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3D Organoid Formation from the Murine Salivary Gland Cell Line SIMS

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[Abstract] Salivary glands consist of multiple phenotypically and functionally unique cell populations, such as the acinar, ductal, and myoepithelial cells that help produce, modify, and secrete saliva (Lombaert *et al.*, 2011). Identification of mechanisms and factors that regulate these populations has been of key interest, as salivary gland-related diseases have detrimental effects on these cell populations. A variety of approaches have been used to understand the roles different signaling mechanisms and transcription factors play in regulating salivary gland development and homeostasis. Differentiation assays have been performed with primary salivary cells in the past (Maimets *et al.*, 2016), however this approach may sometimes be limiting due to tissue availability, labor intensity of processing the tissue samples, and/or inability to long-term passage the cells. Here we describe in detail a 3D differentiation assay to analyze the differentiation potential of a salivary gland cell line, SIMS, which was immortalized from an adult mouse submandibular salivary gland (Laoide *et al.*, 1996). SIMS cells express cytokeratin 7 and 19, which is characteristic for a ductal cell type. Although adult acinar and myoepithelial cells were found *in vivo* to preserve their own cell population through self-duplication (Aure *et al.*, 2015; Song *et al.* 2018), in some cases duct cells can differentiate into acinar cells *in vivo*, such as after radiation injury (Lombaert *et al.*, 2008; Weng *et al.*, 2018). Thus, utilization of SIMS cells allows us to target and analyze the self-renewal and differentiation effects of ductal cells under specific *in vitro* controlled conditions.

Keywords: Salivary gland, Epithelial, SIMS, Differentiation assay, Mouse cell line

[Background] *In vitro* differentiation assays are a remarkable tool that enables us to analyze the potency of cells without relying on *in vivo* models. Cells in these assays can be manipulated for gene expression to, for instance, analyze their differentiation ability. While previous protocols have described the differentiation potential of primary salivary gland cells *in vitro*, our protocol is optimized for the use of the SIMS cell line (Laoide *et al.*, 1996; Maimets *et al.*, 2016). SIMS cells express cytokeratin 7 and 19, which is characteristic for a ductal cell type. Although adult acinar and myoepithelial cells were found *in vivo* to preserve their own cell population through self-duplication (Aure *et al.*, 2015; Song *et al.* 2018), in some cases duct cells can differentiate into acinar cells *in vivo*, such as after radiation injury (Lombaert *et al.*, 2008; Weng *et al.*, 2018). Thus, utilization of SIMS cells allows us to target and analyze the self-renewal and differentiation effects of ductal cells under specific *in vitro* controlled conditions. Our recent work shows that SIMS cells have the ability to differentiate into unique populations of acinar, myoepithelial, and duct cells in 3D differentiation conditions, that normally produce, modify and secrete saliva *in vivo* (Lombaert *et al.*, 2011; Athwal *et al.*, 2019). This protocol

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describes the workflow necessary for the generation and analysis of 3D differentiated SIMS cells. SIMS cells are cultured in a 3D matrigel/collagen matrix over a period of 7-9 days. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which is rich in several growth factors and extracellular matrix proteins, such as laminin-1, collagen IV, and heparin sulfate proteoglycans (Patel *et al.*, 2016). Matrigel has been shown to induce differentiation of stem/progenitor cells and outgrowth of already differentiated cell types by causing polarization of cells embedded in or on top of it. For SIMS grown in this 3D matrix, organoid formation post single cell culture is apparent around day 5 of culture. These organoids are also stained either via cryosectioning or in 3D to analyze for differentiation markers and cell populations. Alterations, such as cell transfections, transductions and/or media changes, to this condition can easily be made to address specific research questions (Athwal *et al.*, 2019). The media components were adjusted using literature from both existing salivary and mammary gland differentiation protocols, which deemed necessary to induce differentiation of the SIMS cells *in vitro*. Here we show the robust use of this cell line to address specific research questions without having to rely on primary salivary gland cells.

Materials and Reagents

1. 500 ml filter units with 0.22 µm filter (Fisher, catalog number: 974102)
2. 24-well tissue culture plates (Thermo Fisher, catalog number: 142475)
3. T75 tissue culture flasks (Thermo Fisher, catalog number: 156499)
4. 2 ml pipettes (Fisher, catalog number: 13-678-25C)
5. 10 cm dishes (Thermo Fisher, catalog number: 150464)
6. 15 ml Falcon tubes (Corning, catalog number: 35296)
7. 1 ml pipettes (Fisher, catalog number: 13-678-25B)
8. Forceps (Fisher Scientific, catalog number: XX6200006P)
9. Razor blade (Fisher Scientific, catalog number: 12-640)
10. Glass slides (VWR, catalog number: 89500-466)
11. SIMS cells (available per request)
12. Sterile DMEM medium + 4.5g/L high glucose + L-glutamine without sodium pyruvate (Invitrogen, catalog number: 11965-092)
13. Sterile 1x PBS-Ca^{+2}-Mg^{+2} free (Thermo Fisher, catalog number: 110010031) or 1x HBSS-Ca^{+2}-Mg^{+2} free (Thermo Fisher, catalog number: 14175079)
14. Penicillin-Streptomycin (Thermo Fisher, catalog number: 15140122)
15. Sterile USDA-approved FBS (Sigma-Aldrich, catalog number: F2442-500ML)
16. 0.25% Trypsin-EDTA (Sigma-Aldrich, catalog number: T4049-100ML)
17. Epidermal growth factor (EGF) (Sigma-Aldrich)
18. Glutamax (Gibco/ThermoFisher, catalog number: 35050061)
19. Fibroblast growth factor-2 (FGF2) (R&D, catalog number: 3139FB025)
20. N2 (Thermo Fisher, catalog number: 17502048)

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21. Dexamethasone (VWR, catalog number: 80056-298)
22. Triiodothyronine (Sigma-Aldrich, catalog number: 709719)
23. Retinoic Acid (Thermo Fisher, catalog number: AA4454077)
24. Hydrocortisone (Sigma-Aldrich, catalog number: H0888)
25. Cholera toxin (Sigma-Aldrich, catalog number: C8052)
26. Calcium, 99%, Granular (Fisher, catalog number: AC201180050)
27. Growth factor reduced Matrigel (BD Biosciences, catalog number: 354230)
28. Collagen Type I, Rat Tail (Millipore, catalog number: 2790888)
29. 4% PFA (VWR, catalog number: AAJ61899-AK)
30. Trypan blue 0.4% (Thermo Fisher, catalog number: 15250061)
31. O.C.T. Compound (Fisher scientific, catalog number: 23-730-571)
32. SIMS subculture media (see Recipes)
33. Differentiation media (see Recipes)

Equipment

1. Water bath (Thermo Scientific, catalog number: TSGP02)
2. Vacuum pump aspirator (standard)
3. CO_2 incubator (standard)
4. Cryotome (standard)
5. Brightfield microscope (standard)
6. Confocal laser scanning microscope (standard)

Software

1. ImageJ software (http://imagej.net, available online for download)

Procedure

A. SIMS subculture
   1. Pre-warm cell medium, 1x PBS-Ca^{+2}-Mg^{+2} free or 1x HBSS- Ca^{+2}-Mg^{+2} free and 0.25% trypsin-EDTA in a water bath at 37 °C.
   2. Aspirate off old medium with vacuum aspirator or use 10 ml pipettes to manually aspirate from the T75 cell culture flask.
   3. Wash twice with 10 ml of sterile 1x PBS/HBSS-Ca^{+2}-Mg^{+}, aspirate off completely-either manually with a 2 ml pipette or with an aspirator and sterile fresh 2 ml glass pipette.
   4. Add 3 ml of pre-warmed 0.25% Trypsin-EDTA per T75 flask.
   5. Incubate plate with cells at 37 °C for 5-7 min. Tap and gently shake the plate to dislodge cells.

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6. Neutralize trypsin by adding fresh ~3-5 ml culture medium using a 10 ml pipette. Pipette gently up and down several times to re-suspend cells and transfer to a 15 ml Falcon tube.
7. Centrifuge at 100 *x g* for 3 min, wash 2 times with 1x PBS-Ca^{+2}-Mg^{+2} free, and take up the cells in desired amount of culture medium.
8. Plate up to 1 million cells to get 95% confluency in 4-5 days in a T75 tissue culture flask.
   Note: Cell viability (~90-95%) is determined by trypan blue cell count.

B. Organoid differentiation culture

1. Resuspend SIMS cells in SIMS medium at 0.4 x 10^6 cells/ml.
2. Thaw out growth factor reduced matrigel matrix on ice for 10 min before culturing cells. Both collagen and matrigel need to remain on ice until they are resuspended with cells for plating.
3. Add 50 µl of cell solution (10,000 cells) to 100 µl of 60:40 ratio of Type I rat tail collagen to growth factor reduced Matrigel on ice. Mix gently while slowly pipetting up and down using a 200 µl pipette.
   Notes:
   a. *Aliquot collagen first then Matrigel. Do not mix with pipette until you add cells to avoid bubbles.*
   b. *Avoid fast aspiration* or suspension of aliquoted matrix mix to avoid bubble formation.
4. Deposit cell/collagen/matrigel mix in the center of 24-well tissue culture plate on ice and incubate for 20 min at 37 °C.
5. Add up to 1 ml differentiation medium.
6. Change medium every 2-3 days.
7. Cultures are maintained up to 14 days as the cells reach maximum confluency within the matrix, and the matrix starts breaking down.
8. Organoid formation can be seen starting Day 5 of culture (Figure 1).

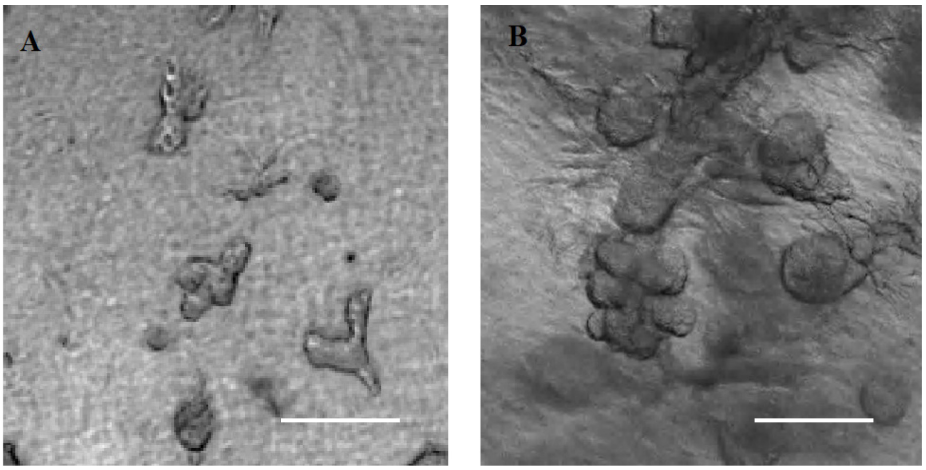

Figure 1. Collagen-matrigel embedded SIMS cells expand and form organoids by Day 5 (A) and 7 (B). Scale bars, 200 µm (4x magnification).

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C. Organoid fixation

1. Wash matrix with ice cold 1x PBS-Ca^{+2}-Mg^{+2} free gently up to 3 times.
2. Cells can be fixed at any timepoint using 2% PFA in 1x PBS-Ca^{+2}-Mg^{+2} free for 20 min on ice or at 4 °C.
3. Rinse the matrix with ice cold 1x PBS-Ca^{+2}-Mg^{+2} free 3 times.
4. Incubate matrix in 1x PBS-Ca^{+2}-Mg^{+2} free for 10 min. Repeat this 3 times for a total of 30 minutes.
5. Using forceps, gently remove the matrix from the tissue culture plate.
   Note: You can cut the matrix into 2-3 pieces using a fine blade if you want multiple cryo blocks.
6. Embed with OCT (a full description of embedding can be found in Campbell *et al.*, 2011).
   Note: Matrix will likely curl up in OCT if it is poured too quickly. Gently pour, and fix any curling using your forceps.
7. Optimal cutting thickness is between 12 and 16 µm.
8. Sections can be post fixed with 4% PFA, ice cold acetone, or acetone/methanol for staining optimization.

D. 3D matrix staining

To conserve the 3D morphology, staining is performed on 1 mm^3 pieces of the organoids embedded in matrix.

1. Post fixation, small pieces of matrix including organoids are cut using a sharp scalpel blade and pointed tweezers. Treat each chunk separately in a plastic dish or 24-well plate.
2. Matrix can be treated again with 2-4% PFA, acetone/methanol, or methanol treatments if necessary.
3. Matrix is treated like tissue slides for staining. An example is described in Athwal *et al.*, 2019.
4. Mount each sample on glass slides with 1-2 spacers depending on the size of the matrix.
5. Capture images using a confocal microscope (Figure 2).
6. This staining can also be performed on cryosections of OCT embedded organoid matrix (Athwal *et al.*, 2019). A complete description of the primary and secondary antibodies used in Figure 2 can be found in above-mentioned manuscript.

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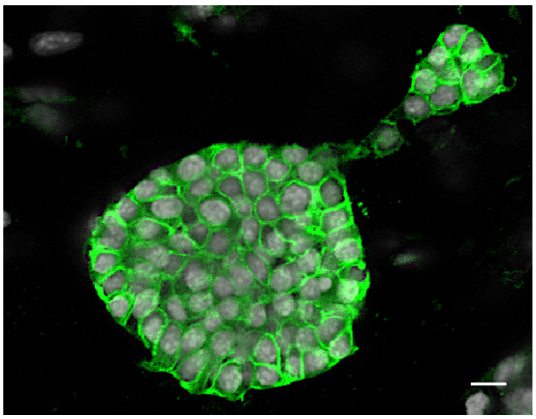

Figure 2. 1 mm^3 collagen/matrigel matrix with 3D grown SIMS organoids were stained with E-cadherin post-fixation with 4% PFA. Scale bar, 10 μm.

Data analysis

Brightfield images are taken starting Day 3 until the end of the experiment. Organoid diameter can be quantified using ImageJ. Each experiment is repeated 3 times with n = 5 images per day for quantification. Details on statistical analysis can be found in our previous manuscript (Athwal *et al.*, 2019).

Notes

1. Cell death is noticed by Day 14 as cells become confluent within the matrix.
2. Serial passage of cells dissociated from organoids has not been performed with SIMS cell organoids.
3. Increase the volume of collagen/matrigel if cell number is increased.
4. Transduced or transfected SIMS cells are selected prior to the 3D culture assay.

Recipes

1. SIMS subculture medium (Table 1)

Table 1. SIMS subculture medium

Reagent	Stock	Final	Volume
Sterile DMEM medium + 4.5 g/L high glucose + L-glutamine without sodium pyruvate	100%		455 ml
Pen-Strep	100%	1%	5 ml
FBS	100%	10%	50 ml

2. Differentiation medium

Table 2. SIMS differentiation medium

Reagent	Original Concentration	Final Concentration	Volume Added
SIMS complete medium			50 ml
Glutamax	100x	1x	500 µl
ITS	100x	1x	500 µl
N2 Supplement	100x	1x	500 µl
EGF	200 µg/ml	20 ng/ml	5 µl
FGF2	25 µg/ml	20 ng/ml	40 µl
Dexamethasone	3.85 mM	1 µM	13 µl
Triiodothyronine	29.7 µM	2 nM	3.4 µl
Retinoic Acid	10 mM --> 1 mM	0.1 µM	5 µl
Hydrocortisone	1 mg/ml	0.4 µg/ml	20 µl
Cholera toxin	100 µg/ml	8.4 ng/ml	4.2 µl
Calcium	50 mM	0.8 mM	0.8 µl

Notes:

a. Make 100 µl aliquots of growth factor reduced matrigel and store at -20 °C.

b. Make 100 µl aliquots of rat tail collagen and store at 4 °C.

Acknowledgments

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Competing interests

There are no conflicts of interest.

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