SIS-ECM Laden with GMSC-Derived Exosomes Promote Taste Bud Regeneration
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APPENDIX

Supplemental Materials & Methods

Cell Cultures and Exosome Purification

According to the approved Institutional Review Board (IRB) protocol at the University of Pennsylvania, gingival tissues were obtained as remnants of discarded tissues from healthy human subjects who underwent routine dental procedures. The gingiva-derived mesenchymal stem cells (GMSCs) were isolated and cultured according to methods as we previously described (Zhang et al. 2009). GMSCs less than 6 passages were used for exosome isolation and animal studies.

Cultured GMSCs were supplemented with complete growth medium (α-MEM supplemented with 1% l-glutamine, 10% FBS, and 1% penicillin/streptomycin) at 37°C with 5% CO_2. When cultured cells reached about 70% of confluence, the medium was replaced with exosome-free medium (α-MEM supplemented with 1% L-glutamine, 5% exosome-depleted FBS (Cat #. EXO-FBS/HIxxx; System Biosciences, Palo Alto, CA), and 1% penicillin/streptomycin) and cells were cultured for 48 hours. Exosomes were purified by using ExoQuick-TC exosome precipitation reagent according to the manufacturer's instructions (System Biosciences, Palo Alto, CA). Briefly, the conditioned media were collected, mixed with ExoQuick-TC reagent (10:1) and then incubated at 4°C for more than 12 hours. Then the mix was centrifuged at 1500g for 30 min and the pellet was resuspended in PBS and the protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (BioVision, Inc., Milpitas, CA, USA). The

purified exosomes were stored at -80°C for future use. The concentration and size distribution of purified GMSC-derived EVs were determined by nanoparticle tracking analysis (NTA) with a NanoSight NS300 (Malvern Instruments, Ltd., Malvern, U. K) (Ragni et al. 2017; Yue et al. 2017). The expression of EV surface markers CD63 and CD9 was determined by Western blot (Khan and Kishore 2017; Phinney and Pittenger 2017).

GMSC/SIS-ECM Construct Preparation and Animal Surgery

6 x 6 mm strips of decellularized porcine small intestinal submucosa extracellular matrix (SIS-ECM 2.0) (Cook Biotech, Inc., West Lafayette, IN) was prepared as described previously (Xu et al. 2017). Animals were randomly divided into the three groups (n = 12/group): (1) Tongue defect transplanted with SIS-ECM; (2) Tongue defect transplanted with SIS-ECM and GMSCs; (3) Tongue defects transplanted with SIS-ECM and GMSC-derived exosomes. GMSCs (1×10^6 per rat) or GMSC-derived exosomes (40μg per rat) suspended in 15μl sterile cold PBS and mixed with an equal volume of cold growth factor-reduced Matrigel. A tongue defect was created using a 6-mm biopsy punch (Miltex, Inc., York, PA) in the left side of anterior dorsal tongue surface at the depth (3mm) of the muscle layers to damage both the epithelium and the underlying soft tissue as previously described (Xu et al. 2017). Then, the mixture of Matrigel was applied to the surface of wounded bed areas, allowing for gelation for 5min. Afterwards, the SIS-ECM membrane was applied on top of the matrigel to cover the wounded bed and stabilized with sutures. At days 0, 1, 3, 5, 7, 14, 21, 28, 56 post-surgery, the wounds were photographed using a Nikon D90 digital camera with an AF Micro NIKKOR 60 mm lens. On day 7, 14, 28 and 56 post-surgery, rats from each group were

sacrificed. Then tongues were harvested by transection at the circumvallate papillae and subsequently prepared for histological and immunofluorescence analyses.

Histological and Immunofluorescence Studies

The tongue samples were fixed in 10% neutralized formalin for 48 h and 5-μm-thick paraffin sections and 8-μm-thick cryosections were cut, respectively. Hematoxylin and eosin staining was performed according to the standard procedures using paraffin sections, while immunofluorescence studies were conducted using cryosections. The cryosections were incubated with primary antibodies for PLCβ2 (Santa Cruz Biotechnology, Dallas, TX), UCH-L1/PGP9.5 (Novus Biologicals, Littleton, CO), BDNF (Abcam, Cambridge, UK), Neurofilament (BioLegend, San Diego, CA), Cytokeratin 14 (Abcam, Cambridge, UK), Cytokeratin 8 (Novus Biologicals, Littleton, CO) (1:200), P2X3, NTPDase 2, and aromatic L-amino acid decarboxinase (AADC) (Bioss) overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. Isotype-matched control antibodies (BioLegend, San Diego, CA) were used as negative controls. Nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA), and slides were observed under a fluorescence microscope (Olympus IX-73). The integrated immunofluorescence density was quantified using ImageJ software.

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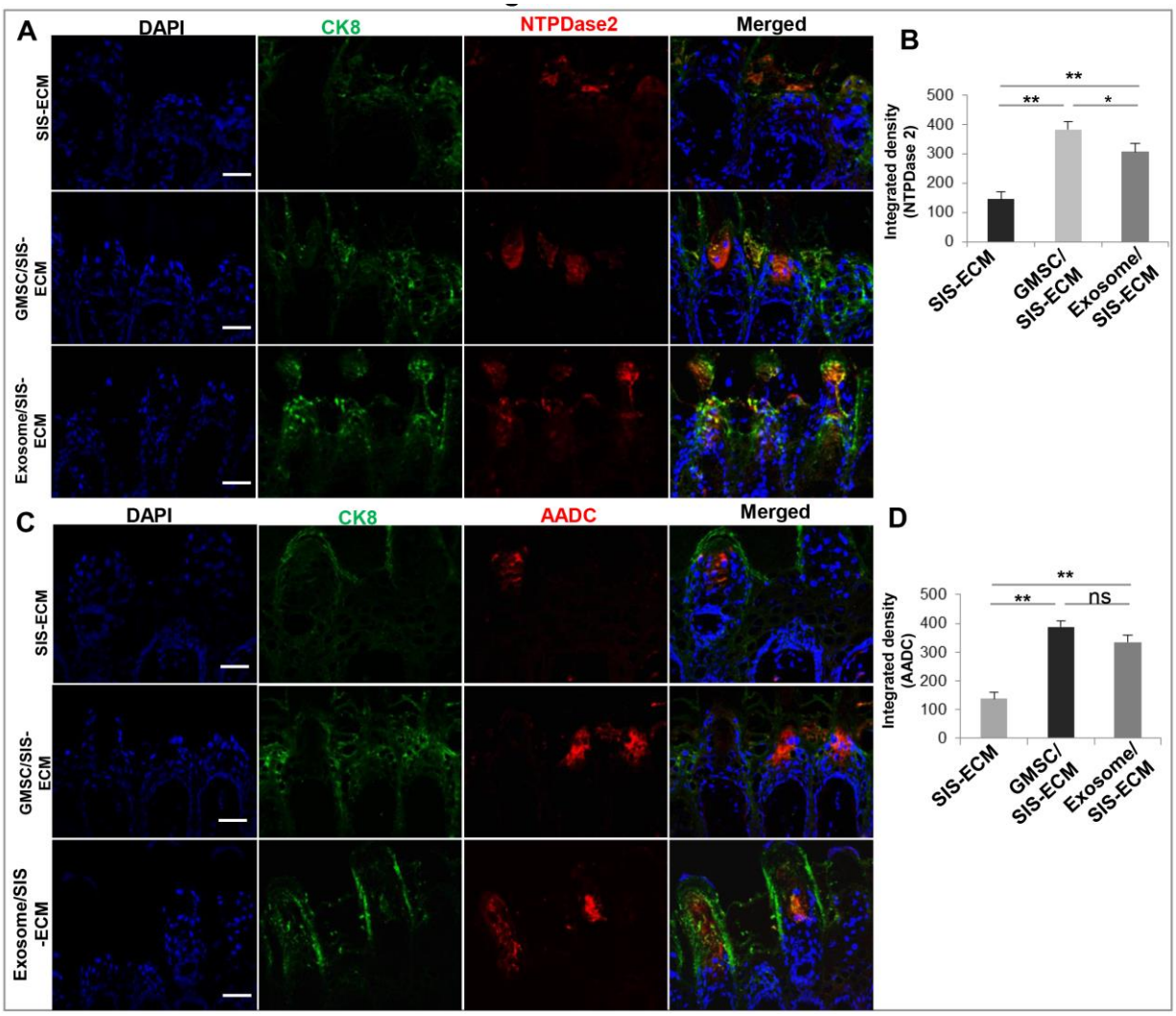
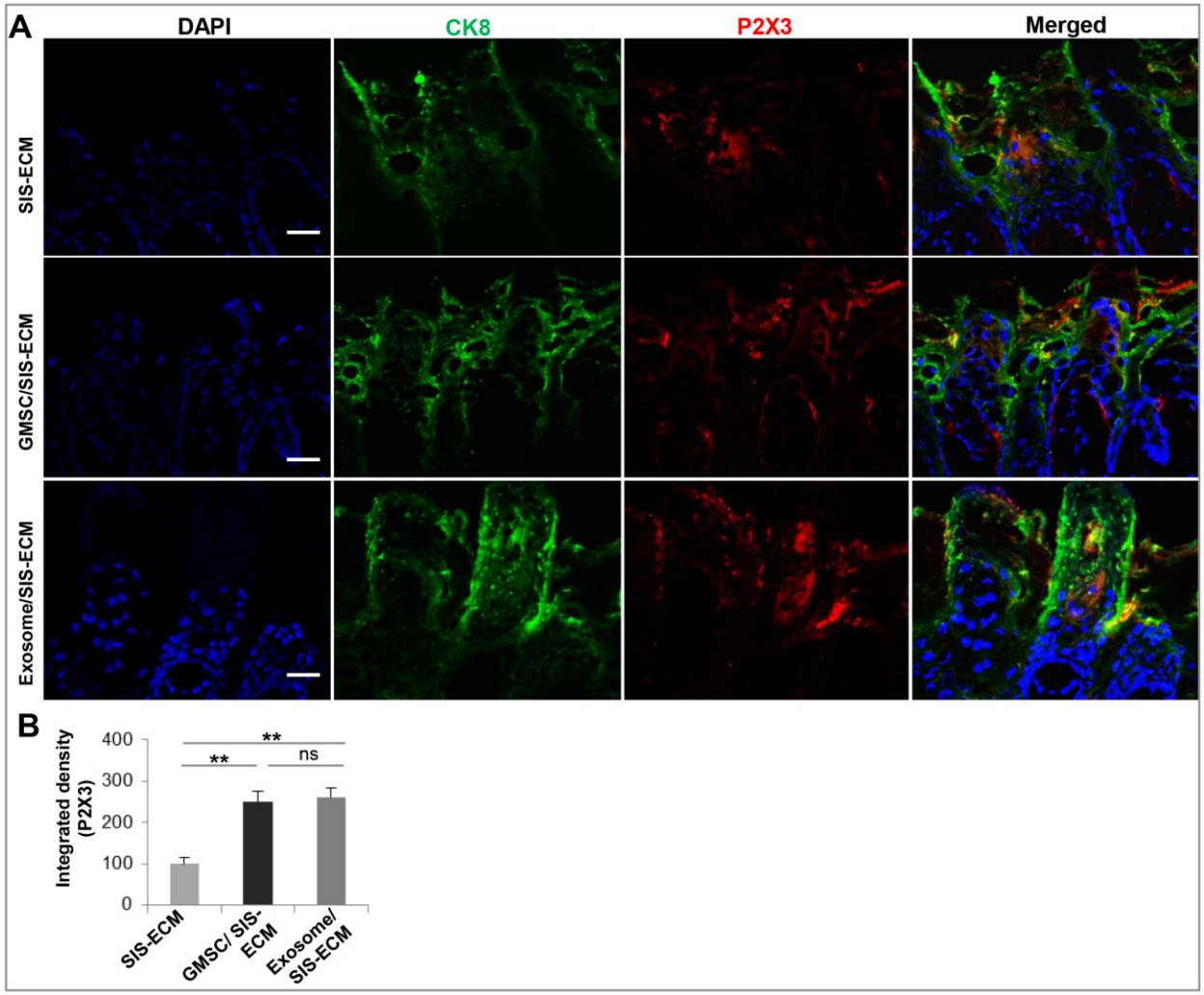
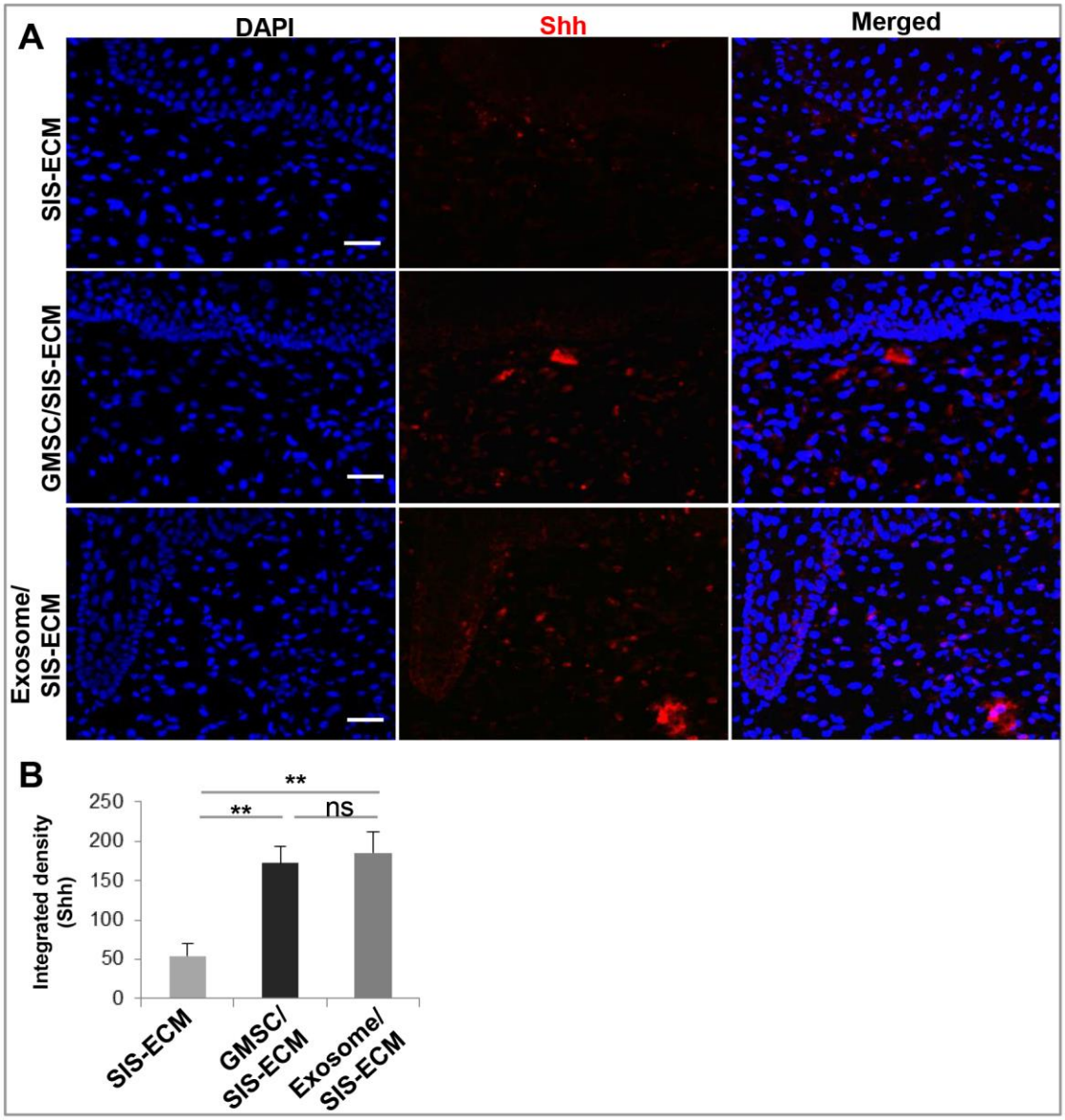


Appendix Figure 1. GMSC/SIS-ECM and Exosome/SIS-ECM promoted regeneration of type I and type III taste bud cells. Co-expression of CK8 (green) and NTPDase 2 (red) (**A, B**) and L-amino acid decarboxylase (AADC) (red) (**C, D**) at local defect healing areas at day 56 post-surgery was determined by immunofluorescence and observed under a fluorescence microscope. The nuclei were counterstained with DAPI (original magnification 20×). *P<0.05; **P<0.01; ns, no significance.



Appendix Figure 2. GMSC/SIS-ECM and Exosome/SIS-ECM upregulated $P2X_3$ receptor expression in regenerated taste buds. Co-expression of CK8 (green) and $P2X_3$ receptors (red) (**A, B**) at local defect healing areas at day 56 post-surgery was determined by immunofluorescence and observed under a fluorescence microscope. The nuclei were counterstained with DAPI (original magnification 20×). **P*<0.05; ***P*<0.01; ns, no significance.



Appendix Figure 3. GMSC/SIS-ECM and Exosome/SIS-ECM upregulated Shh expression in tongue defect areas. The expression of Shh (red) (**A, B**) at local defect healing areas at day 14 post-surgery was determined by immunofluorescence and observed under a fluorescence microscope. The nuclei were counterstained with DAPI (original magnification 20×). *P<0.05; **P<0.01; ns, no significance.