Stem Cells from Deciduous Tooth Repair Mandibular Defect in Swine

INTRODUCTION

Reconstruction of orofacial defects secondary to tumors and trauma relies on different sources of bone grafts with inherent morbidity. Stem-cell-based tissue engineering is a promising alternative for bone regeneration (Petite et al., 2000; Bianco et al., 2001; Rose and Oreffo, 2002). The stem-cell-based therapeutic approach can restore bone defects without incurring graft donor site morbidity. Bone marrow mesenchymal stem cells (BMMSCs) have emerged as an important cell source for bone regeneration (Gronthos et al., 2003; Mankani et al., 2006; Mastrogiacomo et al., 2007), as has been demonstrated clinically in femur fracture repair and regeneration (Shao et al., 2006). Previous studies have indicated that the orofacial bone and dental tissues contain a variety of stem cells, including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), and orofacial mesenchymal stem cells (Akintoye et al., 2006). These orofacial stem cells possess a higher proliferation capacity when compared with BMMSCs, and their origin may be associated with neural crest cells (Gronthos et al., 2000; Shi et al., 2002; Shi and Gronthos, 2003; Seo et al., 2004; Akintoye et al., 2006). When transplanted subcutaneously into immunocompromised mice, SHEDs, an easily accessible stem cell source, are capable of generating robust amounts of bone in vivo (Miura et al., 2003; Seo et al., 2008), suggesting a potential for bone regeneration. To explore the feasibility of using SHED-based bone regeneration to treat orofacial bone defects, we utilized swine as a pre-clinical animal model to test the regeneration of critical-size mandibular defects using SHEDs. Our previous studies demonstrated that miniature pigs (minipig) are appropriate large-animal models for oral and craniofacial tissue engineering and therapies (Shan et al., 2005; Sonoyama et al., 2006; Wang et al., 2007; Yan et al., 2007; Liu et al., 2008). In this study, we utilized minipigs as a large-animal model to examine the feasibility of using autologous stem cells derived from miniature pig deciduous teeth—that is, SPD—to repair critical-size mandibular bone defects.

MATERIALS & METHODS

Animals

Sixteen inbred female minipigs (4–6 mos old, weighing 20–30 kg each) were obtained from the Institute of Animal Science of the Chinese Agriculture University. Minipigs were kept under conventional conditions, with free access to water and a regular supply of soft food diet. The study was performed in accordance with a protocol approved by the Animal Care and Use Committees of Capital Medical University.

Cell Culture

Deciduous incisor pulp tissues from first and second deciduous incisors were harvested from 16 inbred female minipigs. SPDs were isolated and cloned following established
protocols (Gronthos et al., 2000; Miura et al., 2003; Seo et al., 2008; Appendix).

**Immunocytochemistry**

SPDs were subcultured in 24-chamber slides. Cells were fixed in 4% paraformaldehyde for 15 min, blocked with non-specific antibodies, and incubated with either anti-STRO-1 (R&D, Minneapolis, MN, USA) at dilutions of 1:200 to 1:500 or anti-vimentin (Chemicon, Temecula, CA, USA) at a dilution of 1:500 for 1 hr according to the manufacturer’s protocol. To test mouse anti-human STRO-1 antibody cross-reactivity with pig tissues, we performed immunocytochemical stain on human SHEDs, minipig SPDs, and lymphocytes, and found that only human SHEDs and pig SPDs were specifically stained with human STRO-1 antibody. Based on these findings, we utilized mouse anti-human monoclonal antibody to STRO-1 in this study. Samples were subsequently incubated with goat secondary antibodies for 45 min, and observed by fluorescence microscopy. Non-immune serum served as negative control. Subsequently, sections were counterstained with DAPI. We used an alkaline phosphatase detection kit (Chemicon) to examine the expression of ALP according to the manufacturer’s protocol, and the result was observed by light microscopy.

**Flow Cytometric Analysis**

Detached cells were permeabilized with PBS containing 0.1% (wt/v) saponin at room temperature for 20 min. After being blocked with normal serum, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated STRO-1 antibodies (R&D Systems; clone STRO-1) or phycoerythrin (PE)-conjugated ALP antibodies (R&D systems; clone B4-78) for 30 min at room temperature. After 3 washes with PBS containing 0.1% saponin, fluorescence was analyzed by a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, Palo Alto, CA, USA). Positive cells were identified by comparison with the corresponding isotype controls (FITC- or PE-conjugated IgG) in which a false-positive rate of less than 2% was accepted.

**Transfection of eGFP Genes**

Conditional retroviral supernatants were produced by the stable retrovirus-producing cell lines PT67/eGFP as described previously (Brazelton and Blau, 2005; Zhang et al., 2005). For transfection, about 1 x 10^6 SPD grown in 6-well plates were incubated for approximately 20 hrs with a mixture of 1 vol of viral supernatant and growth medium at equal vols and in the presence of 8 μg/mL polybrene (Sigma, St. Louis, MO, USA). A repeated transfection was performed in a period of 72 hrs, and the transfected cultures were selected with G418 (100 μg/mL, Sigma). The transfection efficiency of the cells was 80%.

**Scanning Electron Microscopy**

GFP-positive SPDs were grown on β-TCP carrier (Biomedical Materials and Engineering Center of Wuhan University of Technology, China) for 7 days and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hrs at 4°C. The samples were examined under a Hitachi S-520 scanning electron microscope (Hitachi, Tokyo, Japan; http://www.hitachi.com/).

**Reconstruction of Mandibular Bone Defects in Swine Model with SPDs**

A critical-size defect of 2.5 x 1.5 x 1.5 cm^3 was surgically created in the parasympyseal region of the mandible in 10 minipigs for a long-term (24 wks) follow-up so that we could evaluate bone regeneration (Henkel et al., 2005). In another 6 minipigs, we created 2 smaller defects of 1.0 x 1.0 x 0.5 cm^3 bilaterally in the parasympysis of the mandible and evaluated short-term post-surgical follow-ups, at 2 wks (n = 3) and 4 wks (n = 3) (Appendix Fig. 1).

**Computed Tomography (CT) Assessment of Bone Formation**

CT imaging of the minipig’s mandible (Siemens Company, Bensheim, Germany) was carried out at 2, 4, 12, and 24 wks after transplantation at a scanning length of 0.75 mm.

**Quantitative and Histological Evaluation of Regenerated Bone**

Bone specimens were fixed in 10% buffered formalin. Half of the specimens were decalcified and embedded in paraffin, while the other half were processed as non-decalcified. Sections of 5- to 6-μm thickness from the embedded specimen were stained with H&E. The extent of ossification within each section was analyzed semi-quantitatively by histomorphometric techniques (Appendix). The non-decalcified sample was sectioned into a 400-μm series of slices (Donath and Breuner, 1982), and GFP-labeled SPDs were observed by fluorescence microscopy (Olympus BX/TF, U-LH100HG, Tokyo, Japan). For pair-wise comparison, data were analyzed by one-way ANOVA and the Bonferroni method.

**RESULTS**

**Isolation and Characterization of SPDs**

In culture, SPDs form adherent clonogenic cell clusters of fibroblast-like cells, similar to the morphology described in SHEDs; approximately 67 single colonies were generated from 10^5 single cells cultured at low cell density. These colony-forming cells were spindle-shaped (Fig. 1A) and positively stained for STRO-1, vimentin, and ALP (Figs. 1B-1D). Flow cytometric analysis showed an average of 10.9% of SPDs positive for STRO-1 and 21.1% positive for ALP (Figs. 1G, 1H), suggesting a heterogeneic population of cells, as previously reported in other post-natal mesenchymal stem cells, including bone marrow (Shi and Gronthos, 2003), dental pulp (Gronthos et al., 2000), and periodontal ligament stem cells (Seo et al., 2004).

**Expression of eGFP Gene and Fabrication of an SPD-Scaffold Construct**

The retroviral transfection was carried out on day 2 in culture, followed by a repeated transfection on day 3. Subsequently, transfected SPDs were selected with G418 (100 μg/mL), and colonies expressing green fluorescence were expanded in growth medium (Fig. 1E). After several passages, the progenies of GFP-positive SPDs continued to express eGFP.

For fabrication of the cell-scaffold construct, approximately 2 x 10^4-4 x 10^5 GFP-positive SPDs at third and fourth passages were seeded onto β-TCP scaffolds and cultured in growth medium for 7 days in vitro. SEM studies showed that GFP'-SPDs were able to grow on β-TCP scaffolds (Fig. 1F).
SPD-mediated Bone Regeneration in the Reconstructed Mandibular Defect

At 24 wks, animals were killed, and the mandibles were harvested for CT, gross morphology, and histological analyses (Fig. 2, Appendix Fig. 2). CT scan analyses showed nearly complete regeneration of initial defect sites, with new bone formation in the SPD/β-TCP treatment group as shown in coronal (Fig. 2A) and axial sections (Appendix Fig. 2A), three-dimensional view (Appendix Fig. 2B), as well as gross bone morphology of a sagittal bone section (Appendix Fig. 2C). Histological sections of the SPD/β-TCP grafted site revealed newly formed lamellar bone and degraded β-TCP scaffold (Fig. 2B). In contrast, in the β-TCP group, the defect site was only partially restored, with significant remaining defect in the lateral cortex of the mandible (Fig. 2C, Appendix Figs. 2D, 2E, and 2F). Histologically, the scaffold-treated defect was partially filled with some new bone formation, connective tissues, and degraded β-TCP (Fig. 2D). In the control, or untreated, group, a marked bone defect remained with predominantly connective tissues (Figs. 2E, 2F). The regenerated defect site displayed a mean of 83.1% of mineralized matrix in the SPD/β-TCP-treated group, significantly higher than the 52.2% in the scaffold group (P < 0.01), and 28.4% higher than in the control group (P < 0.01) (Fig. 2G).

At 4 wks post-transplantation, CT scan revealed partial bridging of the lateral cortical continuity defect and moderate bone regeneration in the SPD/β-TCP-treated group, in conjunction with early scaffold degradation (Fig. 3A). The gross view of the reconstructed defect site displayed marked absorption of scaffold at the junction of native bone and graft in the SPD/β-TCP-treated group at the early stage of bone healing (Appendix Fig. 3B). Relatively intact scaffold was observed in the β-TCP group (Appendix Fig. 3C), and a large bony defect remained in the untreated group (Appendix Fig. 3D). Histological sections of decalcified specimens showed abundant new islands of bone and blood vessels amid degraded scaffold in the SPD-treated group at 4 wks post-reconstruction (Figs. 3B, 3C). In contrast, in the β-TCP group, the lateral cortical rim defect remained, with minimal β-TCP absorption (Fig. 3D). The bone void at the defect site remained unfilled in the control group (Fig. 3G, Appendix Fig. 3D). Histological sections showed a lack of new bone formation at the junction of β-TCP scaffold (Figs. 3E, 3F) and abundant connective tissue proper in the untreated group (Figs. 3H, 3I). These findings demonstrated that bone regeneration was significantly higher in the SPD/β-TCP-treated group as compared with the β-TCP group. In the blank (control) group, limited bone regeneration was observed in the defect area, which was filled predominantly with connective tissue proper (Figs. 3G, 3H, 3I, Appendix Fig. 4D).

Detection of Transplanted GFP-positive SPD in Regenerative Bone

To identify if the GFP-positive SPDs engrafted at the transplanted bone defect sites had differentiated into osteoblasts, we first screened non-decalcified sections using light microscopy for location of osteoblasts and bone lacunae in the new bone at 2 wks and

Figure 1. Characterization of stem cells derived from miniature pig deciduous teeth (SPD). (A) Single colonies of SPD showed typical fibroblast-like morphology under light microscopy. (B) SPDs expressed STRO-1 by immunohistochemical staining with anti-STRO-1 antibody and (C) ALP-positive staining with the alkaline phosphatase detection kit under light microscopy. (D) Immunohistochemical staining with antivimentin antibody showed 99.5% positive staining. (E) GFP-expressed SPDs showed fibroblast-like morphology. (F) Scanning electron microscopy revealed GFP-labeled SPDs grown on β-TCP scaffold. (G-H) An average of 10.9% of the 3rd-passage SPDs were positively stained for STRO-1 (G) and 21.1% for ALP by flow cytometric analysis (H). Abbreviations: SPD, stem cells from pig deciduous teeth; ALP, alkaline phosphatase. Scale bars: 100 μm in A; 30 μm in B, C, D, E; 4 μm in F.
Figure 2. SPD-mediated bone regeneration of critical-size mandibular defect at long-term follow-up. (A, B) SPD/β-TCP-treated group: CT coronal image showed a large quantity of new bone formation filling the bone defect at 24 wks post-transplantation; yellow dot area indicates the original site of the bone defect (A); the histological section shows that the defect was filled with new bone (B). (C, D) β-TCP-treated group: CT coronal image shows remaining smaller bone defect at the reconstructed defect site; histological section shows that the defect was partially filled with connective tissues, β-TCP scaffold, and new bone formation (D). (E, F) Untreated, or control, group: CT coronal image shows limited bone regeneration and large bone defect remaining; histological section shows that the defect was filled primarily with connective tissues. (G) Semi-quantitative analysis of bone formation showed a statistically significant increase in mineralized matrix formation at the regenerated defect site, 83.1 ± 5.75% (mean ± SD, n = 4) in the SPD/β-TCP treated group, compared with 52.2 ± 4.54% (n = 3) in the scaffold group (P < 0.01), and 28.4 ± 2.79% (n = 3) in the control group (P < 0.01). Scale bars: 1 cm in A, C, E; 50 μm in B, F; 30 μm in D. BT, bone tissue; BV, blood vessel; CT, connective tissue.

Figure 3. SPD-mediated bone regeneration of small-size mandibular defect at the short-term (wks) follow-up. (A, B, C) SPD/β-TCP-treated group: CT axial image showing replacement of β-TCP scaffold with new bone restoration [red arrow], and bridging of the lateral cortical defect with new bone formation (blue arrow). H&E stain of the regenerated defect site at 10X (B) and 40X (C) revealed several new islands of bones and blood vessels amid degraded scaffold. (D, E, F) β-TCP-treated group: CT axial scan showing a partial bridging of the cortical defect and no visible bone formation; H&E stains (E, F) showing sparse new bone formation at the junction of β-TCP scaffold and partially degraded β-TCP. (G, H, I) Untreated, or control, group: CT axial scan showing a continuity defect in the lateral cortex and lack of bone regeneration. Histological sections show primarily connective tissues filling the defect of the control group (H and I). Scale bars: 1 cm in A, D, G; 1 mm in B, E, H; 100 μm in C, F, I. BT, bone tissue; BV, blood vessel; CT, connective tissue.

DISCUSSION

Craniofacial tissue engineering by stem cells is a fast-moving field with considerable potential clinical applications (Mao et al., 2006; Kaigler et al., 2006; Zhao et al., 2007). Deciduous tooth stem cells are an easily accessible stem cell source and capable of robust ex vivo expansion for several potential clinical applications (Miura et al., 2003; Mao et al., 2006; Seo et al., 2008). Embryologically derived from the neural crest cell, SHEDs and SPDs may share similar tissue origin with the mandibular bone cells, and therefore, may serve as a better cell source for the regeneration of alveolar and orofacial bone defects. A previous study showed that the bone-regenerative capacity of SHEDs was similar to that of bone marrow mesenchymal stem cells (BMMSC) when transplanted into immunocompromised mice at 8 wks post-transplantation (33% mineralized matrix/area in the SHED group vs. 31% mineralized matrix/area in the BMMSC group) (Seo et al., 2008). Similar to SHEDs and other post-natal mesenchymal stem cells derived from bone marrow (Shi and Gronthos, 2003), dental pulp (Gronthos et al., 2000), and periodontal ligament stem cells (Seo et al., 2004), it is not unexpected that the SPDs are a heterogeneous population of cells which, following ex vivo cultures, may display different percentages of cells positive for STRO-1, or form mineralized nodules, or be positive for OI1 red O under differentiation inductive conditions.

In this study, to investigate SPD-mediated bone formation in vivo, we labeled SPDs with GFP and used SPDs to repair critical-size bone defects in the mandibles in swine. At 2 and 4 wks post-operatively (Figs. 4A-4D). Using the same visual field, we captured GFP expressing SPDs under a fluorescence microscope (Figs. 4B, 4D). When both images in Figs. 4C and 4D were overlaid, the locations of some GFP-positive SPDs were superimposed with the osteoblasts and bone lacunae (Fig. 4E, yellow arrows showing GFP-negative osteoblasts, red arrows showing GFP-positive osteoblasts), suggesting that GFP-positive SPD cells might have differentiated to osteoblasts. As expected, the normal bone tissue section showed a uniformly distributed green fluorescence signal without local accumulation of GFP-positive cells under immunofluorescent microscopy (Fig. 4F).
green fluorescence signals were detected by fluorescence microscopy within newly formed woven bone. Photomicrographs of the same visual fields confirmed that GFP-labeled SPDs had differentiated directly into new bone, while the normal bone tissue section was non-specifically labeled with green fluorescence. These findings suggested that SPDs were engrafted to some extent at the treated site and contributed to new bone regeneration in the restoration of the bone defect in the swine mandible model. The results of CT scan, gross view, and histological analyses consistently showed that the SPD group had the earliest and strongest capacity of bone regeneration compared with other groups. At 4 wks post-transplantation, β-TCP was partly degraded and was replaced with a large quantity of new bone formation. At 6 mos post-reconstruction, the defects in the SPD group were markedly restored with new bone, while in the β-TCP group and control group, much less bone regeneration and predominantly connective tissue granulation were evident at the defect sites. Further studies to regulate bone regeneration are under way to optimize the transplanted stem cell numbers, scaffolds, and their immediate niche component.

Overall, our study provides the first evidence that SPDs are capable of regenerating critical-size defects in the orofacial bone in a large-animal model, specifically swine, and may potentially serve as an alternative stem-cell-based approach in the reconstruction of alveolar and orofacial bone defects.

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