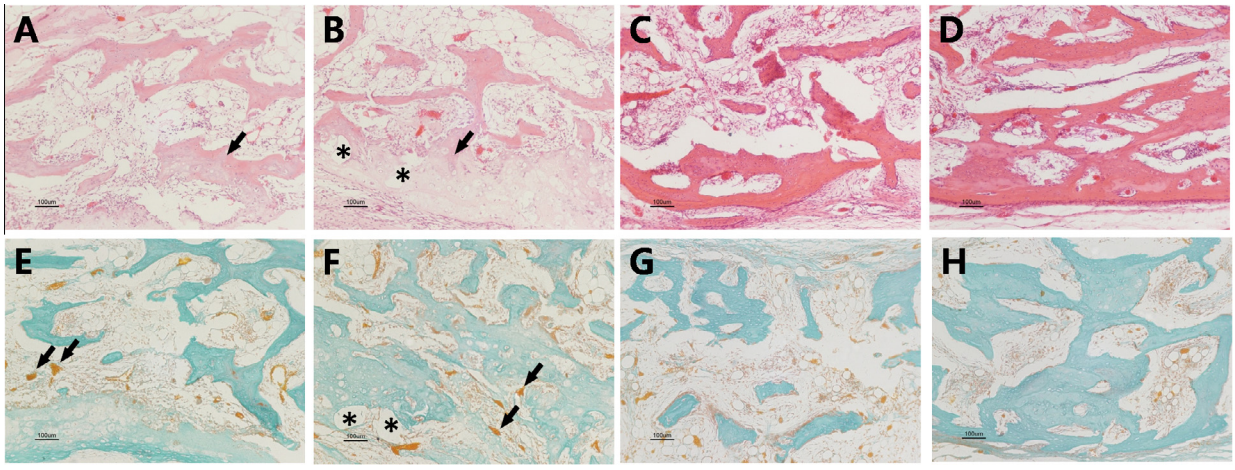

Fig. 4. Histological examination of the *in vivo* constructs in the different implantation groups. (A–D) Hematoxylin-and-eosin staining. A construct in the cell sheet group at 4 weeks (A) and 8 weeks (C) post-implantation, and a construct in the composite group at 4 weeks (B) and 8 weeks (D) post-implantation. The asterisks indicate coral particles and the arrows indicate endochondral bone formation. (E–H) Masson's trichrome staining of the constructs in the different implantation groups. A constructing the cell sheet group at 4 weeks (E) and 8 weeks (G) post-implantation, and a construct in the composite group at 4 weeks (F) and 8 weeks (4H) post-implantation. The asterisks indicate coral particles and the arrows indicate blood vessels.

ties of cell sheets hamper using them to fabricate three-dimensional grafts of adequate shape and size. Combining MSC sheets with custom-shaped scaffold to create tubular bone grafts has been reported previously [4,12,14,15].Although these scaffold-based grafts were strong and could provide mechanical support during the initial stage of bone regeneration, the scaffold restricted cell

migration and the cell viability in the core of the scaffold was low. Our current study demonstrated the feasibility of engineering tubular bone grafts by a novel strategy based on cell sheets and coral particles. The grafts had a maximal size of 30-mm long and 10-mm diameter and therefore were sufficient to repair phalangeal defects. Furthermore, our current approach did not use complex scaffolds, thus minimizing the drawbacks associated with them.

Natural coral (calcium bicarbonate) is commonly used as a bone graft substitute material since it is biocompatible, osteoconductive and biodegradable [16]. Previous studies have shown that the *in vivo* resorption of coral involves a biphasic process: The edges of the coral block become powdery and then are dissolved in the extracellular fluid and phagocytosed [16]. Fricain et al. concluded that contact between the coral crystals and cultured cells is necessary to trigger the process of coral dissolution *in vitro* [17]. We found that the particles added to the MSC sheets were surrounded by ECM and well integrated into the MSC sheets (Fig. 1). In the current study, the initial purpose of incorporating coral particles into the MSC sheets was for them to serve as volume extenders and improve the handling properties of the cell sheets. The coral particles would also increase the surface area and roughness of the cell sheets, making the cell sheets adhere to each other and creating a thick construct. The dissolution of the coral particles would then provide more space for new bone formation. In the experiments, we observed that the composite cell sheets were stronger and had better handling properties than did the cell sheets alone.

Interestingly, our experiments also revealed that the coral particles improved the osteogenic capacity of the cell sheets, as demonstrated by the improvement in bone mineral density and compressive strength. After the coral particles had been completely absorbed, the bone mineral density and compressive strength reached 125.5 ± 8.9 HU and 10.57 ± 1.59N by 8 weeks, respectively, significantly higher than 75.2 ± 6.9 HU and 7.75 ± 0.92N values of the control constructs. There are several possible explanations for this finding. First, enhanced osteoblast differentiation occurred in the presence of dissolved Ca^{2+} in the cell culture media [18]. As demonstrated in our *in vitro* study, the Ca^{2+} concentration increased in the culture medium after coral particles were added to the MSC sheet. This finding indicated that the coral particles were degraded by the cells, releasing Ca^{2+} into the surrounding medium and consequently affecting cell behavior. This result was in agreement with that of a previous study, in which it was demonstrated that an optimal extracellular Ca^{2+} concentration had a beneficial effect on the growth and osteogenic differentiation of MSCs [19]. Second, the oligoelements present in coral have been known to play a critical role in the bone mineralization process and in the activation of enzymatic reactions in osteoid cells [16]. As shown in Fig. 2H, osteoid tissue formation was observed around the coral particles. Moreover, the use of the coral particles likely increased the ECM stiffness of the cell sheets and, therefore, improved the differentiation of MSCs into osteoblasts, because it is known that ECM stiffness influences stem cell differentiation [20].

The widespread application of bone tissue engineering will be highly dependent on the high efficiency of new bone regeneration. To address this issue, we used bioreactors to create the bone grafts *in vitro*. As shown in Fig. 2, after 4 weeks of *in vitro* incubation, the constructs maintained their tubular shape well and possessed sufficient radiological density and compressive strength for implantation, indicating that it is feasible to generate bone grafts using a spinner-flask bioreactor *in vitro*. These observations are consistent with the findings of previous studies in terms of engineering bone using a rotating bioreactor [15,21]. *In vivo* experiments further revealed that the engineered bone grafts had high osteogenic capacity, and by 8 weeks after implantation in subcutaneous sites, the composite sheet constructs had developed into mature tissues, that possessed a mineral density similar to that of the mouse spine. The composite sheets more efficiently improved bone regeneration in the *in vivo* environment than did the cell sheets alone.

The mechanical evaluation showed that integration of coral particles increased the compressive strength from 0.24 ± 0.09N before implantation to 0.55 ± 0.14N. Although this property improved the handling convenience of the graft and maintained the initial tubular shape, the composite graft may not provide sufficient mechanical support at the early stage. However, the grafts exhibited enhanced mechanical properties 8 weeks after implantation. Therefore, if rigid fixation devices are used to provide the initial mechanical support, our tubular bone graft still has a great potential for application even in a load-bearing environment.

Acknowledgment

This study was funded by the National Natural Science Foundation of P.R. China (grants 30770542 and 81170938).

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