Regeneration of dentin-pulp complex with cementum and periodontal ligament formation using dental bud cells in gelatin-chondroitin-hyaluronan tri-copolymer scaffold in swine

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Abstract: The purpose of this study is to use a tissue engineering approach for tooth regeneration. The swine dental bud cells (DBCs) were isolated from the developing mandibular teeth, expanded *in vitro*, and cultured onto cylinder scaffold gelatin-chrondroitin-hyaluronan-tri-copolymer (GCHT). After culturing *in vitro*, the DBCs/GCHT scaffold was autografted back into the original alveolar socket. Hematoxylin and eosin (H&E) staining combined with immunohistochemical staining were applied for identification of regenerated tooth structure. After 36-week post-transplantation, tooth-like structures, including well-organized dentin-pulp complex, cementum, and periodontal ligament, were evident *in situ* in two of six experimental animals. The size of the tooth structure ($1 \times 0.5 \times 0.5$ cm^3 and $0.5 \times 0.5 \times 0.5$ cm^3 size) appeared to be dictated by the size of the GCHT scaffold ($1 \times 1 \times 1.5$ cm^3). The third swine was demonstrated with irregular dentin-bony like calcified tissue about 1 cm in diameter without organized tooth or periodontal ligament formation. The other three swine in the experimental group showed normal bone formation and no tooth regeneration in the transplantation sites. The successful rate of tooth regeneration from DBCs/GCHT scaffolds' was about 33.3%. In the control group, three swine's molar teeth buds were removed without DBCs/GCHT implantation, the other three swine received GCHT scaffold implants without DBCs. After evaluation, no regenerated tooth was found in the transplantation site of the control group. The current results using DBSs/GCHT scaffold autotransplantation suggest a technical breakthrough for tooth regeneration. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 86A: 1062–1068, 2008

Key words: tooth regeneration; dental bud cells; scaffold; gelatin-chondroitin-hyaluronan tri-copolymer

INTRODUCTION

Tooth development is a process which involves the reciprocal and sequential signals between epithelial and mesenchymal tissue.[1] Tooth loss has many causes including physical trauma, gum disease, and tooth decay. Missing teeth can result in movement of the remaining teeth, difficulty with chewing and a lack of self confidence.[2] Currently, there are several approaches to replace teeth including the use of dentures, bridges, and implants, all of which are based on nonbiological techniques and none of which is without problems.[2] Developing a therapy technique to allow individuals to grow replacement teeth *in situ* would provide a remedy for many problems encountered with false teeth and implants such as poor fit and implant rejection.

Regenerative tissue engineering requires integration of three key elements: responding progenitor/stem cells, indicative morphogenetic signals (Morphogens), and extra cellular matrix scaffolds.[3] Adult stem cells are highly plastic and multipotent.[4] Dental pulp stem cells and bone marrow stromal cells could be reprogrammed into odontogenic process and

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participated in tooth formation.[4] *In vitro* and *in vivo* studies have shown that dental pulp stem cells can undergo differentiation into odontoblast-like cells and regenerate dentin-pulp-like structures.[5,6] However, a drawback of the procedure is the inability to determine whether the tooth will function when transplanted in the jaw.[7] Therefore, the present study was designed to evaluate whether transplantation of dissociated dental bud cells (DBCs) into its microenvironment would induce tissue-engineered tooth growth in the swine jaw.

Tooth tissue engineering studies have used polyglycolic acid (PGA), poly-L-lactic acid (PLA), or a copolymer of PGA and PLA (PLGA) to make scaffolding.[8–10] These materials have certain shortcomings in that their degradation products are acidic and lower the pH value around the tissue after *in vivo* implantation which can cause severe inflammation.[11] Gelatin-chondroitin-hylauronan tri-copolymer scaffold had been developed for cartilage regeneration.[12] This tri-copolymer formed from gelatin, chondroitin, and hyaluronan had a uniform pore size of 180 μm and a porosity of 75%. It has good biocompatibility, biodegradable, and produces nontoxic metabolites. Previous study had shown that this scaffold had provided information for cell attachment to meet the requirement for dynamic reciprocity for cartilage tissue engineering. Therefore, we utilized gelatin-chondroitin-hylauronan tri-copolymer scaffolding for this tooth regeneration investigation.

MATERIALS AND METHODS

Preparation of gelatin-chondroitin-hyaluronan tri-copolymer scaffold

Preparation of Gelatin-Chondroitin-Hyaluronan Tricopolymer Scaffold was performed as described previously,[12] briefly, gelatin powder (0.5 g, G-2500; Sigma Co., St. Louis, USA), sodium hyaluronate (HA) (5 mg, 0.5 mL; Seikagaku Co., Tokyo, Japan), and chondroitin-6-sulfate (C6S) powder (0.1 g; Sigma Co., St. Louis, USA) were mixed with 7 mL of double-distilled water and crosslinked for 2–3 min at 25°C using 2 mL of 1% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), the pH of the solution being 5–6. The solution was frozen at −20°C for 1 h, frozen at −70°C for 1 h, and then lyophilized for 72 h. The dried scaffold was recrosslinked for 24 h at room temperature, using 10 mL of 0.2% EDAC, and then lyophilized for 72 h. A tri-copolymer scaffold disc about 5 cm in diameter and 1 cm thick was produced which was cut into small scaffold cylinders sized about $1 \times 1 \times 1.5$ cm^3 for the experiment.

Culturing of DBCs

Surgery was used to remove the preerupted molar tooth from a 1.5-month-old swine (weight: 10 kg). All experiments involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at National Taiwan University, Taiwan. The animals were anesthetized by intramuscular injection of atropine (0.07–0.09 mg/kg) and Zoletil® (0.55–0.8 mg/kg), and then intravenous injection of Citosol® (1.11–1.66 mg/kg) to maintain anesthesia. In addition, a local anesthetic, Lidocaine (2.0 mg/kg) was administered by infiltration at the buccal aspects of the mandibular ridge. A crestal incision was performed with a vertical releasing incision, and a full thickness flap was elevated exposing the lateral wall of the mandibular bone. Anterior and posterior releasing incisions were made at the suspected position around the unerupted molar tooth bud (based on X-rays: 0.06 s, 46–50 kvp, 150 mA) [Shown by Fig. 1(a)]. X-ray was used to carefully check that no tooth tissue remained in the alveolar socket [Fig. 1(b)]. Dental bud tissues were washed by PBS (Phosphate Buffer Solution) and then the calcified tissues, including dentin and enamel tissues, were removed from the tooth bud with scalpel and forceps in laminar flow. All tooth bud tissues were then minced into <1 mm^3 pieces and cultured in Dulbecco's modified eagle's medium (DMEM) + 1.5% Penicillin/streptomycin + 20% Fetal Cal Serum (FCS) at 37°C in 5% CO_2. Cells released from the tissue fragment were grown to confluence in ~7 days. After cultured for 3 weeks, about 1×10^7 cells were obtained. In this study, DBCs in the third passage were used for further experiment.

DBCs seeded onto gelatin-chondroitin-hyaluronan tri-copolymer scaffold

Fabricated gelatin-chrondroitin-hyaluronan-tri-copolymer (GCHT) scaffold cuboids were sterilized with 75% ethanol. The DBCs were isolated by 2 m*M* EDTA/PBS and resuspended at a concentration of 1×10^6 to 1×10^7 cells/mL DMEM. Then 1 mL of cell suspension was injected into $1 \times 1 \times 1.5$ cm^3 size GCHT scaffolding. The DBCs/GCHT scaffold was then cultured in a spinner flask bioreactor for 1 week. The spinner flask was stirred at 70 rpm for 10 h to distribute the cells more evenly, and then at 50 rpm during the remaining culture period.

Implantation in experimental animals

The autotransplantation was performed, meaning that the cells of the donor animal in the DBCs/GCHT scaffold were the same as in the recipient animal. For six swine in the experimental group, the DBCs/GCHT scaffold was implanted into the swine's original alveolar socket. In addition, some DBCs/GCHT scaffold samples were fixed individually with 10% neutral formalin and 2.5% glutaraldehyde for further scanning electron microscopy investigation of the cell-scaffold interactions.

In six swine of the control group, three swine's molar tooth buds were removed but no DBCs/GCHT scaffolds were implanted, the other three swine received GCHT scaffold implants without DBCs.

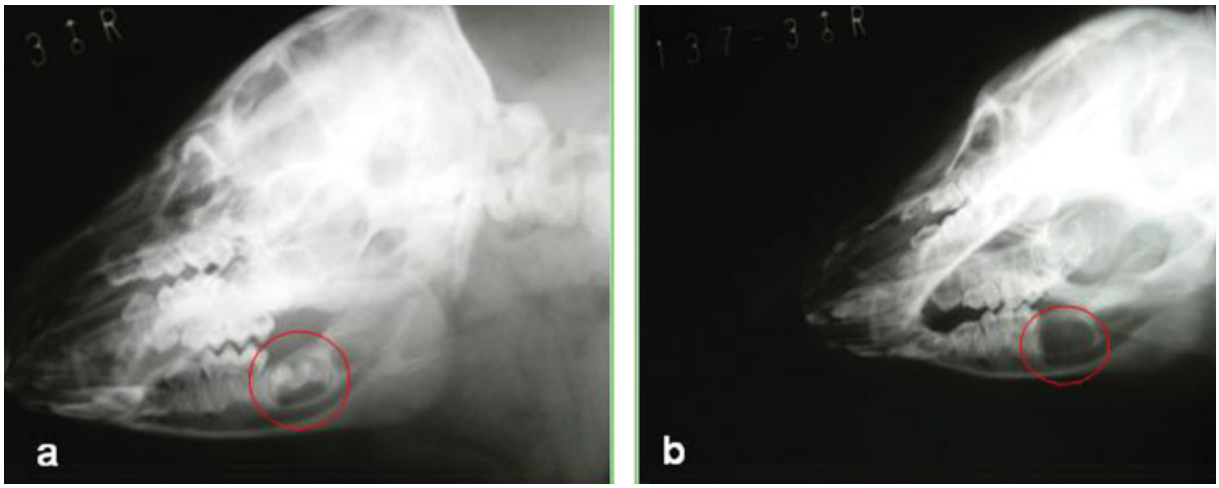

Figure 1. Radiograph image of the operation site of swine demonstrated (a) the pre-operation view of the second molar tooth bud in the lower jaw bone position, and (b) the post-operative view of the site of surgical removal of tooth bud in the same area. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

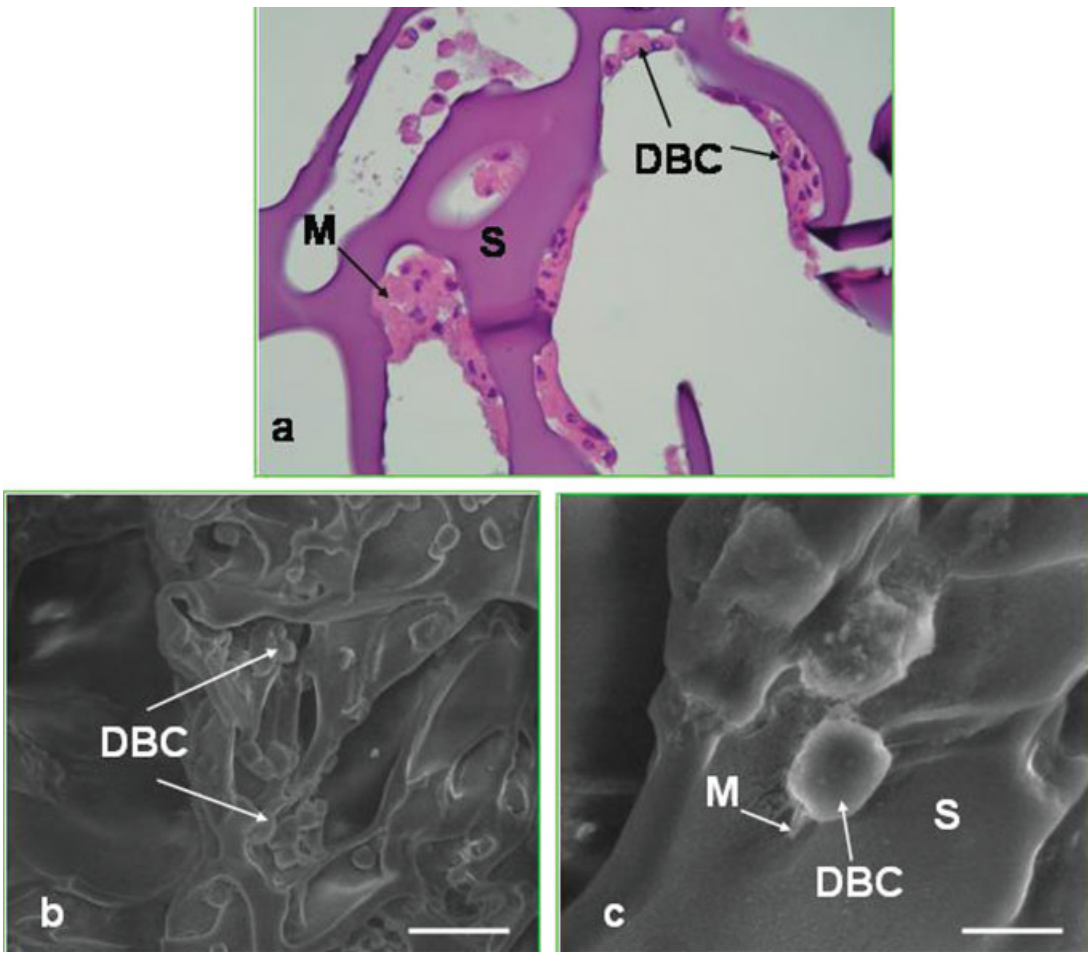

Figure 2. Dental bud cells seeded in GCHT scaffold and cultured in spinner flask for 1 week and viewed by (a) microscopy investigation after stained with hematoxylin and eosin showing that the pericellular matrix(M) around dental bud cells (DBC) stains light eosinophilic, while the GCHT scaffold (S) stains darker eosinophilic (×400), (b) ESEM investigation demonstrates even distribution of cells in the scaffold (scale bar: 20 μm) and (c) ESEM examination in higher magnification demonstrates the attachment of dental bud cells (DBC) on the GCHT scaffold (S) with extracellular matrix (M) (scale bar: 10 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

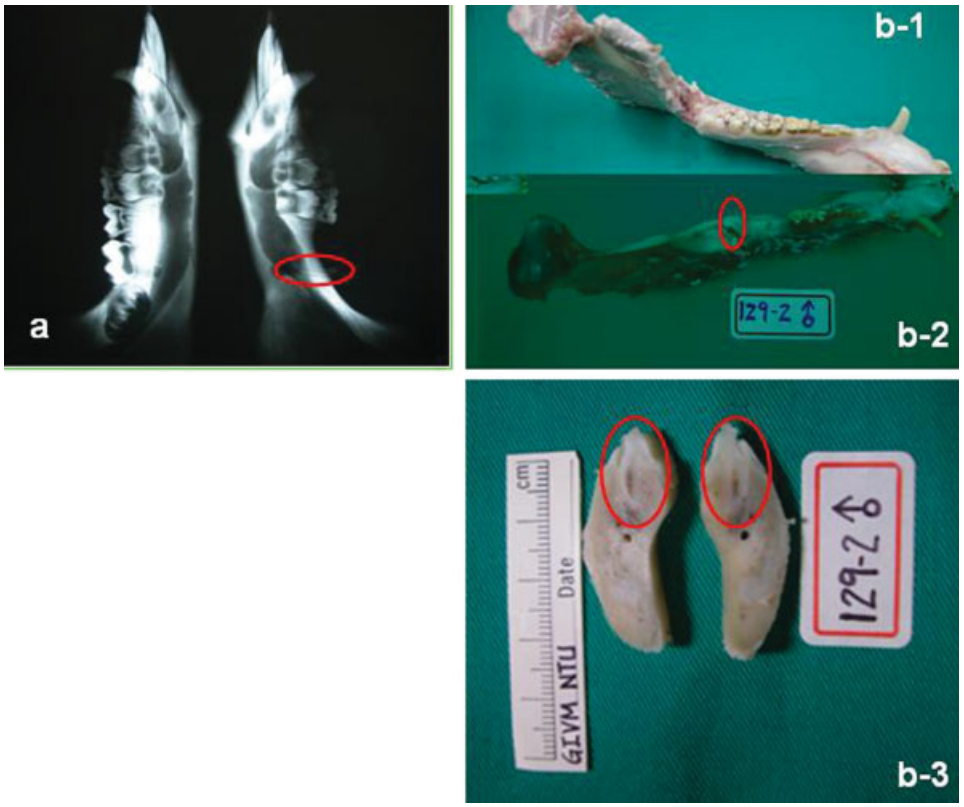

Figure 3. (a) Radiograph images of swine jaw bone demonstrated normal growth of the teeth in the left control site without removal of tooth buds and visible radio-opaque region (red circle region about 1×0.5 cm^2) in the right experimental site indicated the possible regeneration of tooth after removal of tooth buds and transplantation of DBCs/GCHT scaffold for 36 weeks. (b) Gross view of dissected jaw bone demonstrated the intact and normal growth of teeth in the left control side without removal of tooth buds (b-1), and the regenerated tooth (indicated with red circle) in alveolus in the experimental side (b-2), and the images of vertical section views of the regenerated tooth in the jaw bone about $1 \times 0.5 \times 0.5$ cm^3 size (b-3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Histology and immunohistochemical analysis

After X-ray examination, a histological sample was taken from each swine that had a region of tooth regeneration in the mandibular bone. Samples were fixed with 10% neutral formalin, embedded in paraffin after decalcification, and stained with hematoxylin and eosin (H&E). Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Primary antibody used for immunohistochemical analysis was affinity-purified rabbit anti-pig bone sialoprotein (BSP) (obtained from Chemicon, USA). Primary antibody was used at 1:200 dilutions. Controls included the omission of primary antibody.

RESULTS

Characterization of cultured DBCs

By explant culture methods, DBCs released from the tissue fragment were grown to confluence in ~7 days. The DBCs appeared to be heterogeneous, consisting of fibrous, mesenchymal-like cells and clusters of smaller, epithelial-like cells. After cultured for 3 weeks, about 1×10^7 cells were obtained.

DBCs/GCHT scaffold constructs analyzed by Environmental Scanning Electron Microscope and H&E stain

Using H&E staining and environmental scanning electron microscope (ESEM) scanning, the growth of DBCs on GCHT scaffolds [Fig. 2(a–c)] were demonstrated with extracellular matrix for attachment on the scaffold [Fig. 2(a,c)]. The tri-copolymer scaffold is biodegradable and had a uniform pore size of 180 μm with a porosity of 75%. This highly porous structure allows cell penetration, growth, and proliferation.

Radiographic analysis

Thirty-six weeks after surgery, evaluation by X-ray (0.06 s, 60–70 kvp, 150 mA) was done. In the experi-

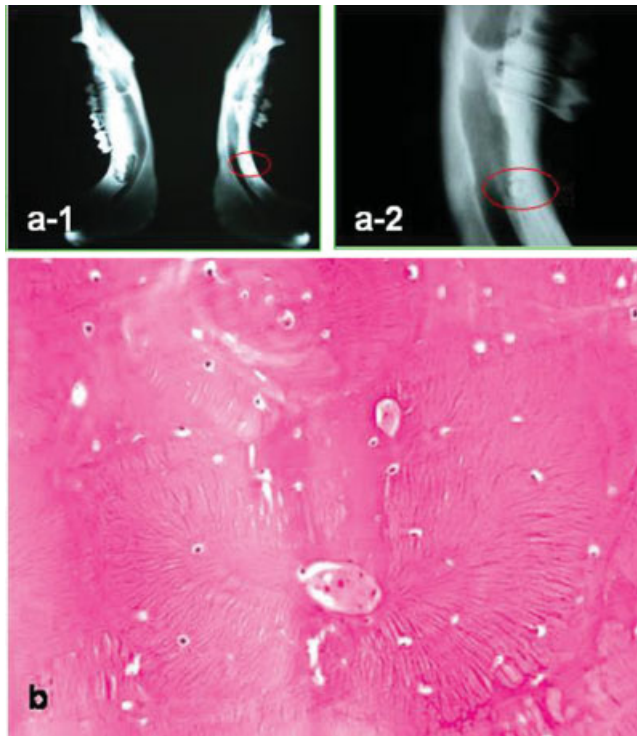

Figure 4. (a-1) Radiograph images of the third swine jaw bone demonstrated normal growth of the teeth in the left control site without removal of tooth buds and visible radio-opaque region (red circle region about 1 cm in diameter) in the right experimental site indicated the possible generated calcified tissue after removal of tooth buds and transplantation of DBCs/GCHT scaffold for 36 weeks. (a-2) Radiograph image of the third swine jaw bone demonstrated visible radio-opaque region (red circle region about 1 cm in diameter) in the right experimental site indicated the possible generated calcified tissue after removal of tooth buds and transplantation of DBCs/GCHT scaffold for 36 weeks. (b) Microscopic views of section of the calcified tissue in the radio-opaque region in the third swine of the experimental group with hematoxylin and eosin staining demonstrated the irregular dentin-bony like calcified tissue without organized tooth or periodontal ligament (magnification ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mental groups, six swine were implanted with DBCs/GCHT scaffolds into the original alveolus. Regenerated teeth were seen by X-ray examination, especially a recognizable tooth formations of 1×0.5 cm^2 size [Fig. 3(a,b)] and 0.5×0.5 cm^2 size (no data shown) in two different swine mandibular alveolus, and the third swine showed a 1 cm diameter radio-opaque region [Fig. 4(a-1,a-2)]. The other three swine had no evident radio-opaque or tooth regeneration results (no data shown). There is also no evident radio-opaque or tooth regeneration in the control group either in the three swine with molar tooth buds removal and no DBCs/GCHT scaffolds implanted or in the other three swine received only GCHT scaffold implants without DBCs.

Histology and immunohistochemical analyzed regeneration teeth

The regenerated tooth containing dentin/pulp-like complex structure a recognizable tooth formations of $1 \times 0.5 \times 0.5$ cm^3 size with evident odontoblasts and blood vessels in the pulp [Fig. 5(b)] and appearance of cellular cementum as well as periodontal ligament attached to surrounding bone was shown by H&E staining [Fig. 5(c)]. We detected bone sialoprotein (BSP) expression in dental pulp and dentin by immunohistochemical methods (Fig. 6). The other regenerated tooth with $0.5 \times 0.5 \times 0.5$ cm^3 size was with similar results. The third swine had a 1 cm diameter radio-opaque region [Fig. 4(a-1,a-2)] stained by H&E Staining presented irregular dentin-bony like calcified tissue [Fig. 4(b)] but no organized tooth or periodontal ligament was found and there was no signs of inflammation or granulation tissue formation in the transplantation site after 36 weeks. The other three swine in the experimental group showed no tooth regeneration, and the transplantation sites were demonstrated with normal bone tissue without inflammation or granulation tissue formation after 36 weeks. In the control groups, swine with molar tooth buds removal but not implanted with DBCs/GCHT scaffolding (three swine) and swine with molar tooth buds removal and GCHT scaffold implanted showed no regenerated tooth and the transplantation site was shown with normal bone tissue without inflammation or granulation tissue formation after 36 weeks.

DISCUSSION

Adult stem cells are highly plastic and mutlipotent.[4] These cells, include dental pulp stem cells and bone marrow stromal cells, can be reprogrammed into an odontogenic fate and participate in tooth formation.[4,5,13] Gronthos et al. have identified a population of postnatal dental pulp stem cells (DPSCs) in human dental pulp, which may be one of the sources of these precursor cells.[5,14] *In vitro* and *in vivo* studies have shown that DPSCs can undergo differentiation into odontoblast-like cells and regenerate a dentin-pulp-like complex when transplanted into nude mice.[5]

Our study demonstrated regeneration of tooth with root formation and dentin, pulp cementum, and even periodontal ligament were evident under H&E staining. We demonstrated the most difficult part of tooth regeneration with root and periodontal ligament formation achieved by using DBCs/GCHT scaffolds implanted into the swine mandibular alveolus. Thirsty-six weeks after implantation, DBCs/

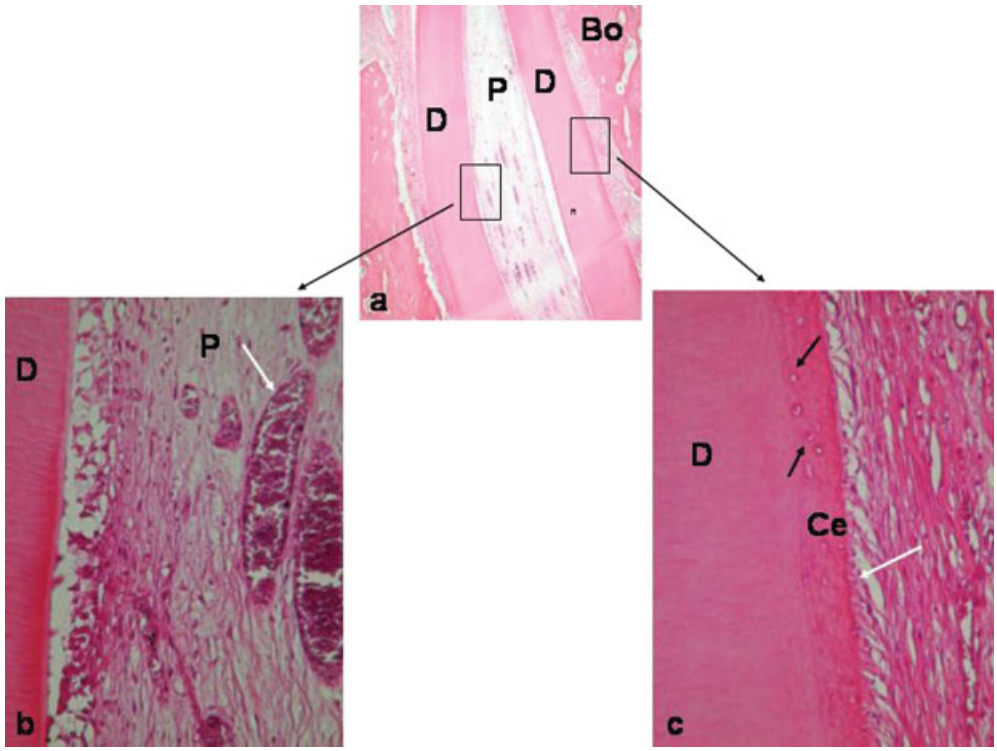

Figure 5. Microscopic views of section of decalcified regenerated tooth structures fixed in the alveolar bone with hematoxylin and eosin staining, (a) demonstrated the regeneration of dentin-pulp structures grown in the aloveolar bone (Bo) with periodontal space and the dental pulp (P) formed in between the dentin (D) (magnification ×100), (b) is the magnified image of (a) in dentin-pulp site demonstrated the distribution of dentinal tubules in the dentin (D) and the distribution of blood vessels (indicated with white arrow) in the pulp (P) (magnification ×200), and (c) is the magnified image of (a) in the dentin-cementum-periodontal site demonstrated the evident structures of dentinal tubules in dentin (D), some lacuna with cells (probably cementoblasts, indicated with black arrows) in cementum (Ce) and different orientated periodontal fibers in the periodontal ligament (indicated with white arrow) (magnification ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

GCHT scaffolds had recognizable, $1 \times 0.5 \times 0.5$ cm^3 and $0.5 \times 0.5 \times 0.5$ cm^3 sized, regenerated teeth in two of six swine examined by X-ray. In the remaining swine, a 1 cm diameterirregular dentin-bony like calcified tissue without organized tooth or periodontal ligament was seen. The other three swine had no tabulatable results. We isolated a clonogenic, rapidly proliferative population of cells from swine dental bud. The proportion of successful regeneration in tooth was 33%.

Our findings indicate that DBCs survive and regenerate tooth structures in the swine jaw, including dental pulp, odontoblasts, dentin, cementum, and periodontal ligament. However, regenerated teeth were with smaller size than normal. Enamel structure was not evident in our section of staining due to the procedure of decalcification. Hence, it is important to bear in mind the role of the dental development stage. Another possibility is that different individual swine, different DBCs viability, and *in vivo* of development time had an influence on the results. Interestingly, Rutherford suggests that the size and shape of the scaffold controls the size and shape of regenerated dentin.[15] In our experiment, the regenerated tooth structures were about $1 \times 0.5 \times 0.5$ cm^3 and $0.5 \times 0.5 \times 0.5$ cm^3 sized, even the swine with radio-opaque region and irregular dentin-bony like tissue formation also with about 1 cm in diameter, the scaffold used in our experiment is about $1 \times 1 \times 1.5$ cm^3 in size which may limit the real size of the regenerated tooth structure.

In other reported study, canine first molar tooth bud cells had been used for seeding on PGA scaffold and then transplanted into the same sockets by Honda et al.[7] Their results showed formation of dentin and bone from dissociated odontogenic cells in the canine jaw.[7] Our results demonstrated not only the dentin-pulp structure, moreover, we had found the well developed root with cementum and periodontal ligament attached to the bone. The presence of dentin tubules in the dentin was evident with expression of bone sialoprotein. In this study, the size of the regenerated tooth is similar to the size of the gelatin-chondroitin-hyaluronan tri-copolymer

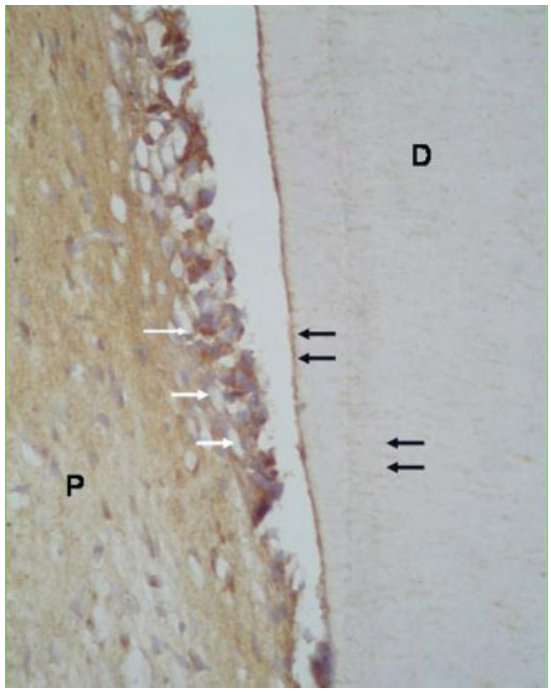

Figure 6. Microscopic view of bone sialoprotein (black arrow) expression in dentin (D), odontoblast (white arrow), and dental pulp (P) of the regenerated tooth after immunohistochemical staining (×400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

scaffold. This is a very important breakthrough; further study would be using different size of the scaffolds for performing the morphology control of the regenerated tooth.

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