

Transplantation of Mesenchymal Stem Cells Is an Optimal Approach for Plastic Surgery

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ABSTRACT

Mesenchymal stem cells (MSCs) are able to differentiate into a variety of cell types, offering promising approaches for stem cell-mediated tissue regeneration. Here, we explored the potential of utilizing MSCs to reconstruct orofacial tissue, thereby altering the orofacial appearance. We demonstrated that bone marrow MSCs were capable of generating bone structures and bone-associated marrow elements on the surfaces of the orofacial bone. This resulted in significant recontouring of the facial appearance in mouse and swine. Notably, the newly formed bone and associated marrow tissues integrated with the surfaces of the recipient bones and re-established a functional bone marrow organ-like system. These data suggested that MSC-mediated tissue

regeneration led to a body structure extension, with the re-establishment of all functional components necessary for maintaining the bone and associated marrow organ. In addition, we found that the subcutaneous transplantation of another population of MSCs, the human periodontal ligament stem cells (PDLSCs), could form substantial amounts of collagen fibers and improve facial wrinkles in mouse. By contrast, bone marrow MSCs failed to survive at 8 weeks post-transplantation under the conditions used for the PDLSC transplantation. This study suggested that the mutual interactions between donor MSCs and recipient microenvironment determine long-term outcome of the functional tissue regeneration. *STEM CELLS* 2007;25:1021–1028

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow MSCs have been identified as a population of hierarchical postnatal stem cells with the potential to differentiate into a variety of cell types including osteoblasts, chondrocytes, adipocytes, cardiomyocytes, myoblasts, and neural cells [1–11]. These cells were initially identified by their capacity to form clonogenic adherent cell clusters with a fibroblastic morphology (colony-forming units-fibroblast [CFU-F]) in vitro [4, 12–16]. Each colony represents a strain of cells derived from the proliferation of a single precursor cell. Previous studies documented that individual bone marrow MSC colonies demonstrated marked differences in their proliferation rates and developmental potential both in vitro and in vivo [4, 8, 13, 17–19]. Although bone marrow MSCs possess the capacity for multipotent differentiation, it appears that they consistently generate bone and associated marrow organ-like structures only in vivo [20]. One of the most striking characteristics of the xenogeneic transplantation system is that bone marrow MSCs organize and support hematopoietic marrow elements during the osteogenic process [21]. This may represent a dynamic process whereby

multipotential MSCs strive to reconstruct the microenvironment from which they were derived [18, 20, 21]. MSCs have also been successfully utilized for the treatment of bone fracture, severe aplastic anemia, and grade IV acute graft-versus-host-disease in humans [22–24], probably through modulation of immune cell responses [25]. Additionally, bone marrow MSCs were used to cure osteogenesis imperfecta [26–29] and generate adipose tissue for soft tissue augmentation and reconstruction [30]. This accumulated evidence highlights a great potential of using MSCs for clinical treatments.

Periodontal ligament stem cells (PDLSCs) were identified as a specific MSC population derived from periodontal ligament with expression of array of osteogenic markers alkaline phosphatase (ALP), matrix extracellular phosphoglycoprotein (MEPE), bone sialoprotein (BSP), and osteocalcin; mesenchymal stem cell marker STRO-1; and tendon marker scleraxis [31]. Although PDLSCs are capable of differentiating into cementogenic, adipogenic, and collagen-forming cells, they are not able to generate bone and associated marrow structures in vivo [31]. Therefore, in this study, we explore the potential of using bone marrow MSC-mediated bone regeneration and PDLSC-mediated collagen regeneration for plastic surgery, in-

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cluding reshaping facial contour and improving facial appearance in animal models.

MATERIALS AND METHODS

Subjects and Cell Culture

Normal human impacted third molars ($n = 18$) were collected from 16 adults (18–20 years of age) at the Dental Clinic of the National Institute of Dental & Craniofacial Research (NIDCR) under approved guidelines set by the National Institute of Health (NIH) Office of Human Subjects Research. Periodontal ligament (PDL) was gently separated from the surface of the root and then digested in a solution of 3 mg/ml collagenase type I (Worthington Biochemicals Corp., Freehold, NJ, <http://www.worthington-biochem.com/>) and 4 mg/ml dispase (Roche Diagnostics, Basel, Switzerland, <http://www.roche-applied-science.com/>) for 30 minutes at 37°C. PDL was used to obtain single-cell suspensions by passing the cells through a 70- μm strainer (Falcon; BD Labware, Franklin Lakes, NJ, <http://www.bdbiosciences.com/>). Single-cell suspensions (1×10^6) of PDLSCs were seeded into 10-cm culture dishes (Costar, Cambridge, MA) with α -modified Eagle's medium (Gibco; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) supplemented with 15% fetal calf serum (Equitech-Bio Inc, Kerrville, TX, <http://www.equitech-bio.com/>), 100 μM L-ascorbic acid 2-phosphate (WAKO, Tokyo, Japan, <http://www.wako-chem.co.jp/english>), 2 mM L-glutamine, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>), then incubated at 37°C in 5% CO_2 . Human bone marrow cells were purchased from commercially available resources (AllCells LLC, Berkeley, CA, <http://www.allcells.com/>). To identify putative bone marrow MSCs, single-cell suspension of 1×10^6 of bone marrow mononuclear cells were seeded into 15-cm culture dishes, and nonadherent cells were removed after 3 hours of incubation at 37°C. The adherent cells were cultured in the same medium used for PDLSC culture. To further confirm the MSCs, individual single colonies were examined to show a positive staining of STRO-1, an identified mesenchymal stem cell marker. All primary cells used in this study were at 1–3 passages.

Antibodies

Rabbit anti-matrixextracellular phosphoglycoprotein (MEPE; LF-155), type III collagen (LF-70), and ALP (LF-47) antibodies (Abs) were provided by Dr. Larry Fisher (NIDCR/NIH, Bethesda, MD). The following antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ, <http://www.bd.com>): mouse monoclonal antibody (mAb) against green fluorescent protein (GFP); R-phycoerythrin (R-PE)-conjugated rat mAbs against CD45, CD9, CD11b, and IgM molecules; and R-PE-conjugated isotype-matched control Abs. Anti-human-specific mitochondria Ab was purchased from Chemicon (Temecula, CA, <http://www.chemicon.com>); anti-STRO-1 Ab was from Dr. Stan Gronthos (Institute of Medical and Veterinary Science, Adelaide, Australia). Rabbit and murine isotype-matched negative control Abs were obtained from Caltag Laboratories (Burlingame, CA, <http://www.caltag.com/>).

Transplantation

Approximately 8.0×10^6 of in vitro expanded human bone marrow MSCs were mixed with 80 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (Zimmer Inc., Warsaw, IN, <http://www.zimmer.com/>) and then transplanted subcutaneously into the calvaria surface of 10-week-old immunocompromised beige mice (NIH-bg- ν/ν -xid; Harlan Sprague Dawley, Indianapolis, <http://www.harlan.com/>) as previously described [32]. For PDLSC transplantation, culture-expanded 4×10^6 PDLSCs were loaded onto 10-mm \times 10-mm collagen-based gelatin sponge Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI) as a carrier vehicle to transplant subcutaneously into immunocompromised mice. These procedures were performed in accordance with specifications of an approved animal protocol (NIDCR 04-317 and the University of Southern California 10874). The transplants were recovered at 4–8 weeks post-transplantation, fixed with

4% formalin, decalcified with buffered 10% EDTA (pH 8.0), and then embedded in paraffin. Sections were deparaffinized and stained with hematoxylin and eosin (H&E).

Immunohistochemistry

The section of MSC transplants was deparaffinized and then blocked and incubated with primary antibodies (1:200–1:500 dilution) for 1 hour. For immunohistochemical detection, Zymed SuperPicTure polymer detection kit (Invitrogen) was used according to the manufacturer's protocol.

Picrosirius Red Staining

Paraffin sections were dewaxed and rehydrated. The sections were treated with 0.2% phosphomolybdic acid for 2 minutes. After they were washed with distilled water, slides were stained with picrosirius red solution (0.1% sirius red in saturated picric acid) for 10 minutes, followed by 0.01 N hydrochloric acid treatment for 2 minutes, and slides were dehydrated and mounted.

Reverse Transcriptase Polymerase Chain Reaction

The polymerase chain reaction (PCR) primers included granulocyte colony-stimulating factor (G-CSF) α -chain, 5'-AAGAGCCCCCT-TACCCACTACACCATCTT-3' (forward primer), 5'-TGCTGT-GAGCTGGGTCTGGGACACTT-3' (reverse primer), 340 base pairs (bp); granulocyte-macrophage colony-stimulating factor (GM-CSF), 5'-CCCATCACTGTACCCGGCCTTGG-3' (forward primer), 5'-GTCCGTTTCCGGAGTTGGGGGC-3' (reverse primer), 279 bp; interleukin (IL)-6, 5'-GACAAAGCCAGATCCTTCAGAGAG-3' (forward primer), 5'-CTAGGTTTGCCGAGTAGATCTC-3' (reverse primer), 229 bp; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AGCCGCATCTTCTTTGCGTC-3' (forward primer), 5'-TCATATTTGGCAGGTTTTTCT-3' (reverse primer), 816 bp. Total RNA isolation, first-strand cDNA synthesis, and PCR processes were described previously [33].

Swine Model

Six inbred male miniature pigs (minipigs; 4–8 months old, weighing 20–40 kg) were obtained from the Institute of Animal Science of the Chinese Agriculture University. Animals were kept under conventional conditions with free access to water and feed. This study was reviewed and approved by the Animal Care and Use Committees of Capital Medical University and the Institute of Dental and Craniofacial Research. Minipigs were anesthetized with a combination of ketamine chloride (6 mg/kg) and xylazine (0.6 mg/kg) before the surgery. Minipig bone marrow MSCs were isolated and cultured under the same condition as described herein for human MSC culture and expansion. The animals were randomly divided into experimental and control groups. A highly porous scaffold HA/TCP block (25 mm in diameter and 10-mm thick with 60% HA and 40% TCP; 200–300- μm pore size; Sichuan University, Chengdu, China) was incubated with 10×10^6 minipig bone marrow MSCs for 90 minutes. Latergrade incision was made on the frontal region subcutaneously, and a suitable tunnel was inactively decohered under the periosteum. HA/TCP-MSC complexes were autologously transplanted under the periosteum of three animals in the experimental group for 8 weeks. Meanwhile, HA/TCP were implanted into the frontal region subcutaneously in the control group ($n = 3$).

Scanning Electron Microscopy

MSC transplant samples were cut into approximately 1-mm cubes and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde for 2 hours. After being washed with sodium dimethylarsenate buffer, they were postfixed in 1% osmium tetroxide, dehydrated with gradient alcohol, and then incubated with isoamyl acetate. After gold coating, the samples were examined under a Hitachi S-520 scanning electron microscope (Hitachi, Tokyo, <http://www.hitachi.com/>).

In Situ Hybridization

Human-specific *alu* and murine-specific *pfl* sequences labeled with digoxigenin were used as probes for in situ hybridization as previously described. Primers included: human *alu*, sense, 5'-TGGCT-

CACGCCTGTAATCC-3' (base number, 90–108), antisense, 5'-TTTTTTGAGACGGAGTCTCGC-3' (base number, 344–364; GenBank accession number, AC004024); and murine *pfl*, sense, 5'-CCGGCAGTGGTGGCGCATGCCTTAAATCCC-3' (base number, 170–201), antisense, 5'-GTTTGGTTTTGAGCAGGGT-TCTCTGTAGC-3' (base number, 275–306, GenBank accession number, X78319). The probes were prepared by PCR containing 1× PCR buffer (PerkinElmer, Waltham, MA, <http://www.perkinelmer.com>), 0.1 mM 2'-deoxyadenosine 5'-triphosphate, 0.1 mM 2'-deoxycytidine 5'-triphosphate, 0.1 mM 2'-deoxyguanosine 5'-triphosphate, 0.065 mM 2'-deoxythymidine 5'-triphosphate, 0.035 mM digoxigenin-11-2'-deoxyuridine 5'-triphosphate, 10 pmol of specific primers, and 100 ng of human genomic DNA as templates. Unstained sections were deparaffinized and hybridized with the digoxigenin-labeled *alu* or *pfl* probe using the mRNAlocator-Hyb Kit (Ambion Inc., Austin, TX, <http://www.ambion.com/>). After being immunoreacted with anti-digoxigenin ALP-conjugated Fab fragments (Roche Diagnostics), the presence of *alu* or *pfl* in tissue sections was detected by using a 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution (Roche Diagnostics).

Bone Marrow Cell Homing

Immunocompromised mice with 8-week MSC transplantation received intravenous injection of 64 mg/kg cyclophosphamide (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) dissolved in phosphate-buffered saline (PBS) once a day for 4 days. Then bone marrow cells (1.5×10^7 all nuclear cells/body) derived from long bones of enhanced GFP (eGFP) transgenic mice (Jackson Laboratory, Bar Harbor, ME, <http://www.jax.org/>) were administered intravenously through the tail vein into the cyclophosphamide-preconditioned immunocompromised mice. Homing of eGFP-positive bone marrow cells into the MSC transplants was examined at 8 weeks postinjection by immunohistochemical and fluorescence-activated cell sorting (FACS) analyses.

FACS Analysis

Cells (1×10^6) isolated from the MSC transplants or the long bones of nontransplanted immunocompromised mice were incubated with 1 μ g of phycoerythrin (PE)-conjugated mAbs for 45 minutes at 4°C. PE-conjugated isotype-matched IgG was used as a control. After being washed with PBS and 0.4% bovine serum albumin, the cells were applied to FACS analysis.

RESULTS

Bone Marrow MSC-Mediated Reconstruction of the Orofacial Region in Mice

To assess the functional role of bone marrow MSC-mediated bone regeneration in plastic surgery, *ex vivo* expanded human bone marrow MSCs were transplanted into the surfaces of the frontal bones of immunocompromised mice using HA/TCP as a carrier vehicle, resulting in a considerable alteration of the orofacial outline (Fig. 1A, 1B). Bone marrow MSCs differentiated into osteoblasts to form new bone and associated hematopoietic marrow elements at 8 weeks post-transplantation (Fig. 1C). Newly formed bone tissue in the MSC transplants connected with the surface of the frontal bone to maintain appropriate position of the transplants (Fig. 1C). Moreover, reconstruction of the hematopoietic niche microenvironments in the MSC transplants preserved the important homeostasis between bone marrow MSCs and hematopoietic stem cells (HSCs), to ensure the formation of a novel bone and associated marrow organ-like structure (Fig. 1C). Conversely, HA/TCP particle transplants led to a limited amount of inductive bone formation at the interface area between the HA/TCP particles and the calvarial bone (Fig. 1D). By further evaluating human MSC-mediated osteogenesis in the orofacial region, we found that newly formed bones could connect with the mouse calvarial

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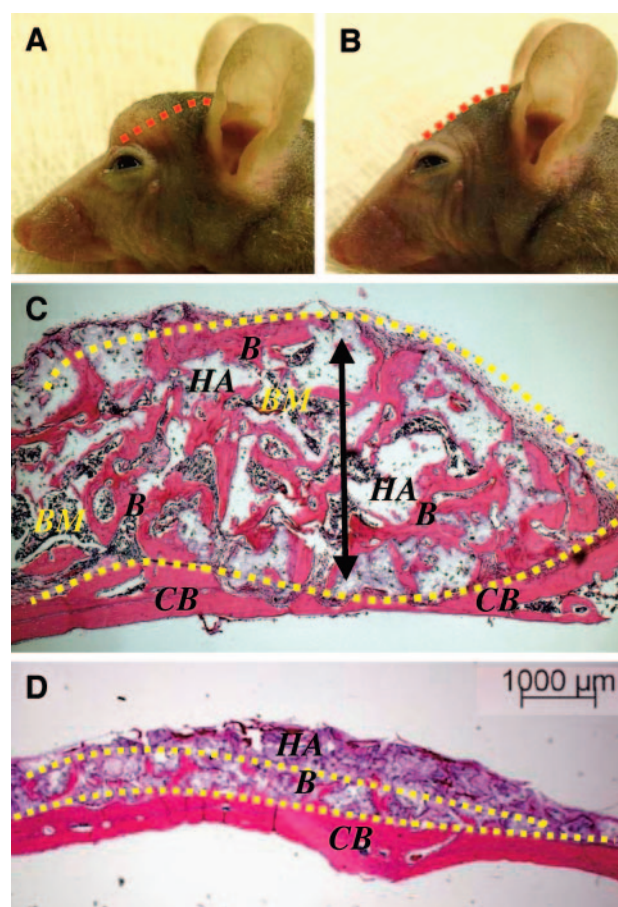


Figure 1. Human BM MSC-mediated reconstruction of the orofacial region in immunocompromised mice. (A): Transplantation of BM MSCs using HA as a carrier vehicle can significantly alter facial appearance in mice. The red dashed line represents the original surface line of the forehead. (B): MSC nontransplanted mice used as a control. (C): Hematoxylin and eosin staining showed that robust amounts of bone and hematopoietic BM were regenerated in BM-MSCTransplant mice. The interface between the newly formed bone and CB was sufficiently integrated, as indicated by the lower yellow dashed line. The black arrow represents the height of the newly formed bone in the MSC/HA transplant. (D): By contrast, transplanted HA particles produced only a limited amount of inductive bone formation in control mice. Abbreviations: B, bone; BM, bone marrow; CB, calvarial bone; HA, HA/TCP.

bone via connective tissues (Fig. 2A) and directly integrate with the HA/TCP particles (Fig. 2B). We also confirmed that osteogenic cells in the MSC transplants expressed osteogenic markers, including ALP, type III collagen, and MEPE (Fig. 2C–2F). These data suggest that MSCs are capable of mediating reliable osteogenesis in the orofacial region.

We previously demonstrated that bone marrow MSC-regenerated bone and associated marrow structures were analogous to regular bone and associated marrow tissues containing functional hematopoietic niches [21]. These findings indicate a practical potentiality for generating bone and associated hematopoietic components for use in plastic surgery of the orofacial region, with hematopoietic niche-like regeneration (Fig. 2G). A surprising phenomenon observed in the MSC transplants was that the recipient hematopoietic marrow was organized to form a chimerical bone and associated marrow organ-like structure, which consisted of donor-originated bone and recipient-originated hematopoietic marrow elements (Fig. 2H). Moreover, osteoblasts, which had differentiated from the MSCs, interacted with hematopoietic progenitors to build up a bone marrow niche microenvironment that was

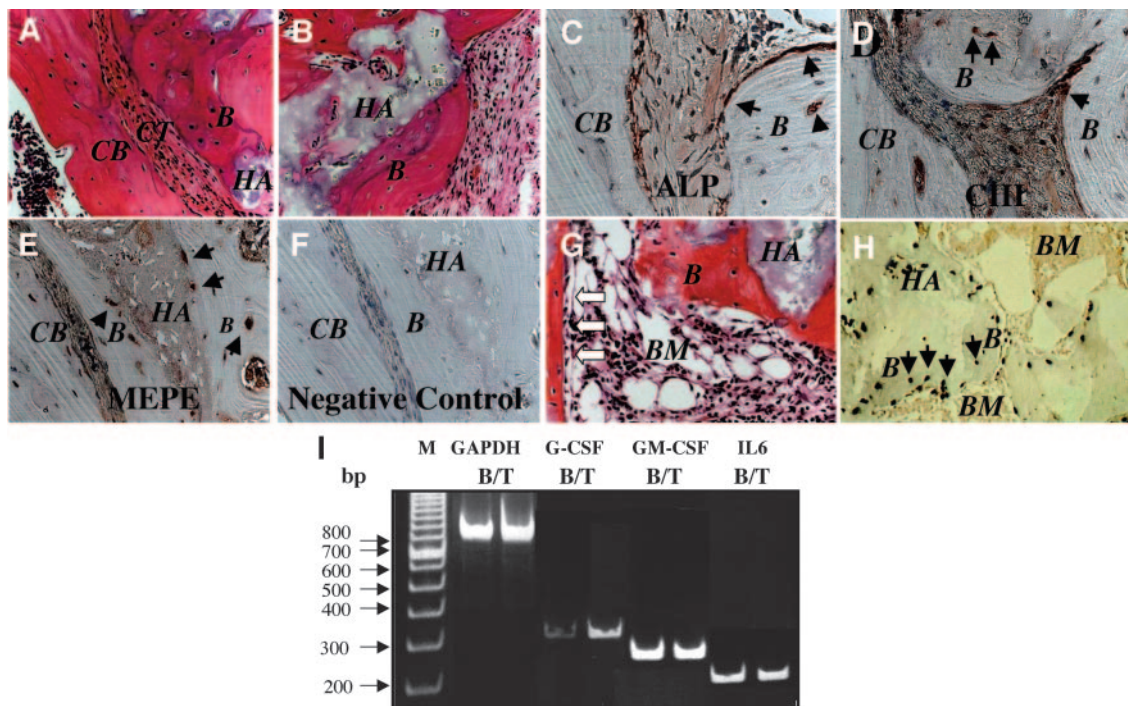


Figure 2. Characterization of MSC-regenerated bone in the orofacial region. (A, B): At 8 weeks post-transplantation, newly formed bone was integrated with HA particles (B) and united with CB (A) by CT; hematoxylin and eosin (H&E) staining. (C–F): Immunohistochemical analysis using anti-ALP (C), type III collagen (CIII) (D), and MEPE (E) antibodies showed osteogenic cells (black arrows) either lining the surface like osteoblasts or settling inside the newly formed bone like osteocytes in the MSC transplants (black arrows). Immunohistochemical analysis using control IgG showed negative staining in the MSC transplants (F). (G): At 8 weeks post-transplantation, the MSC transplants contained well-organized bone and BM elements, which exhibited a typical hematopoietic niche structure, containing osteoblasts (open arrows) and hematopoietic marrow cells; H&E staining. (H): In situ hybridization with probes specific for human *alu*. Human *alu*-positive osteogenic cells (black arrows) were detected on the surface of newly formed bones, but the BM components were negative for *alu* staining. (I): BM cell-derived MSC transplants showed expression of hematopoietic cytokines G-CSF, GM-CSF, and IL-6 as compared with long BM cells as a positive control group (B) by reverse transcriptase polymerase chain reaction (PCR). *GAPDH*, a housekeeping gene, served as a PCR amplification control. Abbreviations: ALP, alkaline phosphatase; B, bone; BM, bone marrow; CB, calvarial bone; CT, connective tissue; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-6, interleukin-6; HA, HA/TCP; M, markers; MEPE, matrix extracellular phosphoglycoprotein; T, transplants.

similar to that seen in long bones (Fig. 2G). To further confirm that these MSC transplants contain hematopoietic cells, we used the reverse transcriptase (RT)-PCR approach to detect expression of hematopoietic cytokines G-CSF, GM-CSF, and IL-6 in MSC-organized bone marrow cells (Fig. 2I).

To gain further insights into the potential mechanisms by which MSC-mediated tissue regeneration is associated with organizing a bone and associated marrow niche, we utilized immunohistochemical staining to demonstrate that GFP-positive bone marrow cells homed to the bone marrow compartment of the MSC transplants 2 months after the tail vein injection of whole bone marrow from GFP mice (Fig. 3A, 3B). Next, we used FACS analysis to characterize the types of cell population that homed to the marrow compartment of the MSC transplants (Fig. 3C). Interestingly, we found that hematopoietic cells, including CD45-, CD9-, and CD11b-positive cells, were capable of long-term homing in the MSC-mediated HSC niche microenvironment 2 months after the systemic infusion of GFP bone marrow (Fig. 3C), suggesting a functional bone and associated marrow organ-like regeneration in the orofacial region.

Bone Marrow MSC-Mediated Reconstruction of the Swine Orofacial Region

Next, we confirmed that bone marrow MSC-mediated tissue regeneration had the potential to be used in human therapies. For this purpose, we selected swine (minipigs) as a translational model in which to transplant autologous bone marrow MSCs to the orofacial region, and utilized HA/TCP as a carrier vehicle.

Prior to use of swine MSCs for tissue regeneration, we verified that swine MSCs are similar to human MSCs in terms of forming single-colony clusters in the culture (Fig. 4A, 4B), expressing mesenchymal stem cell marker STRO-1 (Fig. 4C), and generating bone and associated marrow organ-like structures when transplanted into immunocompromised mice using HA/TCP as a carrier vehicle (Fig. 4D). Analogous to the transplantation experiment in the mouse model, we found that swine bone marrow MSCs were capable of reshaping the orofacial appearance (Fig. 4E, 4F). Clinical image analysis suggested that the MSC transplants formed bony integrations with the frontal bone surfaces of the recipients (Fig. 4G–4L), thereby providing preclinical evidence of the suitability of this system for use in plastic surgery. Autologously transplanted swine bone marrow MSCs showed a significant amount of bone regeneration in comparison to HA/TCP particle transplants (Fig. 5). On the basis of the findings of the scanning electron microscopy and histological analysis, it was clear that autologous swine MSCs generated high-quality bone tissue on the different surfaces of the MSC transplants (Fig. 5).

Human PDLSC-Mediated Collagen Formation for Wrinkle Removal in Mice

To examine whether other types of MSCs could be used for plastic surgery, PDLSCs were selected for removing facial wrinkles in immunocompromised mice by a subcutaneous transplantation (Fig. 6). When PDLSCs were transplanted with HA/TCP as a carrier vehicle, they formed collagen fibers and ce-

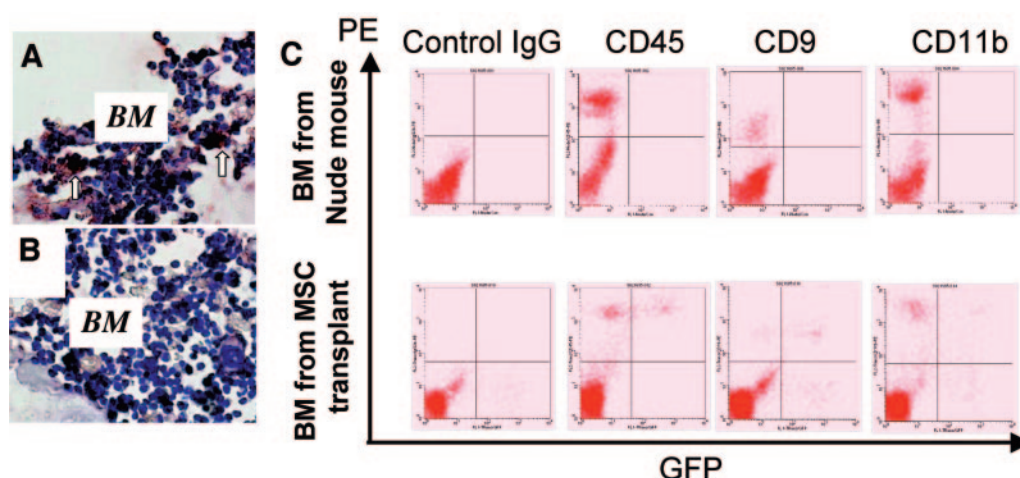


Figure 3. GFP-positive BM cells home to the MSC-organized BM compartment. (A, B): GFP BM was injected into the tail vein of the MSC/HA/TCP transplant-recipient mice. Immunohistochemical analysis using an anti-GFP monoclonal antibody showed that GFP-positive BM cells (open arrows) homed to the BM of the MSC transplants 2 months after GFP BM injection (A). Immunostaining with control IgG showed negative staining for MSC transplants (B). (C): Flow cytometric analysis for the cells harvested from the MSC transplants or from the long bones of nontransplanted immunocompromised mice. GFP-positive cells were coexpressed with hematopoietic cell markers (upright squares) including CD45, CD9, and CD11b in BM cells derived from the MSC transplants (lower panel). BM cells from untreated nude mice (upper panel) were used as a negative control. Abbreviations: BM, bone marrow; GFP, green fluorescent protein; PE, phycoerythrin.

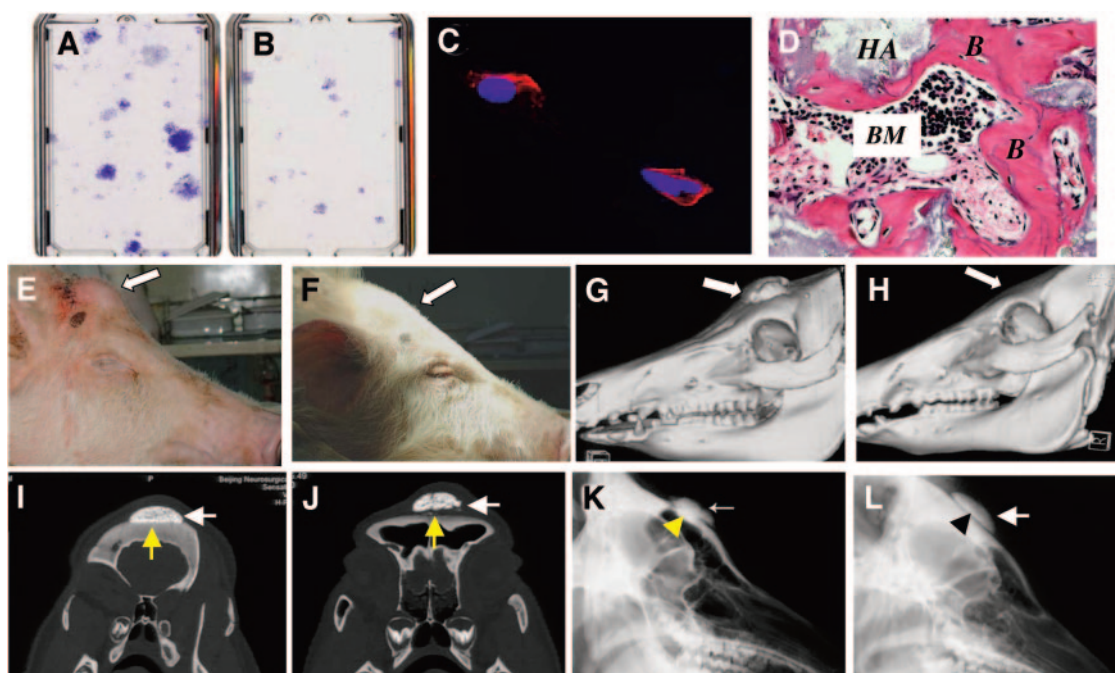


Figure 4. Autologous transplanted BM MSCs can reshape orofacial appearance in swine. (A, B): 0.1% toluidine blue staining showed that swine BM MSCs were capable of generating single-colony clusters when plated at 5×10^4 cells/25-cm flask (A) and 2.5×10^4 cells/25-cm flask (B). (C): Immunocytochemical staining showed that cultured swine MSCs express STRO-1, early mesenchymal progenitor markers. (D) At 8 weeks after transplantation, swine MSCs are capable of forming new bone on the surfaces of HA particles and organizing hematopoietic marrow elements. (E, F): Facial appearance. Transplantation of BM MSCs with HA can significantly reshape facial appearance in swine after 8 weeks (open arrows) (E). By contrast, there was no appearance change on a nontransplanted minipig (F). (G, H): A three-dimensional reconstruction images display the bony appearance of an MSC/HA transplant (open arrow) (G) and of a nontransplanted swine (H). (I, J): Coronal computed tomography scan images. A close integration was found between the MSC transplant (white arrow) and calvarial bone (yellow arrow) (I). By contrast, there was no bony connection between the HA transplant (white arrow) and calvarial bone (yellow arrow) (J). (K, L): X-ray images. A strong connection was observed between the MSC transplant (white arrow) and calvarial bone (yellow arrowhead) (K). The radiolucent line (black arrowhead) between the HA transplant (open arrow) and the calvarial bone suggests a nonbony connection between them (L). Abbreviations: B, bone; BM, bone marrow; HA, HA/TCP.

mentum in vivo [31]. In the current study, we found that PDLSCs showed unique tissue regeneration capacities, which enable them to form significant amounts of collagen fibers when a collagen-based gelatin (Gelfoam) was used as a carrier vehicle (Fig. 6A, 6B). Therefore, we hypothesize that PDLCSs may be

a distinctive population of collagen-forming cells for cosmetic approach. Previously, collagen gels [33] and stabilized hyaluronic acid [34] have been utilized for cosmetic nonsurgical procedures, and dermal fibroblasts have been applied for soft tissue augmentation [34]. Surprisingly, we found that the trans-

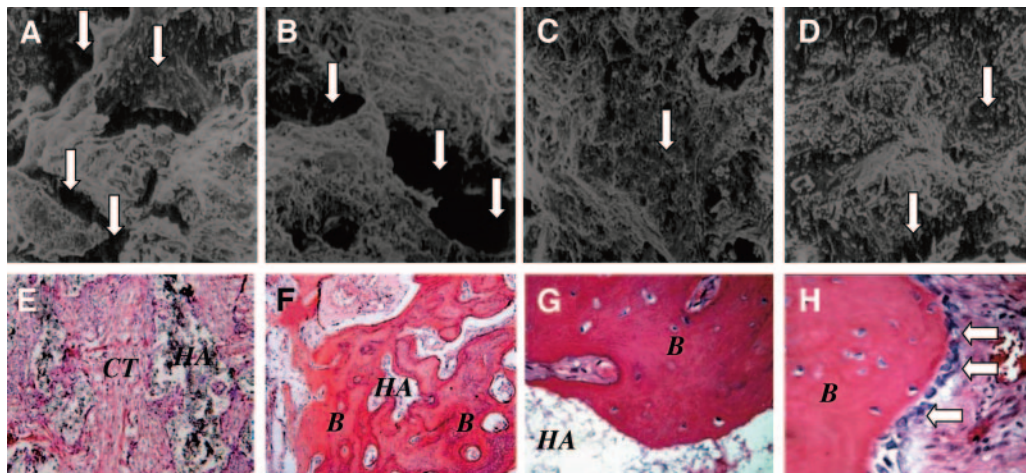


Figure 5. Characterization of MSC-regenerated bone in swine. (A–D): Scanning electron microscopy analysis of MSC/HA and HA transplants. Numerous micro holes (open arrows) exist on the different fractured surfaces, including the inner part of the HA transplants (A) and the exterior part of the HA transplants (B). At 8 weeks after the MSC transplantation, there are significantly fewer spaces on the different fractured surfaces in comparison to the HA transplants, including the inner part of the MSC transplants (C), and the exterior parts of the MSC transplants (D). (E–H): At 8 weeks post-transplantation, hematoxylin and eosin staining showed that the HA transplant showed CT mixed with HA/TCP particles (E). By contrast, robust amounts of bone were regenerated in the MSC transplants at a low magnification (F). High-magnification images showing the inner parts of the MSC transplant (G) and the exterior parts of the MSC transplant (H). Osteoblasts were present (open arrows in [H]) on the surface of the newly formed bone. Abbreviations: B, bone; CT, connective tissue; HA, HA/TCP.

plantation of human bone marrow MSCs and gingival fibroblasts using Gelfoam as a carrier vehicle failed to mediate any tissue formation 8 weeks after transplantation, perhaps due to the rapid disappearance of the delivered MSCs (Fig. 6, gingival fibroblast data not shown). In contrast, PDLSC/Gelfoam transplants showed a long-term cell survival, along with the important advantage of long-term living collagen fiber regeneration (Fig. 6A, 6B). Evidence that transplanted human PDLSCs were responsible for the collagen formation came from human-specific *alu* and mouse-specific *pfl* in situ hybridization (Fig. 6E–6H). Picosirius red staining further confirmed that PDLSCs are capable of forming condensed collagen fibers (Fig. 6M). Our results imply that PDLSCs possess the unique ability to produce collagen fibers, and maintain this capacity in vivo. To further assess the collagen-fiber regeneration capability, single colony-derived PDLSCs were transplanted subcutaneously in the immunocompromised mice using a collagen scaffold as a carrier vehicle (Fig. 6I–6L). Unexpectedly, all of the single colony-derived PDLSCs showed collagen-fiber regeneration capabilities, implying that the collagen-forming potential might be a universal trait of PDLSCs. We, therefore, took advantage of the PDLSC-mediated collagen regeneration to remove wrinkles and improve facial appearance in immunocompromised mice (Fig. 6N, 6n, 6O, 6o).

DISCUSSION

MSCs have been proposed as new therapeutic agents for repairing critical-size bone defects that normally cannot undergo spontaneous healing [3, 20, 34–37]. Histological analysis demonstrated that the MSC-mediated bone regeneration in the orofacial region was similar to that previously observed at subcutaneous sites [18, 20]. Consistent with previous studies on the ectopic tissue regeneration of MSCs, we found that they showed potential for use in craniofacial bone regeneration in the facial area, which was correlated with HSC niche maintenance. These results strongly suggest that MSCs are a promising candidate for use in orofacial tissue regeneration.

Permanent tissue regeneration might require a niche-like homeostasis microenvironment to sustain a long-term cell survival through regular function and prevent the loss of newly formed tissue. Previous studies have shown that the osteoblasts, which have differentiated from MSCs, govern the HSC niche through the bone morphogenetic protein receptor, parathyroid hormone, and the Tie2/angiopoietin-1 signaling pathways [38–40]. Our previous study indicated that MSC-generated bone and associated marrow structures contain bone sialoprotein-positive osteoblasts, B220-positive lymphoid cells, and TER 119-positive erythroid cells [21]. Here, we confirmed that bone marrow MSCs were able to re-establish an HSC niche environment ectopically. MSC-mediated bone formation organizes recipient hematopoietic marrow elements, leading to bone and associated marrow organ-like structure regeneration, in which reciprocal interactions between bone marrow hematopoietic cells and osteogenic cells exert their functional roles in the newly formed ectopic organ for up to 18 months in immunocompromised mice [21]. Although artificial materials, such as alloplastic implants, are also capable of altering the facial contours, MSC transplants are capable of regenerating functional organ-like structures, which are eventually incorporated into the recipient's body. The advantage of this kind of tissue regeneration is that it produces a long-term reconstruction by achieving a balanced biological tissue homeostasis between different components of the transplant and the recipient host microenvironment. Although osteoconductive bone regeneration was found in HA/TCP particles implanted in control-group mice, the amount of bone formation was limited. Moreover, no hematopoietic marrow component regeneration was detected in the control transplants. These results confirmed that the mechanisms of MSC-mediated tissue regeneration were distinctive from osteoconductive tissue regeneration.

Transplanted PDLSCs produced significant amounts of collagen fibers, which mimicked the tissue structures seen in the original PDL compartments. By contrast, transplanted MSCs and gingival fibroblasts failed to generate any tissues, perhaps because they were unable to exploit an optimal niche for their functional survival. Collagen-fiber regeneration was greatly affected by the type of scaffold. For instance, Gelfoam could

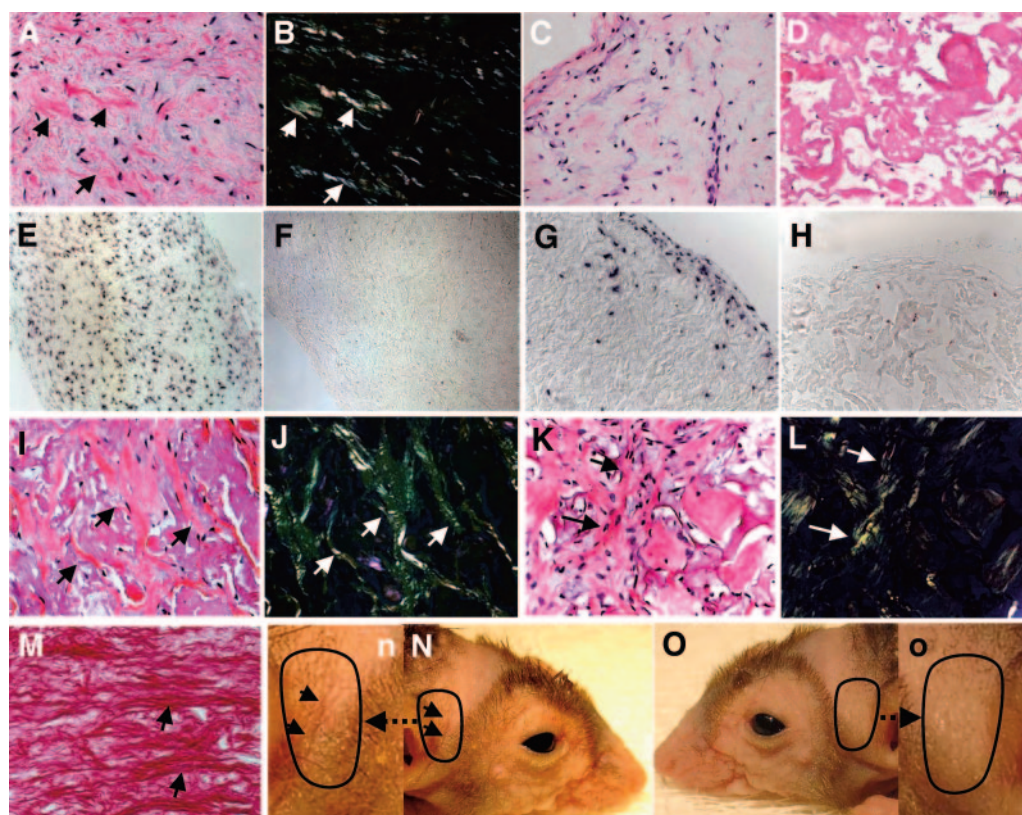


Figure 6. Human MSC-mediated improvement of wrinkles in nude mice. (A, B): When periodontal ligament stem cells (PDLSCs) were transplanted into nude mice subcutaneously for 4 weeks using Gelfoam (collagen-based gelatin sponge) as a carrier vehicle, significant amounts of collagen fibers were regenerated according to hematoxylin and eosin (H&E) staining (black arrows in A). The same polarized light view suggested that these collagen fibers were condensed (white arrows in B). (C, D): In contrast, bone marrow MSCs could not generate collagen fibers using the Gelfoam as a carrier vehicle for 2 (C) and 4 weeks (D). Although some cellular components appeared at 2 weeks post-transplantation, they were not capable of forming collagen fibers (C). At 4 weeks post-transplantation, there were limited numbers of cells in the MSC/Gelfoam transplant (D). (E–H): In situ hybridization with probes specific for human *alu* (E) and mouse *pfl* (G) in the MSC transplants. Many human *alu* positive cells (black dots) were detected inside the PDLSC/Gelfoam transplant at 4 weeks post-transplantation (E). Mouse *pfl* positive cells (black dots) were detected only on the exterior parts of the PDLSC/Gelfoam transplants (G). In situ hybridization using sense mRNA probes showed negative staining for *alu* (F) and *pfl* (H). (I–L): Single colony-derived PDLSCs were analogous to mixed-colony PDLSCs capable of forming condensed collagen fibers, as shown by H&E staining (black arrows in [I] and [K]) and the same field of polarized light (white arrows in [J] and [L]). (M): Picrosirius red staining showed that dense collagen fibers (arrows) were generated in the PDLSC transplant. (N, O): PDLSCs were transplanted subcutaneously under the wrinkle area using Gelfoam as a carrier vehicle. (n, o): High magnification images indicated by circled area in (N) and (O). There was a significant improvement in wrinkle appearance (O, o) in comparison to nontreated control mice (N, n).

support PDLSCs to produce collagen fibers in vivo without any sign of mineralized tissue formation, whereas both collagen fibers and cementum were formed in HA/TCP-PDLSC transplants [31]. The mechanism by which PDLSC-mediated tissue regeneration pattern is dependent upon the supporting material is unknown; however, it might be related to subtle differences in biocompatibility or in the capacity to initiate MSC differentiation. This specific characteristic of PDLSCs provides a unique opportunity for improving facial appearance. Recent studies have suggested that dermal fibroblasts can be utilized for cosmetic purposes to improve wrinkles, and the combined application of the fibroblasts with a collagen gel might promote new collagen formation in vivo. However, our current data showed that the delivery of MSCs or fibroblasts failed to achieve long-term cell survival and tissue regeneration. This discrepancy in terms of the potential therapeutic effects of dermal fibroblasts remains to be clarified by further studies, which will be crucial because of their significant potential clinical impacts.

Although MSCs have previously been used for regenerative medicine, the current study explored a potential new application for cosmetic purposes. Our results suggested that the transplan-

tation of MSCs to the orofacial area might not compromise the original environment of the recipient. Importantly, our in vivo xenogeneic transplantation model demonstrated that MSCs generated normal bone tissues, with associated hematopoietic marrow elements, in the orofacial region. Consistent with our findings, a previous study [41] suggested that MSCs had the potential to be used for cosmetic purposes by forming adipose tissue. Our results might provide the first evidence that MSCs can be utilized for plastic surgery to improve facial appearance. Although a variety of approaches have been proposed for improving current plastic surgery techniques, it is clear that more studies will be necessary to further explore new therapies and achieve better clinical consequences, especially when utilizing using living autologous stem cells.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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