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Stem Cell–Soluble Signals Enhance Multilumen Formation in SMG Cell Clusters

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Appendix

Materials and Methods

Experimental Animals

Female C57BL/6 mice at 5 wk of age were anesthetized via intraperitoneal (IP) injection with 80 to 100 mg/kg ketamine and 5 mg/kg xylazine. Mice were euthanized by abdominal exsanguination, and submandibular glands were removed, processed, and plated as described below. All animal usage, anesthesia, and surgery were conducted with the approval of the University of Utah Institutional Animal Care and Use Committee, in accordance with their strict guidelines.

Preparation of Mouse Submandibular Gland Cell Clusters

Freshly dispersed cell clusters from C57BL/6 mouse submandibular glands (mSMGs) were prepared as described previously (Leigh et al. 2014) with some modifications as follows: mSMGs were minced and then placed in dispersion medium consisting of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1; HyClone, Logan, UT, USA), 0.2 mM $CaCl_2$, 1% (wt/vol) bovine serum albumin (BSA), 50 U/mL collagenase (Worthington Biochemical, Freehold, NJ, USA), and 400 U/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 min with aeration (95% air, 5% CO_2). Pipetting was carried out at 20 and 30 min to disperse the glands. mSMG cell aggregates were washed with enzyme-free assay buffer (120 mM NaCl, 4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], pH 7.4) containing 1% (wt/vol) BSA. After washing, cells were filtered through a 200-μm nylon mesh. A secondary wash was performed in DMEM/Ham's F-12 (1:1) containing 2.5% (vol/vol) fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) along with the following supplements: 2 nM triiodothyronine, 0.1 μM retinoic acid, 0.4 μg/mL hydrocortisone, 80 ng/mL epidermal growth factor (EGF), 5 ng/mL sodium selenite, 5 mM glutamine, 5 μg/mL insulin, 5 μg/mL transferrin, and freshly added 100 μg/mL Normocin (InvivoGen, San Diego, CA, USA). Then, cells were centrifuged at 37 °C and 700 rpm for 5 min. The pellet was suspended in 5 to 10 mL DMEM/Ham's F-12 (1:1) with supplements as described above. Cells were subsequently filtered through 70-μm and 40-μm nylon meshes and plated as described below.

Plating of mSMG Cell Clusters

mSMG cell clusters were dissociated as described above, and approximately 8,500 cell clusters were plated into each well. mSMG cell clusters were plated on the following combinations of extracellular matrix (ECM), feeder layer, and medium: growth factor–reduced Matrigel (GFR-MG; BD Biosciences, San Jose, CA, USA), laminin-1 (L1; Trevigen, Gaithersburg, MD), L1 on top of a human hair follicle–derived mesenchymal stem cell (hHF-MSC) feeder layer, hHF-MSC feeder layer alone, or L1 combined with hHF-MSC conditioned medium (L1 + hHF-MSC CM). In other experiments, mSMG cell clusters were plated on top of a mouse 3T3-J2 fibroblast feeder layer, a human foreskin fibroblast feeder layer, or a mouse embryonic fibroblast feeder layer. Then, mSMG cells were allowed to grow for 6 d at 37 °C with 95% air, 5% CO_2 in supplemented DMEM/Ham's F-12 medium (as described above). The medium was changed every 2 d. In experiments using L1 + hHF-MSC CM, the supplemented DMEM/Ham's F-12 medium was replaced after 24 h by a 2:1 ratio of hHF-MSC CM to complete proliferation medium (described below).

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Preparation of L1 Gel

In total, 100 µL L1 gel (Trevigen) was plated on 8-well chambers mounted on #1 German borosilicate cover glass (Nalge Nunc International Corporation, Naperville, IL, USA). L1 gel was plated at a concentration of 6 mg/mL and allowed to solidify at 37 °C for 1 h.

Preparation of GFR-MG

In total, 100 µL GFR-MG (8 mg/mL; 2:1 GFR-MG [BD Biosciences]: DMEM/Ham's F-12 [1:1] serum-free medium [HyClone]) was plated on 8-well chambers mounted on #1 German borosilicate cover glass (Nalge Nunc International Corporation) and allowed to solidify in a 37 °C incubator for 1 h.

Preparation of hHF-MSC Feeder Layer

hHF-MSCs were obtained as previously described (Liu et al. 2010) and cultured in proliferation medium (DMEM [high glucose; Life Technologies], supplemented with 10% (vol/vol) MSC-qualified fetal bovine serum [MSC FBS; Life Technologies], 1% (vol/vol) of antibiotic/antimycotic [Life Technologies], and 1 ng/mL basic fibroblast growth factor [bFGF; BD Biosciences]); the medium was replenished every other day unless otherwise indicated. Eighty percent confluent cell monolayers were trypsinized with 0.25% trypsin-EDTA (Life Technologies) and plated at 3,000 cells/well. Then, cells were treated with 15 µg/mL mitomycin C (Sigma-Aldrich) for 3 h.

Preparation of Mouse 3T3-J2 Fibroblast Feeder Layer

Cells were originally provided by H. Green (Harvard Medical School, Boston, MA, USA) and cultured in a proliferation medium consisting of DMEM (high glucose; Life Technologies), supplemented with 10% (vol/vol) bovine calf serum (Life Technologies) and 1% (vol/vol) antibiotic/antimycotic (Life Technologies). Cultures were incubated in a humidified 10% CO_2 atmosphere at 37 °C. Eighty percent confluent cell monolayers were treated with 15 µg/mL mitomycin C (Sigma-Aldrich) for 3 h.

Preparation of Human Foreskin Fibroblast Feeder Layer

Human foreskin fibroblast (hFB) cells derived from neonatal foreskins were isolated and grown as described previously (Bajaj et al. 2001). Cells were cultured in DMEM (high glucose; Life Technologies) supplemented with 20% (vol/vol) fetal bovine serum (Life Technologies) and 1% (vol/vol) antibiotic/antimycotic (Life Technologies). Cultures were incubated in a humidified 10% CO2 atmosphere at 37 °C and medium was changed every 3 to 4 d.

Preparation of Mouse Embryonic Fibroblast Feeder Layer

Mouse embryonic fibroblast cells (MEFs; Chemicon, Temecula, CA, USA) were cultured in human embryonic stem cell medium consisting of DMEM/F-12 (Life Technologies) 20% knockout serum replacer (Life Technologies), 1% nonessential amino acid supplements (Life Technologies), 100 mM β-mercaptoethanol (Sigma-Aldrich), and 10 ng/mL bFGF (BD Biosciences). Medium was changed every day (Bajpai et al. 2012). Eighty percent confluent cell monolayers were treated with 15 µg/mL mitomycin C (Sigma-Aldrich) for 3 h.

Preparation of L1 with an hHF-MSC Feeder Layer

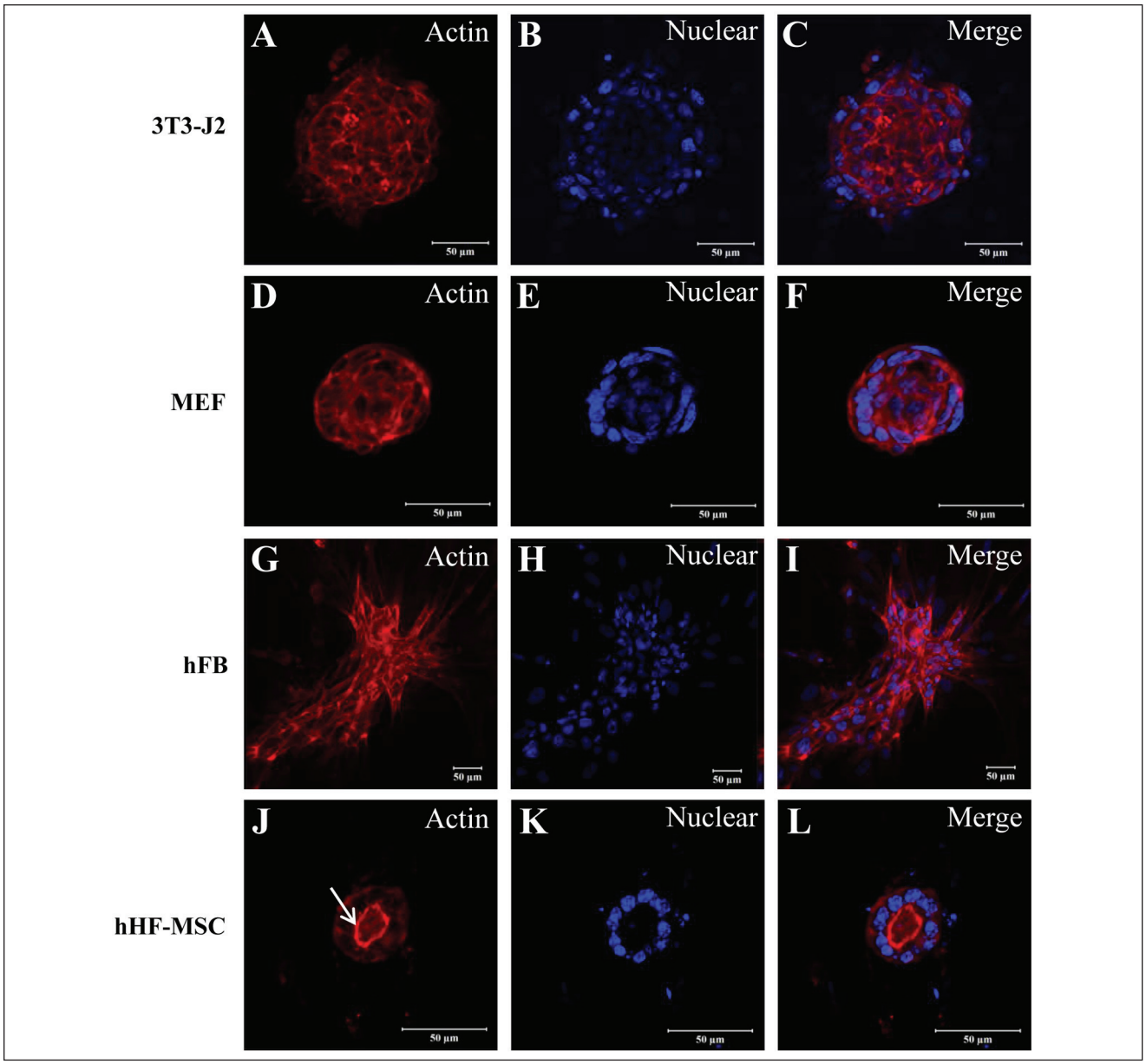
hHF-MSCs treated with mitomycin C (Sigma-Aldrich) were plated at 3,000 cells/well on 8-well chambers mounted on #1 German borosilicate cover glass (Nalge Nunc International Corporation) coated with 100 µL L1 gel (Trevigen), which fully covers 1 well of an 8-well chamber, and allowed to solidify in a 37 °C incubator for 1 h.

Preparation of hHF-MSC CM

Tissue culture medium from passage 8 hHF-MSCs (DMEM high glucose, supplemented with 10% (vol/vol) MSC FBS [Life Technologies], 1% (vol/vol) of antibiotic/antimycotic [Life Technologies], and 1 ng/mL bFGF [BD Biosciences]) was collected after cells reached a confluence of 40%. hHF-MSC CM was combined with complete proliferation medium (1:1 DMEM/Ham's F-12, 2 nM triiodothyronine, 0.1 µM retinoic acid, 0.4 µg/mL hydrocortisone, 80 ng/mL EGF, 5 ng/mL sodium selenite, 5 mM glutamine, 5 µg/mL insulin, 5 µg/mL transferrin, and freshly added 100 µg/mL Normocin [InvivoGen]) in a 2:1 ratio (see Appendix Fig. 2).

Preparation of L1 with Different Ratios of hHF-MSC CM to Complete Medium

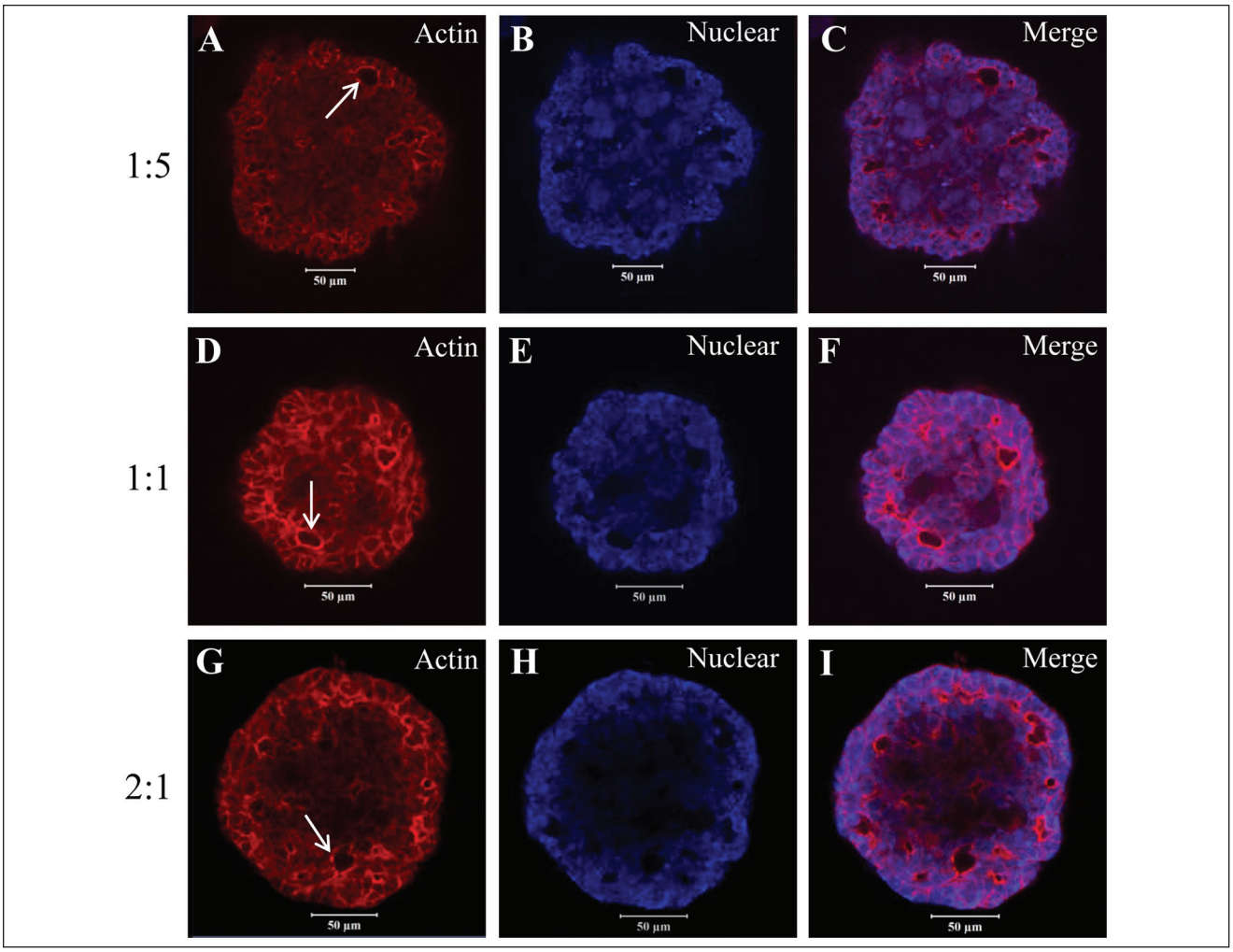
In total, 100 µL L1 gel (Trevigen) was plated on 8-well chambers mounted on #1 German borosilicate cover glass (Nalge Nunc International Corporation) at a concentration of 6 mg/mL (manufacturer's instructions) and allowed to solidify at 37 °C for 1 h. mSMG cell clusters were dissociated as described above and plated on top of the L1 gel. Plated mSMG cell clusters were cultured in varying ratios of hHF-MSC CM (passage 8, 40% confluence) to complete medium (1:1 DMEM-Ham's F-12, 2 nM triiodothyronine, 0.1 µM retinoic acid, 0.4 µg/mL hydrocortisone, 80 ng/mL EGF, 5 ng/mL sodium selenite, 5 mM glutamine, 5 µg/mL insulin, 5 µg/mL transferrin, and freshly added 100 µg/mL Normocin [InvivoGen]). The following ratios of CM to complete medium were tested: 1:5, 1:1, and 2:1.



Appendix Figure 1. The effect of different feeder layers on mouse submandibular gland (mSMG) cell cluster organization. mSMG cell clusters were grown on different feeder layers for 6 d and fixed as described in the Appendix Materials and Methods. mSMG cell clusters were grown on the following feeder layer cells: mouse fibroblast cell line (3T3-J2; **A–C**), mouse embryonic fibroblasts (MEFs; **D–F**), human foreskin fibroblasts (hFBs; **G–I**), and human hair follicle–derived mesenchymal stem cells (hHF-MSCs; **J–L**). Localization of filamentous actin (F-actin) was determined using phalloidin staining (A, D, G, J; red) and propidium iodide nuclear stain (B, E, H, K; blue). 3T3-J2 and MEF feeder layers produced round structures that were approximately 150 and 75 µm in diameter but lacked well-defined lumens (A–F), and structures grown on an hFB feeder layer were completely disorganized (G–I). The hHF-MSC feeder layer produced the most organized mSMG structures that were approximately 50 µm in diameter and formed small central lumens (J–L). The xy cross-section images were obtained using a Carl Zeiss 510 confocal microscope. White arrow indicates apical expression of F-actin.

Confocal Microscopy

mSMG cell clusters were fixed in 4% (vol/vol) paraformaldehyde for 20 min at room temperature, incubated with 0.1% (vol/vol) Triton X-100 in phosphate-buffered saline (PBS) for 5 min, and washed with PBS. Cells were then incubated with 5% (vol/vol) goat serum, containing 10 µM digitonin (Invitrogen) for 2 h at room temperature and washed 3 times with PBS. Cells were incubated for 2 wk at 4 °C with rabbit anti–zonula occludens-1 (ZO-1; Invitrogen), mouse anti–E-cadherin (BD Biosciences), mouse anti–cytokeratin-14 (Abcam, Cambridge, MA, USA), or mouse anti-proliferating cell nuclear antigen (PCNA; Abcam) at 1:50 dilutions in 5% (vol/vol) goat serum containing 10 µM digitonin. Then, mSMG



Appendix Figure 2. The effect of different ratios of human hair follicle–derived mesenchymal stem cell conditioned medium (hHF-MSC CM) to complete proliferation medium on mouse submandibular gland (mSMG) cell cluster organization. mSMG cell clusters were grown on laminin-1 (L1) with different ratios of hHF-MSC CM to complete proliferation medium for 6 d as described in the Appendix Materials and Methods. The following ratios of hHF-MSC CM to complete proliferation medium were tested: 1:5 (**A–C**), 1:1 (**D–F**), and 2:1 (**G–I**). Localization of filamentous actin (F-actin) was determined using phalloidin staining (A, D, G; red) and propidium iodide nuclear stain (B, E, H; blue). The structures grown on a 2:1 ratio were about 190 µm in diameter and showed optimal cell organization with strong apical F-actin expression showing many small lumens (G–I). The 2:1 ratio was optimal, as it allowed the formation of bigger structures compared to the other dilution factors. The xy cross-section images were obtained using a Carl Zeiss 510 confocal microscope. White arrows indicate apical expression of F-actin.

cell clusters were warmed to room temperature for 20 min and washed 3 times for 5 min with PBS. mSMG cell clusters were incubated for 1 wk with either Alexa Fluor 488–conjugated goat anti-rabbit or Alexa Fluor 488–conjugated goat anti-mouse (1:500 dilution in 5% goat serum containing 10 µM digitonin; Invitrogen) at 4 °C. The cell clusters were then warmed to room temperature for 20 min and washed 3 times for 5 min with PBS. mSMG cell clusters were stained for 15 min with Alexa Fluor 633–conjugated phalloidin F-actin stain (1:400 dilution in PBS; Sigma-Aldrich) and for 5 min with either a propidium iodine nuclear stain (1:3,000 dilution in 2× saline-sodium citrate buffer; Invitrogen) or TO-PRO-3 iodide nuclear stain (1:1,000 dilution in PBS; Life Technologies). Images were obtained using either a Carl Zeiss 510 or LSM 700 confocal microscope (Oberkochen, Germany).

Western Blot Analysis

mSMG cells clusters grown on L1 and L1 + hHF-MSC CM as well as minced SMG (used as positive control) were lysed in 200 µL of 2× Laemmli buffer with 10 mM dithiothreitol (DTT), and lysates were sonicated for 20 s with a Fisher Scientific Sonic Dismembrator (Pittsburgh, PA, USA; model FB-120, microtip; output level 5; duty cycle 50%) and boiled for 3 min. The lysates were subjected to 4% to 15% (wt/vol) sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad,

Hercules, CA, USA) on mini-gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 3% (wt/vol) BSA in Tris-buffered saline (0.137 M NaCl, 0.025 M Tris [hydroxymethyl]-aminomethane, pH 7.4) containing 0.1% (vol/vol) Tween-20 (TBST) and immunoblotted overnight at 4 °C with a rabbit anti–aquaporin-5 (Boster Bio, Pleasanton, CA, USA), anti–α-smooth muscle actin (Abcam) or mouse anti–cytokeratin-14 (Abcam), and anti-PCNA (Abcam) primary antibodies in 1:500 dilution in TBST containing 3% (wt/vol) BSA. After incubation with the primary antibodies, membranes were washed 3 times for 15 min each with TBST and incubated with peroxidase-linked goat anti-rabbit IgG antibody (1:5,000 dilution in TBST containing 3% [wt/vol] BSA; Cell Signaling Technology, Beverly, MA, USA) or goat anti-mouse IgG antibody (1:5,000 dilution in TBST containing 3% [wt/vol] BSA; Santa Cruz, Dallas, TX, USA) at room temperature for 1 h. The membranes were washed 3 times for 15 min each with TBST and treated with Clarity chemiluminescence detection reagent (Bio-Rad). The protein bands were visualized using a Bio-Rad ChemiDoc MP imager, and quantification of the bands was performed using Image Lab 4.1 software (Bio-Rad). For signal normalization, membranes were treated with stripping buffer (0.2 M glycine, 1% [vol/vol] Tween-20, and 3.5 mM sodium dodecyl sulfate, pH 2.2) and reprobed with a polyclonal rabbit anti–β-tubulin primary antibody (1:500 dilution; Abcam) followed by incubation with peroxidase-linked goat anti-rabbit IgG antibody as described above. During the SMG cell isolation prep, a portion of the whole mSMG was lysed in 2× Laemmli buffer with 10 mM DTT, sonicated, and run as the "mSMG Fresh" positive control.

Intracellular Free Ca^{2+} Concentration Measurements

Relative intracellular free Ca^{2+} concentration was visualized in mSMG cell clusters grown on different ECMs and feeder layers. Cells were preloaded with Fluo-2 AM (TEFLabs, Austin, TX, USA), a Ca^{2+}-sensitive fluorescent dye, for 20 min at 37 °C and washed with assay buffer (136 mM NaCl, 4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM 137 HEPES [pH 7.4], and 0.1% [wt/vol] BSA). The cells were stimulated with carbachol (1 μM; Sigma-Aldrich) or 1× PBS (negative control) at room temperature, and the release was measured using a Leica DMI6000B fluorescence microscope (Leica Microsystems, Mannheim, Germany). Fluo-2 AM fluorescence images were captured with an ORCA-R2 camera (Hamamatsu Photonics KK, Hamamatsu City, Japan). The graph was obtained using the average pixel intensity values of images that were taken over time and were normalized to changes in background intensity.

Cyclic Nucleotide Measurements

Cyclic adenosine monophosphate (cAMP) levels in mSMG cell clusters grown on L1 and L1 + hHF-MSC CM were measured at different times (1 and 5 min) in response to isoproterenol (1–100 μM; Sigma-Aldrich) using a cAMP XP assay kit (Cell Signaling) as indicated by the manufacturer's instructions and analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Secretory Granule Visualization

mSMG cell clusters grown for 6 d on L1 and L1 + hHF-MSC CM were fixed (1% [vol/vol] glutaraldehyde, 2.5% [vol/vol] paraformaldehyde, 100 mM cacodylate buffer [pH 7.4], 6 mM $CaCl_2$, 4.8% [wt/vol] sucrose) overnight at 4 °C. The following day, the samples were washed 3 times for 5 min with cacodylate buffer. Cell clusters were fixed a second time with 2% osmium tetroxide for 45 min at room temperature. Then, cell clusters were washed twice for 5 min with cacodylate buffer and once for 15 min with distilled water. Cell clusters were then stained with uranyl acetate for 45 min at room temperature and washed 3 times for 5 min with distilled water. Cells were dehydrated with a graded ethanol series (30%, 50%, 70%, 95% 2 times, 100% 3 times for 15 min each) and then with absolute acetone 3 times for 10 min. Next, EPON™ epoxy resin (Hexion, Columbus, OH, USA) was infiltrated into the cells as follows: 30% for 5 h, 70% overnight, 100% for 8 h with 3 changes, and 100% fresh resin for embedding. Then, the fresh resin was polymerized for 48 h at 60 °C. Ultracuts were obtained using a Leica UC 6 ultratome (Leica Microsystems) and sections were taken at a 70-nm thickness. Sections of cell clusters were stained with 1% toluidine blue in 1% sodium borate and imaged on a Leica DMI6000B inverted microscope (Leica Microsystems).

Results

mSMG Cell Clusters Grown on Different Feeder Layers

To test whether a feeder layer could improve salivary cell growth and differentiation, we grew mSMG cell clusters on 3T3-J2, MEF, hFB, and hHF-MSC feeder layers. As shown in Appendix Figure 1A–F, 3T3-J2 and MEF feeder layers produced round structures that were approximately 150 and 75 μm in diameter, respectively. However, these structures lacked a defined apical F-actin ring and failed to form lumens (Appendix Fig. 1A–F). Structures grown on an hFB feeder layer were completely disorganized (Appendix Fig. 1G–I). The hHF-MSC feeder layer produced the most organized mSMG structures that were approximately 50 μm in diameter and formed small central lumens (Appendix Fig. 1J–L).

mSMG Cell Clusters Cultured in Varying Ratios of hHF-MSC CM to Complete Medium

To determine the optimal growing conditions for mSMG cell clusters, we tested different ratios of hHF-MSC CM to complete medium. While the hHF-MSC CM contains critical growth factors, many of the nutrients have been consumed by the hHF-MSCs, thus requiring supplementation with complete medium. The ratio of 1:5 hHF-MSC CM to complete medium

yielded large acinar structures (Appendix Fig. 2A–C), about 200 μm in diameter; however, they displayed low F-actin expression and decreased quality of shape. A 1:1 ratio of hHF-MSC CM to complete medium resulted in slightly smaller structures (Appendix Fig. 2D–F), approximately 150 μm in diameter. Last, a 2:1 ratio of hHF-MSC CM to complete medium yielded structures intermediate in size (Appendix Fig. 2G–I). The structures were about 190 μm in diameter and showed optimal cell organization with strong apical F-actin expression showing many small lumens (Appendix Fig. 2G–I).

Discussion

The hHF-MSCs used in the present study are hair follicle derived, but multipotent MSCs (adult stromal cells capable of self-replicating and differentiating) can be derived from many other sources, including adipose tissue, lung tissue, and bone marrow (Wei et al. 2013). Our initial studies compared the ability of 3 other feeder cell lines (in addition to the hHF-MSCs) to support mSMG cell differentiation in vitro. Specifically, we tested the mouse 3T3-J2 fibroblast cell line, MEFs, and hFBs (Appendix Fig. 1). Although this preliminary study yielded promising results for hHF-MSCs as a feeder layer, these results were inconsistent, and we ultimately concluded that the cell lines tested were unable to produce organized salivary cell clusters (Fig. 1G–L and Appendix Fig. 1A–I). These results indicate that the direct cell-cell contact of feeder cells and mSMG cell clusters may be detrimental to organized cluster formation.

Appendix References

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