Labial Stem Cell Extract Mitigates Injury to Irradiated Salivary Glands

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Appendix

Materials and Methods:

Multilineage differentiation of LSCs

Osteogenesis differentiation LSCs were cultured with alpha-MEM with 15% FBS, 1% penicillin/streptomycin, 2 mM Glutamine, 10^{-8} M Dexamethasone sodium phosphate (D-8893, Sigma-Aldrich, St-Louis, MO, USA), 55 µM 2-Mercaptoethanol (21985-023, Gibco), 0.1 mM L-ascorbic acid phosphate (013-12061, Wako chemicals, Richmond, USA), and 2mM Beta-Glycerophosphate disodium salt hydrate (G9422, Sigma). After 4 weeks in culture, cells were characterized with Alizarin Red S (A5533, Sigma) staining.

Adipogenesis differentiation LSCs were cultured with Alpha-MEM with 15% FBS, 1% penicillin/streptomycin, 2 mM Glutamine, 0.5 mM Isobutylmethylxanthin (I5879, Sigma), 60 µM Indomethacin (I7378, Sigma), 0.5 µM Hydrocortisone (H0888, Invitrogen, San Francisco, CA, USA), 10µg/ml insulin (I-9278, Sigma), 0.1 mM L-ascorbic acid phosphate and 2-ME. Adipogenesis was characterized by Oil Red O (O1391, Sigma) staining after 21 days in culture.

Chondrogenesis differentiation LSCs were cultured with Mesenchymal Stem Cell Identification Kit (SC006, R&D Systems, MN, USA), and characterized with immunofluorescence staining for collagen type II after 28 days. Volocity Image Analysis Software TM (Version 4.5.1) was used to capture images.

Flow cytometry

Labial gland stem cells (LSCs) were harvested by Accutase (423201, Biolegend, San Diego, CA, USA); and 1 x 10^6 cells were used for each test. Cells were filtered through a 70 µM cell strainer. BD Horizon™ Fixable Viability Stain 450 (FVS450, 562247, BD Biosciences, California, USA) was added (1:1000 dilution) and incubated for 15 minutes at 4 °C. After washing twice with

Staining Buffer (420201, Biolegend), cells were incubated with Fc Receptor block (564219, BD Biosciences) for 20 minutes at 4 °C. Then hMSC analysis kit (562245, BD Biosciences, San Jose, CA, USA) was used. All procedures were done according to the manufacturer's protocol. The antibodies used for flow cytometric analysis were as follow: PE Mouse Anti-Human CD44 (Clone: G44-26); FITC Mouse Anti-Human CD90 (Clone:5E10); PerCP-Cy™5.5 Mouse Anti-Human CD105 (Clone:266); APC Mouse Anti-Human CD73 (Clone: AD2); hMSC Positive Cocktail:(CD90, CD105, CD73); PE ***hMSC Negative Cocktail*** (CD34 PE (Clone 581), CD11b PE(Clone:ICRF44), CD19 PE(Clone:hib19), CD45 PE (Clone: HI30) and HLA-DR PE (Clone: G46-6)); ***hMSC Positive Isotype Control Cocktail*** (mIgG1, κ FITC (Clone: X40); mIgG1, κ PerCP-Cy5.5 (Clone: X40); mIgG1, κ APC (Clone: X40); BD Bioscience), and PE ***hMSC Negative Isotype Control Cocktail*** (mIgG1, κ PE (Clone: X40); mIgG2a, κ PE (Clone: G155-178)). The antibodies were incubated with cells for 30 minutes. After washing twice with the Staining Buffer, cells were resuspended in 300 µl of Staining Buffer. Data was recorded on 3 lasers, 11 detectors LSR Fortessa (BD Biosciences) equipped with BD FACS Diva Software (v6, BD Biosciences). Post-acquisition analysis was performed using FlowJo (version 10, Tree Star Inc., OR). Isotype-matched control antibodies were used in the antibody analysis.

Salivary flow rate (SFR) and lag time

Before anesthetizing the mice, the body weight was measured. 0.3 µl/g body weight of 60 mg/ml Ketamine and 8 mg/ml Xylazine were used as the anesthetic to measure the salivary flow rate (SFR) of mice at week 4, 8, 12 and 16 post-IR. After subcutaneous injection of 0.5 mg/kg body weight of pilocarpine (P6503, Sigma-Aldrich, ST. Louis, USA), stimulated saliva secretion was collected for 10 minutes. As previously described, we assumed the density of saliva is 1g/ml, which means the weight of saliva can represent the volume, for example, 0.5g of saliva is equivalent to 0.5 ml volume. Meanwhile, the lag time of saliva was counted for each mouse according to the time from stimulation to the first drop of saliva secreted.

H&E staining

Submandibular glands and parotid glands were fixed in 4% PFA overnight at 4°C and embedded into paraffin. Specimens were sliced into 8 µm thickness followed by staining with Hematoxyline and Eosin (H&E). Image J software (NIH) was used to calculate the percentage of surface area

occupied by acinar cells/total area. The magnification was 200 X, and 5-8 fields were randomly chosen for counting per gland/mouse.

PCNA staining.

Zymed PCNA staining kit (931143, Invitrogen, Carlsbad, CA, USA) was used to test the cell proliferation of SG tissue. After deparaffinization and rehydration, specimens were treated with 10 mM Citrate Buffer solution (pH 6.1) in a 95°C water bath for half an hour and then cooled down to room temperature for 30 minutes. The rest of the procedures followed the manufacturer's instruction. The number of positive cells was counted under 400 X magnification of 5-8 fields/gland with Image J software (NIH).

Immunofluorescent staining.

Submandibular and parotid glands were embedded into optimal cutting temperature (OCT) and cut into 6-8 μm thickness frozen sections. After fixing with 4% PFA (P6148, Sigma-Aldrich, ST. Louis, USA) for 15 minutes, slides were blocked with 10% donkey serum for 1 hour. The primary antibodies used were rabbit anti-aquaporin 5 (1:200, AQP5, ab92320, Abcam, Cambridge, MA, USA); mouse anti-alpha smooth muscle actin (1:200, α -SMA, ab7817, Abcam); rabbit anti-cytokeratin 5 (1:400, CK5, Sigma-Aldrich, Oakville, ON, Canada); goat anti-GFRα -2 (1:200, AF429, R&D systems, Minneapolis, USA); goat anti-CD31 antibody (1:200, AF3628, R&D Systems, Minneapolis, USA); PBS was used as negative control. SG tissues were incubated overnight with primary antibodies or PBS at 4°C. In the next day, slides were incubated with secondary antibodies (1:200) for 1 hour at room temperature. These secondary antibodies used were: donkey anti-rabbi-Rhodamine Red™-X- conjugated, anti-mouse-Alexa Fluor® 594-conjugated, anti-goat-Alexa Fluor 488-conjugated. 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, Ottawa, ON, Canada) was used to label nucleus of the cells. A Leica DM4000 fluorescent microscope was used to take 5-8 pictures for each tissue, and intensity of the fluorescence signal was analyzed by ImageJ software (NIH).

Quantitative real- time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the submandibular glands with RNeasy Plus Minikit (74134, Qiagen, Valencia, CA91355, USA). First strand cDNA was synthesized with the High-Capacity

cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific, MA02451, USA). qRT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal Master Mix II (4440040, Applied Biosystem, Foster City, Canada). The cycles were programmed as follow: 50°C for 2min, 95°C for 20s, then 40 cycles at 95°C for 1 s and 60°C for 20s. The probes used in this study were epidermal growth factor (EGF) (Assay ID: Mm00438696), vascular endothelial growth factor (VEGF) (Assay ID: Mm01281449), insulin-like growth factor 1 receptor (IGF-1R) (Assay ID: Mm00802841), hepatocyte growth factor (HGF) (Assay ID: Mm01135193), basic fibroblast growth factor (FGF2) (Assay ID: Mm01285715), the GDNF family receptor-α2 (GFRα2) (assay ID: Mm00433584), cytokeratin 5 (CK5) (assay ID: Mm00503549), and aquaporin-5 (AQP5) (assay ID: Mm00437578). Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH, Assay ID: Mm99999915) was used as the endogenous reference. Three experimental replicates were conducted for each sample. Results were expressed as fold changes in relative gene expression.