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Transplantation of human placenta-derived mesenchymal stem cells in a silk fibroin/hydroxyapatite scaffold improves bone repair in rabbits

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The main requirements for successful tissue engineering of the bone are non-immunogenic cells with osteogenic potential and a porous biodegradable scaffold. The purpose of this study is to evaluate the potential of a silk fibroin/hydroxyapatite (SF/HA) porous material as a delivery vehicle for human placenta-derived mesenchymal stem cells (PMSCs) in a rabbit radius defect model. In this study, we randomly assigned 16 healthy adult New Zealand rabbits into two groups, subjected to transplantation with either SF/HA and PMSCs (experimental group) or SF/HA alone (control group). To evaluate fracture healing, we assessed the extent of graft absorption, the quantity of newly formed bone, and re-canalization of the cavitas medullaris using radiographic and histological tools. We performed flow cytometric analysis to characterize PMSCs, and found that while they express CD90, CD105 and CD73, they stain negative for HLA-DR and the hematopoietic cell surface markers CD34 and CD45. When PMSCs were exposed to osteogenic induction medium, they secreted calcium crystals that were identified by von Kossa staining. Furthermore, when seeded on the surface of SF/HA scaffold, they actively secreted extracellular matrix components. Here, we show, through radiographic and histological analyses, that fracture healing in the experimental group is significantly improved over the control group. This strongly suggests that transplantation of human PMSCs grown in an SF/HA scaffold into injured radius segmental bone in rabbits, can markedly enhance tissue repair. Our finding provides evidence supporting the utility of human placenta as a potential source of stem cells for bone tissue engineering.

[**Key words:** Silk fibroin; Hydroxyapatite; Human placenta derived mesenchymal stem cells; Tissue engineering; Bone]

Current approaches to treat large bone defects involve transplantation of autologous bone grafts from the iliac crest, allogenic bone graft, autologous fibula graft transfer, and salvage procedures. However, large bone defects are difficult to treat, and all of the above techniques are limited by inadequate supply of donor bone tissue. Tissue engineering is a promising alternative approach to injury repair that uses biomaterials to restore function of damaged or dysfunctional tissues (1,2). An important goal in bone tissue engineering is to devise methods that can enable bone repair in cases where the loss/damage is significantly high, using biomaterial scaffolds to deliver vital cells to the defective site (3). Scaffolds for osteogenesis should mimic the morphology and structure of the bone, in order to achieve optimal integration of transplanted cells into the surrounding tissue and facilitate normal function (4). Specifically, scaffolds for bone tissue engineering should possess several essential properties, including biocompatibility, controllability, biodegradability, excellent mechanical integrity, and three-dimensional structural properties like surface roughness, porosity, and osteoconductivity, similar to that found *in vivo* in bone tissues (5–7).

Silk biomaterials have received increasing attention as promising scaffolds for tissue engineering, although their biological suitability remains to be established (8–11). In particular, the silk fibroin (SF) secreted by the silkworm *Bombyx mori* has certain desirable properties, including biocompatibility and low immunogenicity (8,9,12). Since it is one of the most abundant naturally found proteins, SF can be obtained easily and at low cost (13,14). Previous studies have shown that SF matrices promote the attachment and proliferation of human bone marrow-derived mesenchymal stem cells (BMSCs), allow calcium deposition on these cells, and facilitate the development of bone-like trabeculae with cuboid cells (15,16). However, despite their potential, because of low hardness and ease of deformation under physical stress, application of pure and unmodified SF materials in bone regeneration is challenging. Hence, there is a need to design new silk-based materials with sufficient hardness, toughness, and other mechanical properties.

The use of stem cells to construct biologically functional tissues or organs has been studied extensively. Currently, the preferred source of mesenchymal stem cells (MSCs) for both experimental and clinical studies is the bone marrow (BM). BMSCs are being evaluated in clinical trials for the treatment of myocardial infarction, and in pre-clinical studies for treating certain vascular and neurological disorders, bone regeneration, and cancer therapy (17,18). Although BM is generally considered to be a safe source of MSCs, the extraction procedure is invasive, and they are not as

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abundant in MSCs as the placenta. Moreover, one major limitation of BMSCs is that their proliferative and differentiation capacity is largely dependent on the age of the donors, and has been shown to decrease significantly in older individuals (19,20). In contrast, the placenta is an excellent source of MSCs for regenerative medicine because of the latter's broad differentiation potential, abundance, and easy accessibility. The lack of ethical concerns associated with the procedure makes it even more favorable (21). We previously found that placental MSCs (PMSCs) can be obtained from human donors and expanded *in vitro*, while maintaining biological characteristics similar to BMSCs (22). In studies that investigated the application of PMSCs in tissue engineering, these cells were shown to retain the biological properties of MSCs (23,24). Finally, bone tissue engineering using PMSCs has enormous applications in bone repair and regeneration (25).

In this study, we expanded human PMSCs *in vitro* and examined their biological properties. Natural bone is a complex tissue comprised of minerals (~60–70% of the total dry weight of bone) deposited in a matrix of type I collagen. The primary mineral found in bone is hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HA) (26,27). Although HA has excellent biocompatibility and bioactivity, the biodegradation of sintered HA ceramics is very slow, and porous HA ceramics are fragile (28). Hence, we propose that the independent limitations of SF and HA as tissue-engineering scaffolds can be overcome by creating a combined SF/HA scaffold. Here, we constructed such a scaffold, used it to deliver PMSCs into defective bone regions, and studied the merits of the approach in bone repair *in vivo*.

MATERIALS AND METHODS

Animal and human tissue Naturally mated 3-month old New Zealand white rabbits were procured from the Laboratory Animal Center of Soochow University. All animal experiments were performed after obtaining permission from the Animal Care Committee of Soochow University. Human placenta from healthy donors were collected during cesarean births ($n = 5$; gestational age: 38–40 weeks) and obtained from the Department of Obstetrics and Gynecology. All women who participated in this study were required to provide written informed consent, which was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Preparation of SF/HA porous scaffold Silk fibers secreted by the domestic *B. mori* were degummed four times with 0.05% (w/w) Na_2CO_3 solution at 90–100°C for 30 min, rinsed thoroughly, and air-dried. The SF thus extracted was then dissolved in a ternary solvent containing $CaCl_2$, CH_3CH_2OH and H_2O in a 1:2:8 molar ratio, at 70 ± 2°C for 1 h. The final regenerated SF solution (~3% wt) was obtained following dialysis and filtration. Next, $Ca(OH)_2$ suspension and diluted H_3PO_4 solution were added to 500 ml deionized water and 100 ml SF solution in a flask. This solution was mixed ultrasonically, resulting in an SF/HA particle suspension after 48 h. Next, short silk fibers (2 mm) were added to the SF/HA particle suspension as reinforcement, at a 1% wt ratio of SF/HA particles to short silk fibers, and subjected to ultrasonic dispersion. Finally, the SF/HA porous material was prepared by pre-shaping the mixture into a column-like mold, followed by isostatic compaction at 250 MPa for 5 min, and air-drying at 60°C. Granular NaCl was added to the material at a 200% wt ratio of SF/HA particles to granular NaCl. The SF/HA scaffolds thus constructed were 4 mm in diameter and 10 mm in height, with an apparent density of 0.74 g/cm^3 and a tensile strength of 10.1 MPa. Also, the scaffolds contained interconnected pores of 30–345 μm diameter (average of 120 μm).

Isolation and culture of PMSCs PMSCs were isolated and cultured as previously described, with some modifications (22). Briefly, the placenta were dissected carefully, into pieces of tissues that were then washed several times with PBS, mechanically minced, and enzymatically digested with 0.25% trypsin-EDTA (Gibco, Invitrogen Corp. Carlsbad, CA, USA) for approximately 10 min at 37°C. The digested tissue was filtered twice through a 100 μm pore size nylon membrane to get rid of undigested pieces. Next, they were centrifuged at 300 ×*g* for 10 min, and the red cells thus collected were lysed in a buffer containing 155 mmol/l NH_4Cl and 20 mmol/l Tris (pH 7.6) for 5 min. The homogenate was then centrifuged and suspended in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/ml penicillin–streptomycin (Gibco, Invitrogen Corp.), and 5 ng/ml basic fibroblast growth factor (b-FGF; Cytolab).

Cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere, with media change once or twice each week. Three to five days after enzymatic treatment, digested residues were removed from the culture. Approximately three to four weeks later, we obtained colonies comprised of ≥50 fibroblast-like cells with >80% confluence. These colonies were recovered with 0.25% trypsin-EDTA and replated at a dilution of 1:3.

Analysis of cell surface markers Trypsinized placental cells were aliquoted into fluorescence-activated cell sorting (FACS) tubes at equal cell numbers (1×10^5 cells per reaction). The following FITC- and phycoerythrin (PE)-conjugated mouse anti-human monoclonal antibodies were added to the cell suspension: CD34-FITC, CD90-PE, CD73-FITC, and HLA-DR-FITC (BD Bioscience), CD45-PE and CD105-FITC (Biolegend). Non-specific background signal was evaluated by staining cells with isotype-matched IgG1-FITC and IgG1-PE control antibodies (BD Bioscience). Cells were incubated with the antibodies in the dark to avoid bleaching, at room temperature (RT) for 20 min. All samples were analyzed using a FACS Calibur (Becton & Dickinson, USA). A minimum of 10^4 gated events were acquired from each sample and analyzed using the CellQuest program (Becton & Dickinson).

Osteogenic differentiation To induce osteogenic differentiation, cells at passage 2–4 (P2–4) were seeded into 6-well plates and cultured using methods described previously in literature, with some modifications (29). Subconfluent cells were treated for 3 weeks with proliferation medium, comprised of 1 μM dexamethasone, 10 mM glycerol 2-phosphate, 50 μM L-ascorbic acid 2-phosphate (Sigma–Aldrich), 10% FBS, and 1× penicillin–streptomycin. Osteogenesis was assessed by von Kossa staining for mineralized calcium phosphate. Briefly, cells were stained with 1% silver nitrate (Sigma–Aldrich) for 45 min under ultraviolet light, followed by 3% sodium thiosulfate (Sigma–Aldrich) for 5 min, and counterstained with van Gieson's stain.

Culturing bromodeoxyuridine-labeled PMSCs on the SF/HA scaffold To perform bromodeoxyuridine (BrdU)-labeling, P3 PMSCs at a concentration of 1×10^5 cells/ml were incubated with BrdU (10 ug/ml) in CM for 48 h. The SF/HA biomaterial was sterilized using ^{60}Co, placed into 24-well plates, and seeded with cells (100 μl of BrdU-labeled PMSC suspension at 1×10^8 cells/ml). The plates were incubated at 37°C and 5% CO_2. After 1 h, the SF/HA biomaterial was turned over, and another 100 μl of cell suspension was added to the upper side, followed by incubation for an additional 1 h. Next, 2 ml of CM was added to each well to immerse the biomaterial, and the cells were cultured for 8 days before transplantation, with media change every 2 days.

Examination of PMSCs-seeded scaffolds under the scanning electron microscope In accordance with previously described methods (30), we cultured PMSCs on SF/HA scaffolds for 1 week, removed and washed gently thrice with PBS, and fixed with 3% glutaraldehyde in PBS at RT for 8 h. To prepare the composites for electron microscopy, they were dehydrated in a graded ethanol series ranging from 50% to 99%, air-dried, and sputter-coated with a 60 nm layer of gold. They were then viewed in the scanning electron microscope (SEM) (S-4700, Hitachi Ltd., Tokyo, Japan).

Animal models and implantation procedure Sixteen rabbits were anesthetized by injecting sodium pentobarbital through an ear vein and fixed in a supine position. To separate the radius, an arc incision was made on the anterolateral side, the radius with periosteum was then cut away 10 mm using a saw. The animals were randomly divided into two groups ($n = 8$ per group), and implanted either with PMSCs + SF/HA composite biomaterials (experimental group) or the SF/HA scaffold alone (control group). Once recovered from the surgery, the rabbits were allowed to move freely in their cages without external fixation.

All rabbits were sacrificed at 12 weeks following surgery. Bone samples from the radius were collected and examined for gross anatomy, histology, and radiographic characteristics. Based on a previous report (31), the radiologic approach was used to assess and evaluate the degree of repair in the defective bone, absorption of the implanted graft, quantity of newly formed bone, and re-canalization of the cavitas medullaris (Table 1).

Immunohistochemical and histological analysis Specimens obtained from the defective bone were fixed in 80% ethanol for 24 h. To prepare for immunostaining, they were dehydrated by passing through a graded ethanol series (70–100%) over 2 weeks, and finally transferred to a methylmethacrylate solution which was allowed to polymerize at 37°C for 24 h. The specimens were then sliced

TABLE 1. Radiographic scoring system.

Characteristic	Points
Appearance of graft	
Resorbed	0
Mostly resorbed	1
Largely intact	2
Reorganizing	3
Quality of union at each end	
Non-union	0
Possible union	1
Radiographic union	2
Total points possible per category	
Graft	3
Proximal union	2
Distal union	2
Maximum score	7