Rat Parotid Gland Cell Differentiation in Three-Dimensional Culture

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The use of polarized salivary gland cell monolayers has contributed to our understanding of salivary gland physiology. However, these cell models are not representative of glandular epithelium *in vivo*, and, therefore, are not ideal for investigating salivary epithelial functions. The current study has developed a three-dimensional (3D) cell culture model for rat Par-C10 parotid gland cells that forms differentiated acinar-like spheres on Matrigel. These 3D Par-C10 acinar-like spheres display characteristics similar to differentiated acini in salivary glands, including cell polarization, tight junction (TJ) formation required to maintain transepithelial potential difference, basolateral expression of aquaporin-3 and Na⁺/K⁺/2Cl⁻ cotransporter-1, and responsiveness to the muscarinic receptor agonist carbachol that is decreased by the anion channel blocker diphenylamine-2-carboxylic acid or chloride replacement with gluconate. Incubation of the spheres in the hypertonic medium increased the expression level of the water channel aquaporin-5. Further, the proinflammatory cytokines tumor necrosis factor- α and interferon- γ induced alterations in TJ integrity in the acinar-like spheres without affecting individual cell viability, suggesting that cytokines may affect salivary gland function by altering TJ integrity. Thus, 3D Par-C10 acinar-like spheres represent a novel *in vitro* model to study physiological and pathophysiological functions of differentiated acini.

Introduction

MAMMALIAN SALIVARY GLANDS are composed of acinar and ductal cells (Fig. 1A). To achieve unidirectional saliva secretion, acinar cells must polarize to differentiate distinct apical and basolateral membranes (Fig. 1B). In current models of saliva secretion, the transepithelial movement of Cl⁻ is the primary driving force for fluid and electrolyte secretion by salivary acinar cells (Fig. 1B).¹ In salivary epithelium, agonist-induced stimulation of the basolateral muscarinic M3 receptor initiates activation of Ca²⁺-dependent Cl⁻ channels on the apical surface and K⁺ channels on the basolateral surface.¹ The stimulated efflux of K⁺ and Cl⁻ down their electrochemical gradients produces a transepithelial potential difference (PD) that drives Na⁺ transport and water diffusion across the tight junction (TJ), creating an isotonic primary saliva secretion in the gland lumen^{2,3} (Fig. 1B).

TJs consist of a narrow belt-like structure in the apical region of the lateral plasma membrane and circumferentially bind each cell to its neighbor, thereby contributing to epithelial polarity.⁴ The major TJ proteins are occludin, claudins, and junctional adhesion molecules.⁵ These proteins associate with intracellular scaffold proteins called zonula occludens

(ZO), which anchor TJ proteins to the actin cytoskeleton⁵ (Fig. 1B).

Salivary cell lines have been useful for studying salivary gland function and differentiation, most commonly the human salivary gland (HSG) cell line that differentiates into an acinar-like phenotype when grown on a basement membrane, such as Matrigel.⁶ However, HSG cell cultures on Matrigel are unable to differentiate into polarized monolayers,⁶ most likely due to inappropriate expression and localization of TJ proteins. A promising alternative to the use of HSG cells is the rat parotid gland (Par-C10) cell line, which forms TJs when grown as two-dimensional (2D) cell monolayers on permeable membranes^{7,8}; however, there are some limitations with 2D cultures, including difficulties pertaining to experimental manipulation, detailed microscopic and biochemical analyses, and detection of essential structural features of glandular epithelium *in vivo*. The aim of the current study was to develop an *in vitro* three-dimensional (3D) cell culture model using Par-C10 cells that more closely resembles salivary gland morphology *in vivo* regarding study of TJs and formation of transepithelial gradients for investigations of physiological and pathological responses in salivary gland acini.

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Materials and Methods

3D culture of Par-C10 cells

The Par-C10 cell line was derived from freshly isolated rat parotid gland acinar cells by transformation with simian virus 40 (SV40) and exhibits characteristics of freshly isolated acinar cells.⁹ Fifty microliters of growth-factor-reduced (GFR) Matrigel (8 mg/mL; 2:1 GFR Matrigel: Dulbecco's modified Eagle's medium [DMEM]–Ham's F12 [1:1] medium; Becton Dickinson Labware, Franklin Lakes, NJ) was allowed to solidify in a 37°C incubator for 1 h in eight-well chambers mounted on #1.5 German borosilicate coverglasses (Nalge Nunc International Corporation, Naperville, IL). Then, Par-C10 cells (1×10^4 cells/well; passage 40–60) were plated on the GFR Matrigel in DMEM–Ham's F12 (1:1) medium with supplements, as defined previously.⁷ Differentiated 3D cultures of Par-C10 acinar-like spheres were used for assays after incubation in a 37°C incubator with 5% air and 95% CO₂ for 2 days. Osmotic stress was induced in some Par-C10 3D cultures to upregulate aquaporin-5 (AQP5) expression, as described previously.^{10,11} Accordingly, cells were incubated for 24 h in hypertonic serum-free DMEM–Ham's F12 medium containing 200 mM sorbitol.

Measurement of carbachol-induced changes in PD in 3D Par-C10 acinar-like spheres

Intraluminal recordings from 3D Par-C10 acinar-like spheres grown on a 35 × 10 mm tissue culture dish (Becton Dickinson Labware) precoated with GFR Matrigel were made using 20–30 MΩ glass microelectrodes filled with 0.3 M KCl with a tip diameter of ~1 μm and an Axoclamp 2A intracellular amplifier (Molecular Devices, Downingtown, PA). Measurements were made of PD between the lumen of the spheres and the bath before, during, and after stimulation of the cells with carbachol (10 or 100 μM, as described in figure legends). Data were acquired with a Digidata 1332A data acquisition board (Molecular Devices) and analyzed with Clampfit software (version 9; Molecular Devices).

Intracellular free Ca²⁺ concentration measurements

The intracellular free Ca²⁺ concentration ([Ca²⁺]_i) was quantified in single cells using an InCyt Dual-Wavelength Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH), as described previously.⁷ Increases in [Ca²⁺]_i are expressed as the peak response obtained under the indicated conditions (see Fig. 5C legend) or after subtracting basal [Ca²⁺]_i from the peak [Ca²⁺]_i (see Fig. 5D legend).

Confocal microscopy analysis

Par-C10 acinar-like spheres were fixed in 4% paraformaldehyde for 30 min at room temperature, incubated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, and washed thrice with PBS. Then, the acinar-like spheres were incubated with 5% goat serum containing 10 μM digitonin for 2 h at room temperature and washed thrice with PBS. The spheres were incubated overnight at 4°C with the following primary antibodies at 1:500 dilution in 5% goat serum containing 10 μM digitonin: rabbit anti-ZO-1 (Invitrogen, Carlsbad, CA), mouse anti-occludin (Invitrogen), rabbit anti-Na⁺/K⁺-ATPase (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit

anti-ATP7A (a gift from Professor Betty Eipper, University of Connecticut Health Center), rabbit anti-muscarinic-3 receptor (Abcam, Cambridge, MA), rabbit anti-Na⁺/K⁺/2Cl[−] cotransporter-1 (NKCC1; Santa Cruz Biotechnology), rabbit anti-aquaporin-3 (AQP3; Millipore, Bedford, MA), rabbit anti-AQP5 (Millipore), or rabbit anti-claudin-3 antibodies. The next day, spheres were warmed to room temperature for 20 min and washed thrice for 5 min with PBS. Spheres were incubated for 45 min with AlexaFluor 488-conjugated goat anti-rabbit or AlexaFluor 594-conjugated goat anti-mouse secondary antibodies (1:500 dilution in 5% goat serum containing 10 μM digitonin) and washed thrice with PBS. Spheres were stained for 5 min with 1:10,000 dilution in PBS of Hoechst nuclear stain and 1:500 dilution in PBS of phalloidin F-actin stain (Sigma, St. Louis, MO). Images were obtained and analyzed using a Carl Zeiss 510 confocal microscope.

Transfection of Par-C10 cells with green fluorescent protein-tagged human P2Y₂ receptor

Par-C10 cells were transfected with cDNA encoding the green fluorescent protein (GFP)-tagged human P2Y₂ receptor (GFP-P2Y₂R; cDNA was a gift from Dr. Fernando A. González, University of Puerto Rico) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommendation. After 7 days, G418-resistant cells were pooled and maintained in media containing 0.5 mg/mL G418. Further selection was achieved by fluorescence activated cell sorting for GFP.

Western blot analysis

Par-C10 cells grown on GFR Matrigel were lysed in 200 μL of 2× Laemmli buffer, and lysates were sonicated for 5 s with a Branson Sonifier 250 (microtip; output level 5; duty cycle 50%) and boiled for 3 min. The lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis on mini-gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline (0.137 M NaCl, 0.025 M Tris [hydroxymethyl]-aminomethane, pH 7.4) containing 0.1% Tween-20 (TBST) and immunoblotted overnight with rabbit anti-AQP5 antibody (1:1000 dilution; Millipore) at 4°C in TBST containing 3% bovine serum albumin and 0.02% sodium azide. After incubation with the primary antibody, membranes were washed thrice for 15 min each with TBST and incubated with peroxidase-linked goat anti-rabbit immunoglobulin G antibody (1:2000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The membranes were washed thrice for 15 min each with TBST and then treated with chemiluminescence detection reagent containing 20 mM Tris buffer (pH 8.5), 250 mM Luminol, and 90 mM coumaric acid (Sigma), and protein bands were observed on X-ray film. For signal normalization, membranes were treated with stripping buffer (0.1 M glycine, pH 2.9, and 0.02% sodium azide) and reprobed with rabbit anti-extracellular signal-regulated kinase antibody (1:1000 dilution; Santa Cruz Biotechnology). All experiments were performed in duplicate and repeated at least thrice.

Pretreatment of 3D Par-C10 acinar-like spheres

Tumor necrosis factor-α (TNFα; 10 ng/mL) and/or interferon-γ (IFNγ; 10 ng/mL) (Becton Dickinson Pharmingen,

San Diego, CA) were added to 3D Par-C10 acinar-like spheres and incubated for 48 h at 37°C, as described previously for Par-C10 cell monolayers.⁷ The anion channel blocker diphenylamine-2-carboxylic acid (DIDS; 100 μ M) was incubated with the 3D Par-C10 acinar-like spheres for 30 min at 37°C before the PD measurements. In some experiments, chloride in the medium was replaced with gluconate, and the Ca^{2+} concentration was increased to 4 mM to counteract Ca^{2+} chelation by gluconate.

Statistical analysis

Data are means $\pm$ standard error of the mean of results from three or more experiments. p -Values <0.05 calculated from a two tailed t -test represent significant differences.

Results from experiments with 100 μ M carbachol alone were analyzed by a one-sample t -test. Data from subsequent PD measurements for spheres incubated with multiple agents were analyzed by one-way analysis of variance followed by pair-wise *post hoc* Tukey's t -test where $p < 0.001$ represents significant differences between experimental groups.

Results

Par-C10 cells form differentiated 3D acinar-like spheres in GFR Matrigel

Par-C10 cells plated on top of a thin layer of GFR Matrigel formed multicellular acinar-like spheres of ~ 30 – 60 μ m diameter after 2 days in culture. Confocal microscopy sections of acinar-like spheres stained for actin (Fig. 1C) indicated a

FIG. 1. (A) Structure of major salivary glands. Salivary glands consist of acinar and ductal cells; primary saliva is formed in acinar cells and modified as it passes through the ducts. (B) Fluid secretion model for salivary acini. Activation of basolateral M3 muscarinic receptors initiates signaling cascades that stimulate apical Ca^{2+} -dependent Cl^- channels. The stimulated efflux of Cl^- produces a transepithelial potential difference (PD) that drives Na^+ transport and water diffusion across the tight junction (TJ; green rectangles), creating a plasma-like primary secretion in the lumen. Transepithelial water transport is mediated by paracellular and transcellular pathways. Salivary acini also express P2Y_2 nucleotide receptors at the apical surface whose activation also increases intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Inset (adapted from Aktories and Barbieri³⁹) indicates that the TJ proteins occludin, claudins, and JAMs are linked to the cytoskeleton via cytoplasmic ZO-1 proteins. (C) Par-C10 cells form three-dimensional (3D) acinar-like spheres. Par-C10 cells grown on growth-factor-reduced (GFR) Matrigel for 2 days were analyzed for sphere formation using immunofluorescence confocal microscopy with phalloidin (green). Serial x-y and x-z plane confocal projections were taken to generate a 3D image using a Carl Zeiss 510 confocal microscope. Images of confocal z-sections of a sphere (from 0 to 40.5 μ m) are shown. Sphere diameter taken at the widest point of the x-y plane (I) is shown in μ m. Color images available online at www.liebertonline.com/ten.

hollow luminal space that resembled a donut hole whose size increased over 2 days to a sphere diameter of approximately 20 μm (Fig. 1C). The luminal region of 3D Par-C10 acinar-like spheres was encircled by actin filaments that were stained intensely with the F-actin fluorescent dye phalloidin (Fig. 1C, green; and Fig. 2H, K, P, S, T, W, X, Z1, red). Fainter actin staining was detected at the plasma membrane (Fig. 1C, green; Fig. 2H, K, P, S, T, W, X, Z1, red). These results indicate that 3D Par-C10 acinar-like spheres form an apical peri-junctional actin ring typical of polarized acinar epithelium. The 3D Par-C10 acinar-like spheres also expressed the TJ proteins occludin (Fig. 2A, red); ZO-1 (Fig. 2B, green) and claudin-3 (Fig. 2E) at the tips of cell-cell contacts surrounding the lumen (i.e., the apical membrane), another indicator of cell differentiation into polarized acinar-like spheres.

The 3D Par-C10 acinar-like spheres expressed M3 muscarinic receptors at the basolateral surface (Fig. 2I, K, green), whereas GFP-P2Y₂Rs expressed in Par-C10 spheres were localized to the apical surface (Fig. 2L, green), a receptor

distribution consistent with previous studies with polarized lung and colonic epithelium,^{12,13} as well as Par-C10 cells grown on permeable supports.⁷ Par-C10 acinar-like spheres expressed AQP3 at the basolateral membrane (Fig. 2M, O, red), consistent with previous studies with rat¹⁴ and HSGs.¹⁵

The 3D Par-C10 acinar-like spheres also expressed the copper transporter ATP7A in the peri-nuclear region (Fig. 2Q, S, green), consistent with the localization of ATP7A to the Golgi apparatus,¹⁶ whereas Na⁺/K⁺-ATPase and NKCC1 localized to the lateral membrane (Fig. 2U, W and 2Y, Z1, respectively, green), as described previously for salivary acini.^{17,18} These findings strongly suggest that the 3D Par-C10 acinar-like spheres are well-differentiated structures.

Osmotic stress upregulates AQP5 protein expression in 3D Par-C10 acinar-like spheres

Par-C10 acinar-like spheres express low levels of the water channel AQP5 (Fig. 3). However, when Par-C10 acinar-like

FIG. 2. Three-dimensional Par-C10 acinar-like spheres express markers of cell differentiation. Protein expression was detected using immunofluorescence microscopy with goat anti-mouse occludin (A, D; red), rabbit anti-ZO-1 (B, D; green), rabbit anti-claudin-3 (E, G; green), rabbit anti-M3 receptor (I, K; green), rabbit anti-AQP3 (M, O; red), rabbit anti-ATP7A (Q, S; green), rabbit anti-Na⁺/K⁺-ATPase (U, W; green), and rabbit anti-Na⁺/K⁺/2Cl⁻ cotransporter-1 (NKCC1) (Y, Z1, green) followed by Hoechst nuclear stain (C, D, F, G, J, K, N, O, R, S, V, W, Z, Z1; blue) and phalloidin staining (H, K, P, S, T, W, X, Z1; red). Fluorescence of GFP-P2Y₂ receptor (L; green) is also shown. Images were obtained and analyzed using a Carl Zeiss 510 confocal microscope. Sphere diameter taken at the widest point of the x-y plane (l) is shown in μm . Color images available online at www.liebertonline.com/ten.

FIG. 2. (Continued).

spheres were exposed to the hypertonic medium for 24h, AQP5 protein expression was upregulated (Fig. 3), consistent with previous studies with human airway epithelium,¹⁰ SV40 transformed submandibular cells,¹¹ rat lung, and salivary and lacrimal glands.¹⁹ The localization of AQP5 could not be determined given that commercially available anti-AQP5 antibodies do not recognize the protein in 3D acinar-like spheres.

Carbachol-induced changes in PD in 3D Par-C10 acinar-like spheres

Using a microelectrode inserted into the lumen of a 3D Par-C10 acinar-like sphere, a PD of approximately -6 to -10 mV was recorded between the lumen and the baso-

lateral medium (Fig. 4B), demonstrating that the acinar-like spheres maintain transepithelial ion gradients required for fluid secretion. Further, there was a significant decrease in PD within ~ 2 min after addition of $100 \mu\text{M}$ carbachol to the basolateral (outer) surface of the spheres ($p < 0.001$; one-sample *t*-test; Fig. 4B). A similar decrease in PD was seen with $10 \mu\text{M}$ carbachol (Fig. 4B, C). These results demonstrate that 3D Par-C10 acinar-like spheres exhibit muscarinic receptor-mediated changes in transepithelial PD required for saliva secretion in intact acini. The carbachol-induced decrease in PD was abolished in spheres pretreated with the anion channel inhibitor DIDS or when chloride in the medium was replaced with gluconate (Fig. 4B, C), suggesting the presence of muscarinic-receptor-mediated apical chloride

FIG. 3. Hypersomotic stress upregulates AQP5 expression in Par-C10 cells grown on Matrigel. Lysates were prepared from Par-C10 cells grown on GFR Matrigel, and AQP5 expression in serum-free Dulbecco's modified Eagle's medium–Ham's F12 medium with (hypertonic treatment) or without (basal) 200 mM sorbitol was detected by Western blot analysis, as described in Materials and Methods section. The experiment was repeated at least thrice with similar results.

efflux into the lumen, the major mechanism underlying fluid secretion in salivary glands.

Effect of $TNF\alpha$ and/or $IFN\gamma$ on 3D Par-C10 acinar-like spheres

We tested whether $TNF\alpha$ and/or $IFN\gamma$ affected TJ integrity or M3 receptor signaling in 3D Par-C10 acinar-like spheres. As shown in Figure 5A, the TJ protein ZO-1 (green) was located at intercellular junctions near the apical surface in untreated Par-C10 acinar-like spheres, indicative of localization to TJs. However, a 48 h treatment with $TNF\alpha$ and/or $IFN\gamma$ caused ZO-1 protein re-distribution throughout the apical membrane, which correlated with the generation of disorganized lumens of decreased size, as compared to untreated controls (Fig. 5A). Luminal disorganization was most pronounced when acinar-like spheres were treated with both $TNF\alpha$ and $IFN\gamma$ (Fig. 5A). The 48 h treatment with $TNF\alpha$ and/or $IFN\gamma$ also caused a significant decrease in

FIG. 4. Three-dimensional Par-C10 acinar-like spheres exhibit changes in PD in response to the muscarinic receptor agonist carbachol. (A) (1) Par-C10 acinar-like spheres grown on GFR Matrigel were untreated or pretreated with 100 μM diphenylamine-2-carboxylic acid (DIDS) or cultured with the medium in which chloride was replaced with gluconate and placed on the stage of a stereomicroscope, and the lumen of the sphere was penetrated with a 20–30 M Ω glass microelectrode filled with 0.3 M KCl (tip diameter $\sim 1 \mu m$); (2) transepithelial PD in spheres was recorded using an axoclamp 2A intracellular amplifier (Molecular Devices); and (3) changes in transepithelial PD in response to carbachol or medium were determined. (B) Changes in transepithelial PD (ΔPD) of Par-C10 acinar-like spheres grown on GFR Matrigel are shown in response to 10 or 100 μM carbachol in the presence or absence of DIDS or in the medium in which chloride was replaced with gluconate. Data are representative of results from three or more measurements, where asterisk (*) indicates significant differences ($p < 0.001$) for a given group, as compared with 100 μM carbachol alone, using one-way analysis of variance followed by pairwise *post hoc* Tukey's *t*-test. (C) Representative traces for each condition shown in (B). Color images available online at www.liebertonline.com/ten.

FIG. 5. Tumor necrosis factor- α (TNF α) and/or interferon- γ (IFN γ) treatment alter ZO-1 distribution at TJs of 3D Par-C10 acinar-like spheres without affecting carbachol-induced calcium signaling. **(A)** Two-day-old 3D Par-C10 acinar-like spheres were treated with TNF α (10 ng/mL) and/or IFN γ (10 ng/mL) for 48 h and subjected to immunofluorescence using rabbit anti-ZO-1 antibody followed by AlexaFluor 488-conjugated goat anti-rabbit immunoglobulin G antibody (green) and Hoechst nuclear stain (blue). The x-y images were obtained and analyzed using a Carl Zeiss 510 confocal microscope. Sphere diameter taken at the widest point of the x-y plane (I) is shown in μm . **(B)** Par-C10 acinar-like spheres were treated with or without TNF α (10 ng/mL) and/or IFN γ (10 ng/mL) for 48 h. Then, spheres were stimulated with carbachol (100 μM) and changes (Δ) in PD were determined, as described in Figure 4A. Data are expressed as means $\pm$ SE where * $p < 0.05$ indicates significant differences from untreated cells. **(C)** Continuous carbachol (100 μM)-induced increases in $[\text{Ca}^{2+}]_i$ in Par-C10 acinar-like spheres were monitored, as described in Materials and Methods section. **(D)** Par-C10 acinar-like spheres were treated with or without TNF α (10 ng/mL) and/or IFN γ (10 ng/mL) for 48 h. Then, spheres were stimulated with carbachol (100 μM) and changes (Δ) in $[\text{Ca}^{2+}]_i$ were monitored, as described in Materials and Methods section. Changes in $[\text{Ca}^{2+}]_i$ are expressed as the peak carbachol-induced increase in $[\text{Ca}^{2+}]_i$ minus basal $[\text{Ca}^{2+}]_i$. Data are expressed as the mean $\pm$ standard error of the mean of triplicate results from three experiments. Color images available online at www.liebertonline.com/ten.

carbachol-induced PD (Fig. 5B). These results suggest that pro-inflammatory cytokines disrupt TJ integrity in 3D Par-C10 acinar-like spheres. Carbachol (100 μM) induced an increase in $[\text{Ca}^{2+}]_i$ in Par-C10 acinar-like spheres in the absence of cytokines (Fig. 5C), consistent with results from previous studies with Par-C10 cells.²⁰ In contrast to the effects of cy-

tokines on TJ integrity or PD in Par-C10 acinar-like spheres (Fig. 5A, B), TNF α and/or IFN γ treatment for 48 h had no effect on increases in $[\text{Ca}^{2+}]_i$ induced by 100 μM carbachol in individual cells of 3D Par-C10 acinar-like spheres (Fig. 5D), indicating that intracellular calcium signaling is unaffected by these cytokines.

Discussion

In this study, we demonstrate that rat parotid Par-C10 cells are capable of differentiating into 3D acinar-like structures that exhibit numerous features of glandular epithelium *in vivo*. These features include the formation of cyst-like spheroids with a hollow lumen, polarization of the cells to differentiate basolateral and apical surfaces (Figs. 1C and 2), and the establishment of a transepithelial PD upon muscarinic receptor activation (Fig. 4B, C) that is essential for saliva secretion *in vivo*.²¹

The apical versus basolateral distribution of several ion transporters and receptors within the 3D Par-C10 acinar-like spheres resembles their distribution in intact salivary glands. Apical expression of the P2Y₂ nucleotide receptor (Fig. 2L) and basolateral expression of the M3 muscarinic receptor (Fig. 2I, K) mimic their distribution in Par-C10 cells grown on permeable supports.⁸ Our previous studies indicate that P2Y₂R mRNA or activity is barely detectable in native salivary glands; however, the P2Y₂R is upregulated in isolated salivary gland cells cultured for 24 h,²² *in vivo* 3 days after ligation of salivary gland ducts in rats,²³ and in established salivary gland cell lines, including Par-C10 cells,^{7,24,25} indicating that the P2Y₂R is upregulated in acinar cells undergoing dedifferentiation. Although the expression levels of the P2Y₂R have not been quantitated in Par-C10 3D cell cultures that express the P2Y₂R on the apical membrane only, it is highly likely that P2Y₂R expression levels are lower in the acinar-like spheres than in undifferentiated Par-C10 cells. These studies suggest that Par-C10 cells are capable of transitioning between an undifferentiated and differentiated state in 3D culture and that P2Y₂R distribution could be used as a marker to determine the state of differentiation.

The expression levels of the water transporter AQP5 in Par-C10 acinar-like spheres were very low under basal conditions (Fig. 3), although expression of AQP5 was upregulated in Par-C10 acinar-like spheres exposed to the hyperosmotic medium (Fig. 3). These data suggest that the Par-C10 acinar-like spheres are capable of mediating increased water permeability associated with saliva secretion. These results are consistent with previous studies with other tissues and species demonstrating that hyperosmotic stress induces increases in aquaporin expression at the mRNA and protein levels.^{10,11,19} Par-C10 acinar-like spheres also express AQP3 on the basolateral membrane (Fig. 2M), consistent with previous studies with rat¹⁴ and HSGs.¹⁵ These results indicate that Par-C10 acinar-like spheres represent a valuable tool for studying mechanisms underlying fluid secretion.

Par-C10 acinar-like spheres in the present study lack amylase expression (data not shown), consistent with previous observations using 2D cultures of Par-C10 cells grown on permeable supports where it was suggested that the activities of signaling molecules downstream of the β -adrenergic receptor may be defective, including G_s, adenylyl cyclase, and CFTR.⁸ This would result in a decrease in cyclic adenosine monophosphate (cAMP)-dependent cellular responses and may relate to the culture conditions or medium constituents used, including the presence of cholera toxin routinely employed to promote a differentiated state of Par-C10 cells. Therefore, it is predicted that the conditions described here for the culture of Par-C10 acinar-like spheres are not conducive to monitoring amylase secretion. However, a later study

demonstrated that SV40-transformed rat parotid gland cells treated with rat serum showed a 16-fold increase in amylase content.²⁶ These studies suggest that Par-C10 3D acinar-like spheres may be capable of amylase secretion in the presence of factors provided by rat serum.

The distribution of TJ proteins to the lateral membrane at the apical surface of acinar-like spheres (Fig. 2A, B, D, E, G) resembles TJ protein distribution in HSGs and rodent salivary glands.^{27,28} Further, the peri-junctional actin ring encircling the apical pole of cells in the acinar-like spheres (Figs. 1C and 2H, K, P, S, T, W, X, Z1) is structurally similar to native polarized acini.²⁹

The Menkes protein (ATP7A) is a copper-transporting P-type ATPase located in the *trans*-Golgi network whose dysfunction causes a fatal neurodegenerative disorder, Menkes disease.³⁰ In the 3D Par-C10 acinar-like spheres, ATP7A is restricted to the peri-nuclear region (Fig. 2Q, S), consistent with localization to Golgi and suggesting that the acinar-like spheres delineate intracellular organelles similar to intact rat parotid glands.³¹ Further, the lateral distribution of Na⁺/K⁺-ATPase (Fig. 2U, W) is similar to the distribution of this active transporter in polarized epithelium.³²

The acinar NKCC1 has been described as the major Cl⁻ uptake mechanism in acinar cells and is critical for driving saliva secretion *in vivo*.¹⁷ In the Par-C10 acinar-like spheres, NKCC1 was observed at the basolateral membrane (Fig. 2Y, Z1), consistent with its distribution *in vivo* in parotid glands.¹⁷

Although salivary gland cells have been characterized in 3D culture,^{33–35} this is the first study to demonstrate that 3D acinar-like cultures are capable of establishing a transepithelial PD that is modulated by the muscarinic receptor agonist carbachol (Fig. 4B, C). This response is inhibited by the anion channel blocker DIDS and when chloride in the medium was replaced with gluconate, consistent with carbachol-induced chloride efflux into the lumen, the major mechanism underlying fluid secretion in salivary glands.^{2,3} Thus, 3D Par-C10 acinar-like spheres apparently exhibit muscarinic-receptor-mediated stimulation of apical Ca²⁺-dependent Cl⁻ channels, which generates an increase in the transepithelial PD that drives the retrograde diffusion of Na⁺ and water across the epithelium (Fig. 1B).¹

Recently, we have shown that Par-C10 cell monolayers exposed to TNF α and/or IFN γ exhibit a variety of responses, including decreases in transepithelial resistance and agonist-induced I_{sc} , increased paracellular permeability to normally impermeant molecules, and altered TJ morphology associated with reduced expression of the TJ protein claudin-1, suggesting a loss in TJ integrity.⁷ Similarly, TNF α and/or IFN γ altered ZO-1 distribution in 3D Par-C10 acinar-like spheres (Fig. 5A) and decreased lumen size (Fig. 5A). Additionally, treatment with TNF α and IFN γ inhibited carbachol-induced changes in PD. Because TNF α and/or IFN γ levels are increased in the salivary glands of patients with Sjögren's Syndrome (SS),^{36,37} the present study supports the hypothesis that cytokines decrease saliva secretion by altering TJ integrity. The 3D Par-C10 acinar-like spheres exhibit increases in [Ca²⁺]_i in response to carbachol, similar to previous studies in Par-C10 cells grown on permeable supports.²⁰ In intact parotid acinar cells or in Par-C10 cells grown on plastic, carbachol-induced intracellular calcium mobilization consists of two phases, transient (which is reached within 1–2 s after carbachol addition³⁸) and sustained (in

which $[Ca^{2+}]_i$ returns toward the basal levels over the next minute and stabilized).^{9,38} In 3D Par-C10 acinar-like spheres, the intracellular calcium levels do not return completely to basal levels at the times studied (Fig. 5C), indicating a difference with native parotid cells or Par-C10 cells grown on plastic. $TNF\alpha$ and/or $IFN\gamma$ had no effect on intracellular calcium signaling induced by carbachol in Par-C10 cell monolayers (Fig. 5D), indicating that cytokines are not affecting cell viability, although they alter TJ protein distribution.

In conclusion, the 3D Par-C10 acinar-like spheres described in this study may represent a useful cell culture model for investigating the physiological and pathological functions of salivary glands, including ion and fluid secretion and TJ structure and function. Additionally, 3D Par-C10 acinar-like spheres may be useful for studying acinar formation in 3D biopolymer scaffolds for tissue engineering applications.

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Disclosure Statement

No competing financial interests exist.

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