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ORIGINAL ARTICLE

Ectopic osteogenesis with immortalized human bone marrow stromal stem cells and heterologous bone

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Summary
To resolve the problem of the insufficient availability of seed cells and to provide seed cells for tissue engineering research, an immortalized human bone marrow stromal stem cell line (MSCxj cells) was established in our department to investigate the ectopic osteogenesis of MSCxj cells.

MSCxjs were grown with a heterogeneous bone scaffold for 48 h. Three groups were included: group A: MSCxjs of 35 PDs were maintained with heterogeneous bone; group B: MSCxjs of 128 PDs were maintained with heterogeneous bone; and group C: heterogeneous bone alone. Tetracycline fluorescence staining, H&E staining, and ponceau staining, immunohistochemistry and bone histomorphometry were performed. At the same time, scanning electron microscopy was conducted to detect the growth of MSCxjs and heterogeneous bone.

Scanning electron microscopy showed favorable adherence of MSCxjs to heterogeneous bone. A large number of newly generated filamentous extracellular matrix and fine granular materials were found to cover the cells. The results from staining showed that the osteogenesis was not obvious in group A/B 4 weeks after transplantation. Eight weeks after implantation, osteoid matrix deposition was noted in and around the heterogeneous bone in group A/B. Twelve weeks after implantation, osteogenesis was increased in group A/B. There were no significant differences in the time course for bone formation and the amount of newly generated bone between group A/B.

Like primary hBMSCs, MSCxj cells have favourable ectopic osteogenesis and can be applied as seeded cells in bone tissue engineering.

Key words: tissue engineering; immortalization; human; bone marrow stromal cells; ectopic osteogenesis; xenografts

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INTRODUCTION

Numerous factors, including trauma, infection and cancer can cause focal or segmental bone loss and generate large gaps between bones, also known as bone defects. A majority of bone defects are difficult to spontaneously heal completely, and bone nonunion is frequently observed in clinical practice. A number of researchers have conducted studies to investigate

bone defects (Zhao et al. 1998, Bauer and Muschler 2000, Byun et al. 2010, Hesse et al. 2010). Recently, as the relevant technology has developed, the repair of bone defects has more often been carried out through tissue engineering. Tissue engineering bone constructed by scaffold, seed cells and growth factors provides a promising strategy for the treatment of bone defects.

Human telomerase reverse transcriptase (hTERT) was introduced into the human bone marrow stromal stem cell line (MSCxj) through liposomes to establish immortalized MSCxjs. The biological features of MSCxjs and functions of adult stem cells have been determined. These MSCxjs have biological features similar to normal human bone marrow stromal stem cells and the plasticity of adult stem cells. MSCxjs can differentiate into osteoblasts *in vitro* (Teng et al. 2007a, b). In the present study, the *in vitro* ectopic osteogensis of MSCxjs was further investigated and the prospect of using MSCxjs in bone tissue engineering was explored.

MATERIALS AND METHODS

Main reagents and materials

The following were used in our study: mouse anti-human osteocalcin monoclonal antibodies (R&D, USA), tetracycline (Sigma, USA), a SM2500E hard tissue microtome and an MPS60 image acquisition and analysis system (Leica, Germany). The reagents used for cell culture were as follows: L-DMEM-low glucose, trypsin (Gibco BRL, USA), fetus bovine serum (FBS; Hyclone, USA), dexamethasone, β-glycerol phosphate, vitamin C (Sigma, USA), Percoll separating solution (Pharmacia, USA) a 24-well plate, a 50 ml flask (Coster, USA), Methyl thiazolyl tetrazolium (MTT; Sino-American Biotechnology Co., Ltd., China) and a CO_2 incubator (Heraeus, Germany). The complete medium was L-DMEM containing 10% FBS, 100 U/ml penicillin and streptomycin. The conditional medium for osteogenesis was the complete medium supplemented with 10^{-8} mol/l dexamethasone, 50 mg/l β-glycerol phosphate, and 10mmol/l vitamin C. The immortalized human bone marrow stromal stem cells were initially prepared by the Orthopaedic Institute of the Fourth Military Medical University and named MSCxj. Antigen-free bovine cancellous bone was developed as the scaffold by the Orthopaedic Institute of the Fourth Military Medical University.

Grouping

Eighteen healthy female nude mice (4–6 weeks) weighing 12–20 g were purchased from the Animal Center of the Forth Military Medical University and were treated as follows: group A: heterologous bone plus MSCxj (35PDs) (n=12); group B: heterologous bone plus MSCxj (128PDs) (n=12); group C: heterologous bone alone (n=12).

Culture of MSCxj

The immortalized MSCxj cells were prepared by the Orthopaedic Institute of the Fourth Military Medical University and labelled. The thawed out MSCxj cells from passage 35 and passage 128 were maintained in an L-DMEM containing 10% serum. Cell adherence and growth were observed under a phase contrast microscope. When the cell confluence reached 90% (after about 5 days of culture), these cells were digested with 0.25% trypsin (1:3 v/v) followed by culture. The cryopreserved cells were thawed and maintained in the medium until 35 population doublings (PDs) or 128 PDs. When the cell number reached 2×10^9, theses cells were digested and then mixed with phosphate-buffered saline (PBS; 5 ml) followed by centrifugation at 250 g for 10 min. These cells were washed with PBS again and the cells were harvested followed by preparation of cell suspension (2×10^9/ml) with 4.5 ml of L-DMEM containing 10% serum.

Preparation of heterologous bones

A bovine heterologous bone scaffold was developed by the Orthopaedic Institute of the Fourth Military Medical University. The degreased and deproteined bovine bone was cut into $3\times3\times3$ mm^3 cancellous bones and washed with double distilled water. These cancellous bones were dried at room temperature for 24 h, wrapped with aluminum foil and then sterilized by cobalt 60 radiation. They were then immersed in PBS for 24 h before use.

Integration of MSCxj and scaffold

The cells were divided into three groups. In group A and B, MSCxj cells of 35 PDs and 128 PDs, respectively, were maintained with the bovine cancellous bone (n=12 per group) when the cell number reached 2×10^9. Group C included the bovine cancellous bone alone (n=12). Under aseptic conditions, the disinfected bovine cancellous bones from the different groups were put into 3 wells (8-well plate) independently. In group A and group B, the MSCxj cell suspension was maintained with bovine cancellous bones through repeated precipitation. Briefly, the cell suspension was dropped onto the pre-moistened bone scaffolds which were then incubated in an incubator. This procedure was repeated once every 4 h for a total of 48 h. In group C, the cell suspension was replaced with PBS.

Forty eight hours later, the cells completely adhered to the scaffolds and cell growth was favourable as demonstrated by the scanning electron microscopy., These scaffolds were then grown in the L-DMEM osteogenic medium (containing 10% FBS, 100 U/ml penicillin and streptomycin, 1×10^{-8} mol/l dexamethasone, 50 mg/l β glycerophosphate and 10 mmol/l vitamin C) for 18 d and the complex of MSCxj cells and bone scaffolds was obtained for further use.

Scanning electron microscopy (SEM) was performed to detect the growth of cells on scaffolds 48 h and 18 d before implantation. The scaffolds were washed with PBS twice and fixed in 3% glutaraldehyde. Dehydration was performed with acetonitrile in a series of concentrations. Critical evaporation and metal spraying were conducted followed by SEM.

Ectopic osteogensis in nude mice

The samples in group A, B and C were paired and a total of 18 subgroups were established. The mice were intraperitoneally anesthetized with 10 g/l sodium pentobarbital (60 mg/kg), a 3 mm incision made at the lateral back of each mouse (n=18) and the complex was implanted. The wound was sutured, the animals numbered and then maintained in cages.

The nude mice were sacrificed 4, 8, and 12 weeks after implantation (6 mice per time point) and each group (A, B and C) had 4 samples at each time point. Ten days before sacrifice at week 4 and 8, animals were fed with chow supplemented with tetracycline as a fluorescence marker for observation of ectopic osteogenesis. Osteoid was observed 8 weeks after implantation. Ten days and 3 days before sacrifice at week 12, animals were intraperitoneally injected with tetracycline hydrochloride (25 mg/kg) for 3 consecutive days for fluorescent double labelling and thereafter observation of ectopic osteogenesis. The animals were killed through cervical dislocation.

Gross observation

The following were noted: contamination by bacteria and fungi, survival and wound healing rate of the mice following implantation, and the color of and coverings on the scaffolds.

Histological examination

Tissues were fixed in 10% formaldehyde and dehydrated with ethanol in a series of concentrations. Transparentization was performed with toluene and then tissues were immersed in methyl acrylic acid methyl ester followed by embedding (Lv and Yang 1999, Lv et al. 2002, 2006). The tissues were cut consecutively into 5 μm sections followed by mounting and observation of tetracycline fluorescence. In addition, H&E staining and ponceau S red staining and immunohistochemistry for osteocalcin were carried out.

Morphometry

The tissues were consecutively cut into 5 μm sections (10 sections) at a low magnification (×10) and after HE staining the levels of osteogenesis were established. The areas with newly generated bones and areas of implanted scaffold were determined with an image analysis system and the proportion of areas with newly generated bones was calculated as follow: proportion (%) = (areas with newly generated bones/ areas of implanted scaffold) ×100%.

Statistics

Data were expressed as means ± standard deviation and analyzed with SPSS statistic software. Comparisons between two groups were performed with t test at the significance level $2\alpha=0.05$.

RESULTS

Features in three-dimensional culture

MSCxjs were maintained with heterologous bones for 48 h, and cells were found on the surface and in the pores of the cancellous bones. The cells were spindle and adherent to the wall. Long cell processes were noted. The cells interacted with each other over time. A large amount of filamentous extracellular matrix and small granular materials were found before implantation and covered these cells (Fig. 1). There was no contamination by bacterial or fungus infection.

Observation of ectopic osteogenesis

Gross features

The complex of MSCxj/ heterologous bone was implanted. The wound healing was acceptable and all mice survived. Four weeks after implantation, fibrous tissues covered the scaffolds and white heterologous bones were frequently observed. Eight weeks after implantation, scaffolds were covered with dark red bone tissues and characterized by red and white bone tissues. Twelve weeks after implantation, the scaffolds were smooth and dark red. However, the scaffolds in control group were white (Fig. 2).

H&E staining, ponceau staining and tetracycline fluorescence staining

Results from H&E staining, ponceau staining and tetracycline fluorescence staining showed that

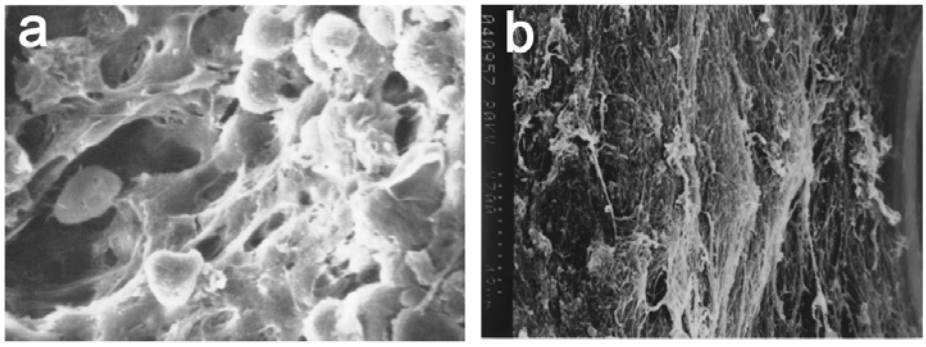

Fig. 1. **Scanning electron microscopy for MSCxjs;** a) MSCxjs were maintained with heterologous bones for 48 h (×1000); b) MSCxjs were maintained with heterologous bones for 18 d. A large amount of filamentous extracellular matrix and small granular materials were found before implantation (×600).

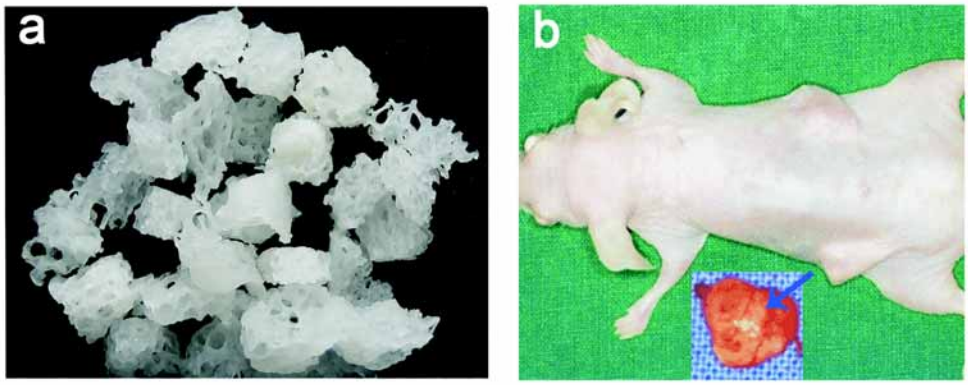

Fig. 2. **Heterologous bones scaffolds and subcutaneous implantation;** a) heterologous bones scaffolds; b) 12 weeks after the complex of MSCxj/ heterologous bone was subcutaneously implanted in the nude mice. The blue arrow indicates the heterologous bone.

osteogenesis was not obvious in group A/B 4 weeks after implantation. Eight weeks after implantation, osteoid matrix deposition was noted around and in the pores of heterogeneous bones in group A/B. In addition, osteoblast-like cells rich in cytoplasm were found and some osteogenesis was observed, but a majority of pores were filled with fibrous tissue. Twelve weeks after implantation, osteogenesis was increased in group A/B. The newly generated bones were located in the pores and nearly normal bone marrow tissues were also found. In the cross section, the scaffolds were surrounded by newly generated bones, and lamellar bones formed. The progressive growth of ectopic bones was noted by tetracycline fluorescence staining. There were no significant differences in the time course for bone formation and the amount of newly generated bone between group A/B. Osteogenesis was not observed in the control group and only a small amount of fibrous tissues were found on the ectopic bones (Fig. 3).

There was no degradation of ectopic bones in group A, B or C.

Immunohistochemistry for osteocalcin

Immunohistochemistry for osteocalcin showed positive staining in the lacunae as shown by arrows (Fig. 4).

Morphometry

Osteogenesis was not obvious in group C at all time points and in groups A and B at 4 weeks after implantation. Therefore, the difference in osteogenesis was not compared between the three groups. The proportion of newly generated bones in group A and group B was 5.64%±2.68% and 4.92%±2.95%, respectively, at 8 weeks after implantation and 13.94%±2.21% and 14.34%±3.46%, respectively, at 12 weeks after implantation. Statistical analysis did not show a significant difference between group A and group B at the same time point (statistically

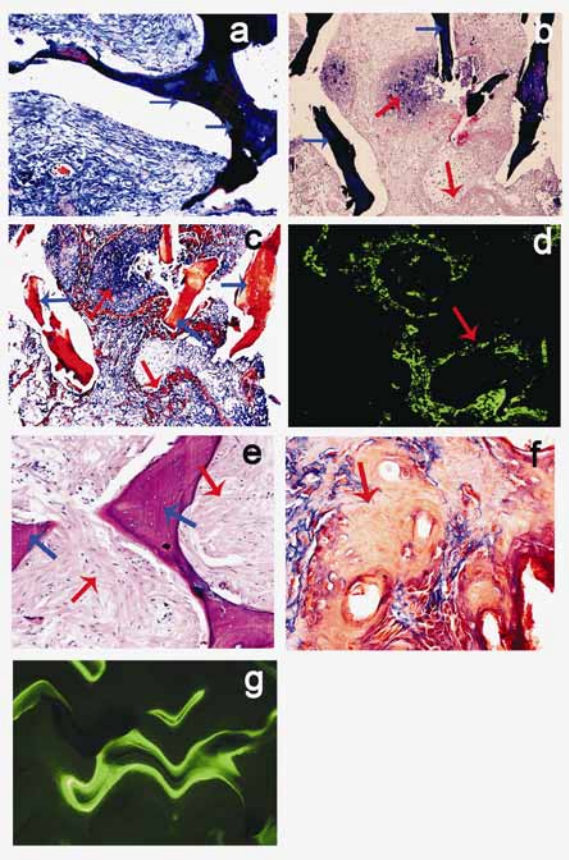

Fig. 3. **Features of ectopic osteogenesis: H&E staining, ponceau staining and tetracycline fluorescence staining;** a) heterologous bone alone (ponceau staining, group C, ×100); b) 8 weeks after implantation of MSCxj/heterologous bone in nude mice (H&E staining, group A, ×100); c) 8 weeks after implantation of MSCxj/heterologous bone (ponceau staining, group A, ×100); d) 8 weeks after implantation of MSCxj/heterologous bone (tetracycline fluorescence staining, group A, ×100); e) 12 weeks after implantation of MSCxj/heterologous bone (H&E staining, group B, ×400); f) 12 weeks after implantation of MSCxj/heterologous bone (ponceau staining, group B, ×400); g) 12 weeks after implantation of MSCxj/heterologous bone (tetracycline fluorescence staining, group B, ×400). The blue arrow indicates the heterologous bone; the red arrow indicates the ectopic osteogenesis.

significant). Furthermore, the proportions of newly generated bones in both group A and group B at 12 weeks after implantation were markedly higher than those at 8 weeks after implantation (statistically significant).

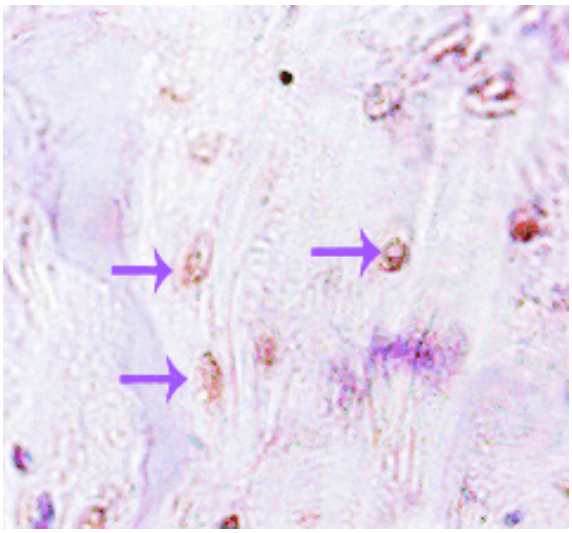

Fig. 4. **Immunohistochemistry for osteocalcin 12 weeks** after the complex of MSCxj/heterologous bone was subcutaneously implanted in nude mice (group B, ×200). The purple arrow indicates the osteocytes.

DISCUSSION

MSCxj act as seed cells in bone tissue engineering, a discipline where seed cells are a key and basic factor. The ideal seed cells should have the following characteristics (Hodgkinson et al. 2009, Cordonnier et al. 2010): (1) It is easy to obtain these seed cells with minimal injury; (2) Seed cells should have the characteristic of directed differentiation into osteoblasts; (3) Seed cells have potent reproductive capacity and an appropriate amount of cells can be obtained. Bone marrow stromal stem cells (BMSCs) have been widely applied in bone tissue engineering in the past decade. However, cell senescence after repeated passaging, the difficulty of collecting a sufficient number of in a timely way and lack of standard cell lines in the laboratories are the key issues in the application of BMSCs (Hodgkinson et al. 2009, Cordonnier et al. 2010, Richardson et al. 2010).

The problem of cell senescence can be resolved through immortalization of bone marrow stromal stem cells (MSCxjs), and the characteristics of adult stem cells of BMSCs are then preserved. In the present study, after osteogenic induction, MSCxjs and ectopic bones were implanted in the nude mice and the ectopic osteogenesis was observed. The results showed that osteogenesis was active and the newly generated bones had nearly normal bone structure.

But osteogenesis was not observed in the mice treated with MSCxjs alone. Immunohistochemistry showed osteocalcin expression in osteoblasts or osteocytes. Our results indicated that MSCxjs cells have the characteristic of ectopic osteogenesis which is similar to primary BMSCs and can be applied as seed cells in the experiments of bone tissue engineering. In addition, a sufficient number of MSCxjs cells can be obtained and these cells may become a standard cell line for research in the laboratories.

The other problem in the application of BMSCs in bone tissue engeering as seed cells is the difficulty of timely collection; the collection of BMSCs from bone marrow puncture to harvesting in sufficient quantities is time consuming and liable to failure (Hodgkinson et al. 2009, Zou et al. 2009, Jukes et al. 2010). MSCxjs can be applied to establish a cell bank which solves this problem, but the transplant rejection of allogeneic cells should be further studied. Some researchers speculate that BMSCs have extremely weak immunogenicity; others even propose that these cells have no immunogenicity, and therefore, allogeneic bone marrow stromal stem cell transplantation is feasible, and favourable results can achieved in animal studies (Itescu et al. 2003, Poncelet et al. 2007, Ren et al. 2010). However, some researchers have an opposite opinion. They postulate that BMSCs still have immunogenicity and results from animal studies cannot be applied to humans. The higher order the animals, the more obvious is the immunological rejection (Spees et al. 2004, Nauta et al. 2006). Therefore, the immunological rejection of MSCxjs, a type of seed cells in bone tissue engineering, should be further clarified. Because of the possibility of immunological rejection, implantation of these cells has been performed in the present study only in nude mice with immunodefficiency. The subcutaneous blood supply in nude mice is not rich and therefore, the osteogenic ability of these cells in unit time is not comparable with that in the large animals described previously.

Scaffold in bone tissue engineering

Currently, the scaffolds are mainly made from (1) synthetic materials including inorganic substances (hydroxyapatite and calcium phosphate) and organic substances (polylactic acid and polyglycolic acid); (2) naturally derived materials including calcined bone, demineralized bone matrix and deproteined bone matrix and other natural coral materials (Liu et al. 2008, Sundelacruz et al. 2009, Cordonnier et al. 2010). The synthetic materials are easy to prepare and not restricted by sources. Recently, with the development of a rapid prototyping technique (He et al. 2010), great progress has been made in research into synthetic materials. However, the effects of the decomposition of organic synthetic materials on cells and the degradation of inorganic materials limit the wide application of scaffolds in clinical practice (Liu et al. 2008, Sundelacruz and Kaplan 2009, He et al. 2010). Numerous studies have show that bio-derived bone after processing not only has low antigenicity but can induce osteogenesis, which facilitates the adherence and growth of seed cells. The development of these materials improves the porosity, pore communication and pore size which are the key problems in the preparation of biomimetic materials. In addition, these materials have a rich source, are easy to prepare and are less costly (Liu et al. 2008, Revell and Athanasiou 2009, Sundelacruz and Kaplan 2009, Bedi et al. 2010, Cordonnier et al. 2010, He et al. 2010). With the development of manufacturing technology and discipline, scaffolds of bio-derived bone will play a critical role in bone tissue engineering.

In the present study, the ectopic bones were developed by our institute. These bones preserve the favourable pore structure of cancellous bone and have low antigenicity. They have been applied as non cell scaffolds in more than 1000 patients in our department, achieving favourable outcomes (Hu and Lu 1990, Yuan et al. 1999, 2003a, b). But evidence on these bones as cell scaffolds is insufficient. In the present study, they were used as cell scaffolds and had good biocompatibility with MSCxj cells. In addition, the cells were well adherent to these scaffolds and acceptable ectopic osteogenesis was observed after implantation of cells and scaffolds. Our results demonstrated these ectopic bones were good scaffolds in bone tissue engineering. However, the degradation of these scaffolds was relatively slow and obvious degradation was not observed even 12 weeks after implantation. Therefore, more studies are required to investigate the degradation of ectopic bones in the bone tissue engineering.

Inoculation of seed cells and scaffold

To tightly and evenly attach numerous seed cells to scaffolds is a problem in bone tissue engineering. Currently, only two methods are adopted (Li et al. 2005, Liu et al. 2008): (1) repeated precipitation, where the cell suspension is dropped onto the scaffolds and then incubated in culture medium in an incubator. These procedures are repeated several times. (2) negative adsorption, where the scaffolds are put into a relative vacuum container containing cell suspension of high density. After negative adsorption, the seed cells are adherent to the scaffolds (Zou et al. 2009, Richardson et al. 2010). However, the negative pressure is hard to regulate and practice should be

performed before application. In the recent years, a bioreactor has been applied in the culture of BMSCs which facilitates the large-scale production of seed cells. In addition, a thermostatic oscillation incubator is used for the inoculation of seed cells and scaffolds. These developments obviously play critical roles in molecular and cellular bioengineering.

CONCLUSION

The present study aimed to investigate the feasibility of MSCxjs as seed cells in bone tissue engineering. Repeated precipitation was applied and ectopic osteogenesis of MSCxjs detected. For small scaffolds, our method is favourable and seed cells could be evenly distributed on and in the scaffolds. Therefore, this method may be applied in thin cancellous bone materials and small granular materials.

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