RESEARCH REPORTS
Biological

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Gingivae Contain Neural-crest- and Mesoderm-derived Mesenchymal Stem Cells

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APPENDIX

MATERIALS & METHODS

Antibodies and Reagents

Anti-runt-related transcription factor 2 (RUNX2) and -Osteocalcin (OCN) antibodies were purchased from Millipore (Billerica, MA, USA). Anti-alkaline phosphatase (ALP), -Nestin, -β-TUBULIN III, -Collagen II, -Sox9, and -Neurofilament Medium (NF-09) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-Sca-1-PE, -CD34-PE, -CD44-PE, -CD45-PE, -CD11b-PE, -CD73-PE, -CD117-PE, -CD4-PerCP, -CD25-APC, -IgG_1-PE, -IgG_{2a}-PE, -IgG_{2b}-PE, -CD3ε, and -CD28 antibodies were purchased from BD Bioscience (San Jose, CA, USA). Anti-Foxp3-PE, -IL17-PE, -CD105-PE, and -CD90-PE antibodies were purchased from eBioscience (San Diego, CA, USA). Purified anti-peroxisome proliferator-activated receptor γ (PPARγ), -lipoprotein lipase (LPL), and -FAS Ligand (FASL) antibodies, as well as secondary antibodies, were purchased from Santa Cruz Biosciences (Santa Cruz, CA, USA). Anti-β-actin antibody was purchased from Sigma (St. Louis, MO, USA). The EdU imaging kit was purchased from Invitrogen (Carlsbad, CA, USA).

Cell Culture

Gingival tissues from the mouse mandibular molar region were gently separated, minced, and digested with solution containing 2 mg/mL collagenase type I (Worthington Biochemical, Freehold, NJ, USA) and 4 mg/mL dispase II (Roche Diagnostics, Indianapolis, IN, USA) in phosphate-buffered saline (PBS) for 1 hr at 37°C. We obtained single-cell suspensions by passing the cells through a 70-μm strainer (BD Biosciences, Franklin Lakes, NJ, USA). All nucleated cells (ANC) were seeded at 1×10^6 into 100-mm culture dishes (Corning, NY, USA) with α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Invitrogen), followed by an initial incubation for 48 hrs at 37°C and 5% CO_2. The cultures were washed with PBS twice to eliminate the non-adherent cells. Attached cells were cultured for another 12 days under the same conditions as in the complete medium mentioned above.

Colony-forming Units-Fibroblastic (CFU-F) Assay

The CFU-F assay was performed as described previously (Yamaza *et al*., 2011). Briefly, independent ANCs ($1\text{-}1.5 \times 10^5$) isolated from gingivae were seeded on 60 mm culture plates (Corning). After 14 days, the culture plates were stained with a mixture of 0.1% toluidine blue (Merck, Darmstadt, Germany) and 2% paraformaldehyde (PFA, Merck) solution. Colonies containing > 50 cells were counted as single colony clusters. The CFU-F count was performed in 5 independent samples *per* experimental group.

Detection of β-galactosidase (lacZ) Activities by X-gal Staining

Cells or slides were fixed for 20 min at room temperature with 0.2% glutaraldehyde in PBS and washed 3 x in rinse solution (0.005% Nonidet P-40 and 0.01% sodium deoxycholate in PBS). Cells or slides were stained overnight at room temperature in the standard staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.4% X-gal in PBS), rinsed twice in PBS, and post-fixed in 3.7% formaldehyde. Sections and cells were then counterstained with Nuclear Fast Red (Sigma).

Population Doublings

Multiple single-colony-derived GMSCs were trypsinized and seeded at 2×10^5 cells in 35-mm dishes (Corning) in complete growth medium at the first passage. Cells were harvested and seeded at the same number when they reached confluence. Population doublings (PD) were calculated by the following formula: $PD = \log_2$ (number of harvested cells/number of seeded cells). PD numbers were determined by the cumulative addition

of total numbers generated from each passage until cells ceased dividing. The PD assay was repeated with 3 independent isolated cells for each group.

Flow Cytometry Analysis

A quantity of 2×10^5 cells with 1 μg antibody or isotype-matched IgG control was incubated for 1 hr at 4°C. All samples were analyzed by FACSCalibur (BD Biosciences, San Jose, CA, USA).

Real-time PCR

Total RNAs were isolated from different GMSCs by means of the TRIzol® Reagent (Life Technologies, Invitrogen). RNA samples (1 μg) were reverse-transcribed in a Reverse Transcription system (QIAGEN). Primers used were: (sox9) forward, 5′-TCGACGTCAATGAGTTTGACCA-3′, and reverse, 5′-ATG CCGTAACTGCCAGTGTAGG-3′; (Col2a1) forward, 5′-GGG CTCCAATGATGTAGAGATG-3′ and reverse, 5′-CCCACTT ACCAGTGTGTTTCG-3′ and (GAPDH as an internal control) forward, 5′-GAAGGTGAAGTTCGGAGTC-3’, and reverse, 5′-GAAGATGGTGATGGGATTTC-3′. PCR conditions were: 95°C 5 min (95°C 10 sec, 55°C 45 sec) x 40, and 90°C 10 sec.

Immunohistochemistry

Tissue sections were treated with 0.3% hydrogen peroxide and 0.1% sodium azide in PBS, pH 7.2, for 30 min, and incubated with indicated primary antibodies, overnight, at 4ºC. After being washed with PBS, the sections were immunostained by means of a VECTASTAIN ABC kit (Vector, Burlingame, CA, USA) according to the manufacturer’s instructions. Finally, samples were counterstained with hematoxylin.

Immunofluorescence Staining

GMSCs at passage 3 were seeded on chamber slides (Nunc) for neurogenetic induction and then fixed with 4% paraformaldehyde (PFA). Slides were incubated with normal serum, which was from the same species of secondary antibody. After being blocked, the slides were first incubated with the specific antibodies overnight at 4°C, followed by incubation with Alexa Fluor® 488-conjugated secondary antibody (1:200, Invitrogen) for 30 min at room temperature in dark. Finally, slides were mounted with VECTASHIELD® Mounting Medium (Vector Laboratories).

EdU Detection Staining

EdU^+ label-retaining cells were detected on sections by means of an EdU imaging kit according to the manufacturer’s instruction.

Western Blot Analysis

Total protein was extracted with M-PER mammalian protein extraction reagent (Thermo, Rockford, IL, USA). A 20-μg quantity of protein was applied and separated on 4-12% NuPAGE gel (Invitrogen), followed by transfer to Immobilon™-P nitrocellulose membranes (Millipore Inc., Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk and 0.1% Tween-20 for 1 hr, followed by incubation with the primary antibodies (1:200-1,000 dilution) at 4°C overnight. Horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biosciences; 1:10,000) was used to treat the membranes for 1 hr, followed by enhancement with a SuperSignal®West Pico Chemiluminescent Substrate (Thermo). Bands were detected on BioMax MR film (Kodak, Rochester, NY, USA). Each membrane was also stripped with a stripping buffer (Thermo) and re-probed with anti-β-actin antibody to quantify the amount of loaded protein.

Multi-lineage Differentiation Assay

For *in vitro* osteogenic assay, GMSCs (passage 2) were cultured to confluence and changed to an osteoinductive medium containing 2 mM β-glycerophosphate (Sigma, St. Louis, MO, USA), 100 μM L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 10 nM dexamethasone (Sigma). After 4 wks of osteoinductive culture, calcium deposits were detected by staining with 1% Alizarin Red (Sigma). The mineralized areas were quantified by ImageJ and shown as a percentage of Alizarin Red-positive area over the total area.

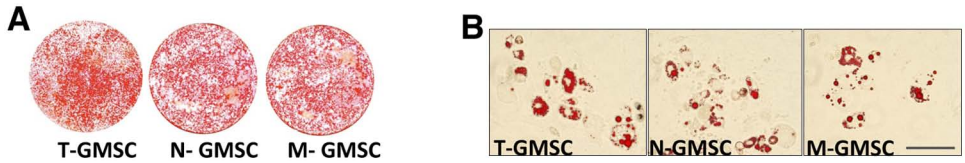
For *in vitro* adipogenic induction assay, GMSCs (passage 2) were cultured to confluence and then induced under adipogenic medium containing 500 μM isobutyl-methylxanthine, 60 μM indomethacin, 0.5 μM hydrocortisone, and 10 μM insulin for 2 wks. Cultures were then stained with 0.3% Oil Red O. The number of Oil-Red O-positive droplet-containing cells was counted and shown as a percentage of Oil Red O-positive cells over total cells. Three independent experiments were performed for this assay.

For *in vitro* chondrogenic induction assay, GMSCs (passage 2) were induced by the “pellet culture” technique (Johnstone *et al*., 1998). Briefly, approximately 1×10^6 cells were placed in a 5-mL polypropylene tube (Falcon), centrifuged to pellet form, and cultured in complete culture medium until the pellets became round. Then, 1 mL of chondrogenic medium containing DMEM (Gibco) with 15% FBS, 2 mM L-glutamine, 1% ITS^+ (BD Bioscience), 100 nM dexamethasone, 100 μM ascorbic acid, 2 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin was added, freshly supplemented with 10 ng/mL of transforming growth factor-β1 (TGF-β1). Medium was changed every 3 or 4 days for 4 wks. After 4 wks, the pellets were fixed in 4% PFA, embedded in paraffin, and cut into 6-μm sections. Chondrogenic differentiation was determined by staining with 0.1% safranin-O (Sigma) and 0.1% toluidine blue (Sigma) solution. Three independent experiments were performed for this assay.

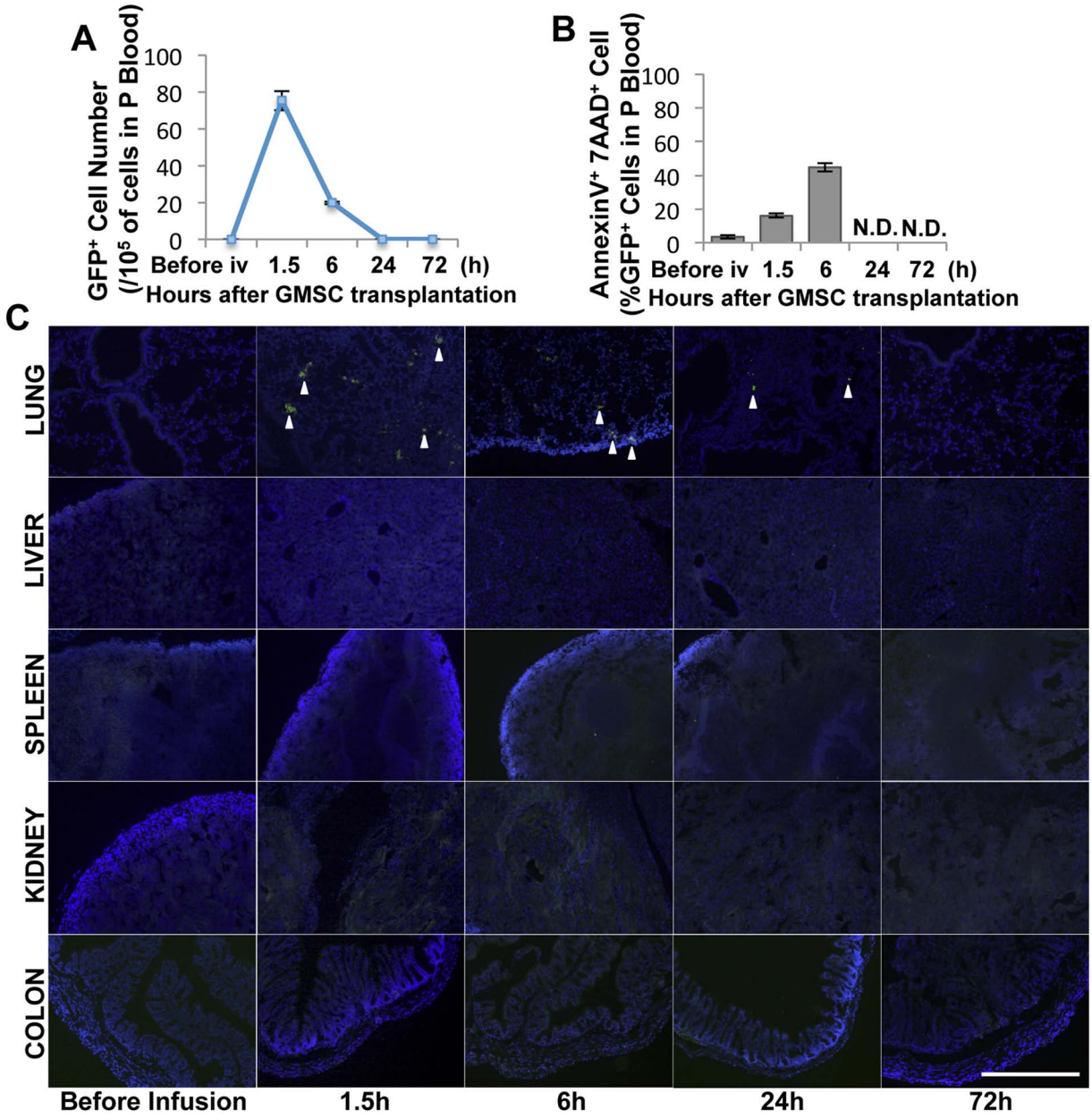
For neuronal differentiation, GMSCs were seeded in 2-well chamber slides (Nunc) and cultured in DMEM/F12 (Invitrogen) supplemented with 10% FBS, 1 × N-2 supplement (Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL fibroblast growth factor 2, and 10 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN, USA) and cultured for 14–21 days. Medium was changed every 3 or 4 days.

Co-culture of GMSCs with Activated Splenocytes

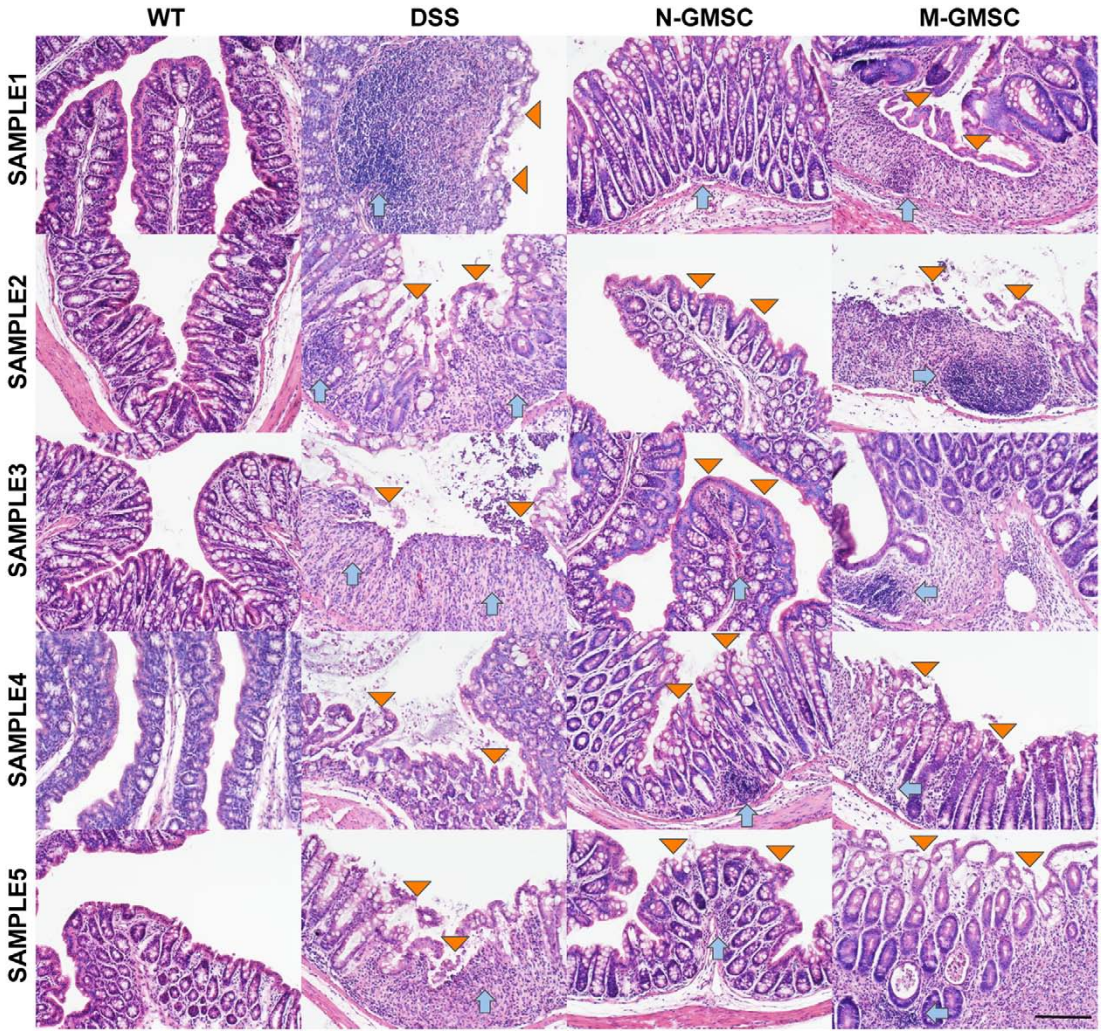
After collection of mouse spleens, red blood cells were removed with ACK lysis buffer (Gibco, Grand Island, NY, USA), and the



Appendix Figure 1. Osteogenic and adipogenic differentiation capacity of the N-GMSCs and M-GMSCs. (**A**) Alizarin red staining showed that N-GMSCs and M-GMSCs form similar amounts of mineralized nodules after 4 wks of culture in osteoinductive conditions. (**B**) Oil red O staining showed that N-GMSCs and M-GMSCs had similar capacity to differentiate into adipocytes after 2 wks of culture under adipo-inductive conditions. Scale bar = 100 μm.



Appendix Figure 2. Apoptosis of transplanted GMSCs in peripheral blood. (**A**) Flow cytometric analysis showed that the number of systemic infused GFP+ GMSCs reached a peak at 1.5 hrs post-transplantation in peripheral blood and then fell to undetectable levels at 24 hrs post-infusion. (**B**) The number of AnnexinV+7AAD+ double-positive apoptotic GMSCs reached a peak at 6 hrs post-transplantation in peripheral blood and then fell to undetectable levels at 24 hrs post-transplantation. (**C**) The sections from the lung, liver, spleen, kidney, and colon at different time-points. GFP+ cells (white triangles) can be detected only in the lung 1.5 to 24 hrs post-transplantation. Error bars represent mean ± SD. Scale bar = 500 μm.



Appendix Figure 3. Histological examination of colon tissue. Hematoxylin and eosin (H&E) staining showed infiltration of inflammatory cells (blue arrows) with destruction of epithelial layer (yellow triangles) in colons of mice with colitis. N-GMSC transplantation resulted in significantly reduced numbers of inflammatory cells and the destruction of the epithelial layer compared with the M-GMSC group. Blue arrows: lymphocyte infiltration. Orange triangles: epithelial margin. Scale bar = 200 μm.

splenocytes (2-3 × 10^6) were activated with plate-bound 1 mg/mL anti-CD3ε antibody and 1 mg/mL soluble anti-CD28 antibody (BD) for 3 days in complete medium containing Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Basel, Switzerland) with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate (Sigma), 1% non-essential amino acid (Cambrex), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Seeding of 2 × 10^5 mouse GMSCs to a 12-well dish was followed by the addition of activated splenocytes (1 × 10^6/well) to co-culture for 3 days. To measure T-cell viability, we used flow cytometry to detect T-cell apoptosis with an Annexin V-PE Apoptosis Detection Kit (BD Pharmingen).

Dextran Sulfate Sodium (DSS)-induced Mouse Colitis

Acute colitis was induced by the administration of 3% (w/v) DSS (molecular mass, 36-50 kDa; MP Biochemicals, Solon, OH, USA) in drinking water for 10 days in C57BL/6J mice (Zhang *et al.*, 2009). After sorting by flow cytometry, 2 × 10^5 GMSCs were re-suspended in PBS and infused intravenously into mice with colitis (n = 5 *per* group) at day 3 post-DSS feeding. At day 10, peripheral blood was collected for Th17, Treg level assay; and then the mice were euthanized, and the entire colon was collected and gently cleared of feces with sterile PBS. For histopathological analysis, colon segments were fixed in 4% PFA, and paraffin-embedded sections were prepared for H&E staining.

APPENDIX REFERENCES

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