Implantable Three-Dimensional Salivary Spheroid Assemblies Demonstrate Fluid and Protein Secretory Responses to Neurotransmitters

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Radiation treatment in patients with head and neck tumors commonly results in hyposalivation and xerostomia due to the loss of fluid-secreting salivary acinar cells. Patients develop susceptibility to oral infections, dental caries, impaired speech and swallowing, reducing the quality of life. Clinical management is largely unsatisfactory. The development of a tissue-engineered, implantable salivary gland will greatly benefit patients suffering from xerostomia. This report compares the ability of a 2.5-dimensional (2.5D) and a three-dimensional (3D) hyaluronic acid (HA)-based culture system to support functional salivary units capable of producing fluid and phenotypic proteins. Parotid cells seeded on 2.5D, as well as those encapsulated in 3D HA hydrogels, self-assembled into acini-like structures and expressed functional neurotransmitter receptors. Structures in 3D hydrogels merged to form organized 50 μm spheroids that could be maintained in culture for over 100 days and merged to form structures over 500 μm in size. Treatment of acini-like structures with the β -adrenergic agonists norepinephrine or isoproterenol increased granule production and α -amylase staining in treated structures, demonstrating regain of protein secretion. Upon treatment with the M3 muscarinic agonist acetylcholine, acini-like structures activated the fluid production pathway by increasing intracellular calcium levels. The increase in intracellular calcium seen in structures in the 3D hydrogel culture system was more robust and prolonged than that in 2.5D. To compare the long-term survival and retention of acini-like structures *in vivo*, cell-seeded 2.5D and 3D hydrogels were implanted into an athymic rat model. Cells in 2.5D failed to maintain organized acini-like structures and dispersed in the surrounding tissue. Encapsulated cells in 3D retained their spheroid structure and structural integrity, along with the salivary biomarkers and maintained viability for over 3 weeks *in vivo*. This report identifies a novel hydrogel culture system capable of creating and maintaining functional 3D salivary spheroid structures for long periods *in vitro* that regain both fluid and protein secreting functions and are suitable for tissue restoration.

Introduction

SALIVA IS AN ESSENTIAL oral lubricant containing an array of vital proteins for maintaining oral health. Radiation therapy (RT) for head and neck cancer often results in irreversible salivary gland damage, leading to salivary hypofunction, dental caries, and fungal infections from loss of protective saliva.^{1–3} Although advanced radiation techniques such as intensity-modulated radiation therapy (IMRT) significantly reduce radiation to the salivary glands compared to conventional radiation, a large percentage of patients de-

velop xerostomia post-IMRT.^{4,5} Postradiotherapy palliative therapies remain largely ineffective for long-term resolution of xerostomia.³

Salivary tissue engineering potentially offers permanent relief of xerostomia. We previously reported the isolation of acinar-like cells from human salivary tissues, and their self-assembly into three-dimensional (3D) acini-like structures when cultured on 2.5-dimensional (2.5D) hyaluronic acid (HA) hydrogels modified with extracellular matrix (ECM)-derived bioactive peptide fragments.⁶ HA is a naturally derived, biocompatible matrix that can be chemically

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functionalized for use as a crosslinkable tissue engineering scaffold.^{7,8} Unlike other hydrogels, HA is uniquely recognized by its cellular receptors, CD44 and RHAMM. We recently employed a 3D HA-based culture system to organize salivary acinar-like cells into spherical structures. Creating functional implantable tissues with these structures would enable autologous restoration strategies to remove healthy tissue before RT, expand the necessary cell populations *in vitro*, and restore salivary function to patients after RT. Crucial to this strategy is the ability of these cells to re-assemble into native functional structures in 3D. This report describes structural reconstitution of salivary structures in 3D hydrogels, and their functional activation of protein and fluid production pathways upon treatment with neurotransmitters.

The autonomic nervous system innervates salivary acinar, myoepithelial, vascular, and intercalated duct cells.³ Salivary acinar cells secrete most of the fluid, electrolyte, and proteins in saliva. Neurotransmitter receptors are present on the basolateral membranes of acinar cells that possess α -adrenergic, β -adrenergic, M3 muscarinic, and cholinergic substance P receptors.³ The major pathway for protein exocytosis occurs via activation of β -adrenergic receptors (sympathetic pathway), while the primary stimulation for fluid secretion occurs through activation of the M3 muscarinic receptors (parasympathetic pathway).^{3,9–12}

The most abundant protein in saliva, α -amylase, comprises 10% of all salivary protein and is actively secreted by stimulated acinar cells.³ Secreted salivary proteins such as α -amylase are stored at high concentrations in specialized secretory granules.^{13,14} Two distinct pathways regulate trafficking of secreted and ECM proteins. Several are secreted by a constitutive pathway that allows for exocytosis of secreted proteins without stimulation.^{14–16} However, exocytosis can be regulated for controlled release of granule contents.¹⁶ The regulated secretory pathway is the major pathway for granule exocytosis.^{14,15} In this pathway, neurotransmitter agonists such as norepinephrine bind to G-protein coupled β -adrenergic receptors that, in turn, activate the adenylyl cyclase/cAMP/Protein Kinase A second messenger pathway to stimulate protein exocytosis.^{3,10–12}

Transport of fluid also occurs in two distinct ways among glandular epithelia. Fluid can move through the plasma membranes via transcellular transport or through the tight junctional barrier via paracellular transport.¹⁷ Studies with rat and rabbit submandibular glands showed that most fluid is transported via the paracellular pathway with less transported via the transcellular pathway.^{18–20} The transepithelial movement of water via the paracellular pathway is the primary mechanism for fluid secretion by salivary acinar cells.^{3,9,21–25}

Fluid secretion can be activated by binding of acetylcholine to M3 muscarinic receptors, and subsequent activation of G proteins. The α -component of the G protein then dissociates and activates phospholipase C, forming inositol triphosphate (IP₃) and releasing calcium from intracellular stores.^{11,23,24,26–28} Calcium then activates the apical calcium-activated chloride channels. As chloride is released into the lumen, sodium from the interstitium follows. The resulting sodium chloride creates an osmotic gradient that drives transepithelial movement of water into the lumen.^{11,23,24,26,29}

In this study, we report our advances to support salivary acinar-like cell assembly and functional neurotransmitter

responses within 2.5D and 3D HA hydrogel systems both *in vitro* and *in vivo*.

Materials and Methods

Cell culture

Parotid gland tissue was collected from patients aged 40–55 years undergoing surgery under an IRB-approved protocol and consent from both Christiana Care Health Systems and University of Delaware. Homogeneous populations of proliferating acinar-like cells were obtained from tissue explant outgrowths and tissue dissociation procedures as previously described.^{6,30} Parotid gland tissue specimens were minced to a slurry that was suspended in serum-free Hepato-STIM™ medium (BD Biosciences Discovery Labware) and distributed in a six-well plate (BD Falcon™) and left untouched for 7 days, and then supplemented with Hepato-STIM™ growth medium until cells migrated out of the tissue explants. Confluent cells were passaged with 0.05% (w/v) trypsin EDTA (Invitrogen). About 0.5 mg/mL of trypsin soybean inhibitor (Sigma) stopped the trypsin activity. Cells were plated at a dilution of 1:10. Cells in passage 3–4 were used for all experiments.

2.5D hydrogel preparation

HA was modified to contain methacrylate groups by reacting it with glycidyl methacrylate (GMA), as described previously.³¹ This photocrosslinkable HA (HAGMA) was crosslinked into hydrogels in the presence of photoinitiator, 30% (w/v) 2,2-dimethoxy-2-phenylacetophenone (DMPA) in 1-vinyl-2-pyrrolidinone (NVP). Hydrogels were generated in cell culture inserts (Millipore; diameter: 12 mm, pore size: 0.4 μ m) and placed in 24-well plates (Corning). Polymerized gels were swollen in 1 $\times$ PBS for 24 h. Salivary acinar-like cells were seeded on the hydrogels (1×10^5 cells/200 μ L hydrogel) and the Hepato-STIM™ growth medium was added both inside and outside the culture insert.

3D hydrogel preparation

An HA-based Hystem™ hydrogel system (BioTime, Inc.) was utilized to encapsulate salivary cells in 3D. Cells (1×10^5 cells/150 μ L hydrogel) were mixed with the HA-thiol component of the kit. Extralink™ [poly(ethylene glycol)-diacrylate] was added directly to this mixture, and mixed thoroughly before plating on 12 mm cell culture inserts (Millipore) placed in 24-well plates. Plates were placed in a 37°C incubator for partial polymerization. After 15 min, a small amount of Hepato-STIM™ growth medium was added to the outside chamber of the culture insert and the gel polymerized for 10 min. Upon complete polymerization, the culture medium was added over the hydrogel.

Analysis of structure growth

About 1×10^5 cells/hydrogel were seeded on the 2.5D and in the 3D HA hydrogels. Acini-like structures were counted from each quadrant of three such hydrogels, for every time point. Hydrogels were supplemented with 300 μ L of the medium every 3 days for the first 12 days. After about 12 days in culture when the hydrogels were full of larger spheroid structures that metabolize rapidly, the medium was exchanged every 3 days.

Cell proliferation measurements

About 1×10^5 cells/hydrogel were seeded in 3D and were maintained as mentioned above. Hydrogels were analyzed at days 1, 6, 12, 21, and 26. On the day of analysis, the medium was removed and the hydrogels were washed with $1 \times$ PBS. About $30 \mu\text{L}$ of 30 kU/mL hyaluronidase type VI-S (Sigma) was added to each gel and incubated at 37°C for $\sim 2 \text{ h}$. The cells then were pelleted and washed with $1 \times$ PBS. The cell pellet was resuspended in $200 \mu\text{L}$ of lysis buffer, and cells were allowed to lyse for 30 min on ice. The solution then was centrifuged at $13,000 \text{ RPM}$ for 20 min, and the supernatant was used for further analysis. The Qubit™ dsDNA HS Assay Kit (Invitrogen) was used to measure dsDNA concentration using the Qubit® Fluorometer (Invitrogen).

Immunofluorescence

Primary antibodies were monoclonal claudin-1 (Zymed Laboratories), polyclonal ZO-1 (Zymed Laboratories), monoclonal β -catenin (BD Transduction Laboratories), monoclonal E-cadherin (Abcam), monoclonal Ki-67 (BD Pharmingen™), polyclonal M3 muscarinic receptor (Santa Cruz Biotechnology), polyclonal $\beta 1$ adrenergic receptor (Santa Cruz Biotechnology), polyclonal $\beta 2$ adrenergic receptor (Abcam), and polyclonal α -amylase (Sigma). Secondary antibodies Alexa 488 and Alexa 568 (Invitrogen) against mouse or rabbit IgG were used. Nuclei were stained with Draq5 (Biostatus).

Staining of tissue sections and 2D cultured cells was performed as previously described.³⁰ Briefly, tissue cryosections ($8 \mu\text{m}$) were fixed with cold 100% methanol, rehydrated with $1 \times$ PBS, and blocked overnight in 3% (w/v) bovine serum albumin (BSA) in PBS. Sections were incubated with primary antibody at 37°C for 45 min and washed in $1 \times$ PBS for 30 min. Secondary antibody incubation was for 40 min at 37°C followed by 10 min treatment with Draq5. Tissue sections then were washed with $1 \times$ PBS for 30 min. Slides were mounted with Gel Mount and imaged on a Zeiss 510 NLO LSM confocal microscope. The 2D cultured cells were stained identically with minor changes. After methanol fixation, cells were permeabilized with 0.2% (v/v) Triton X-100 for 10 min and washed twice with $1 \times$ PBS before blocking. Stained cells were covered with Gel Mount (Biomedica Corporation) and imaged by confocal microscopy. The 2.5D hydrogels were stained in the same way as cultured cells. After staining, hydrogels were removed from culture inserts and placed cell side down in 8-well chamber slides (Lab-tek® Products, Nalge Nunc International), covered with Gel Mount. The 3D hydrogels were stained similarly. Each step during the staining of 3D hydrogels was prolonged by 50% to allow complete diffusion of liquids through the hydrogel. Cells on hydrogels were imaged by confocal microscopy. Negative controls omitted primary antibody. Primary antibodies specific for antigens expressed by other cell types present in the same tissues or cells served as negative controls for nonspecific binding.

Functional response: exocytic granule production

Cells seeded (1×10^5 cells/gel) on/in hydrogels were treated with varying concentrations (10 to $100 \mu\text{M}$) of isoproterenol hydrochloride (Santa Cruz Biotechnology) and norepinephrine hydrochloride (Sigma), each of which induce granule production and exocytosis, for various time points

from 15 min to 2 h. After treatment, the culture medium was removed and cells were fixed immediately with 100% methanol and stained with α -amylase for microscopic analysis.

Functional response: calcium release studies

Intracellular calcium [Ca_i] release studies utilized the calcium indicator dye, Fluo-4 (Invitrogen). Cells were seeded at 1×10^5 cells/gel in cell culture inserts as before. Cells on 2.5D and in 3D hydrogels were cultured for 12 days. Fluo-4 was suspended in 0.8% (w/v) pluronic acid and mixed with 8 mL of $1 \times$ Hanks' balanced salt solution (HBSS) without calcium. Cell-seeded hydrogels were incubated with Fluo-4 suspended in pluronic acid for 30 min at 37°C . After 30 min, cells were washed twice with $1 \times$ HBSS, and incubated for 30 min in HBSS. After loading and recovery, hydrogels were imaged using a Zeiss LSM 5-live high-speed confocal microscope. Baseline fluorescence was established for 30 s at 488 nm. Cell-seeded hydrogels then were treated with various concentrations (ranging from 50 to $500 \mu\text{M}$) of the muscarinic agonist acetylcholine chloride. The 2.5D hydrogels were flipped so that cells were on the bottom of the chamber slides. About $50 \mu\text{L}$ of the agonist was added to the bottom of the chamber containing $50 \mu\text{L}$ medium. The 3D hydrogels were suspended in $100 \mu\text{L}$ of medium and treated with $100 \mu\text{L}$ of agonist. Resulting increases in Ca_i levels were captured in a series of image scans and by graph plot.

Animal studies

Animal studies were performed as approved by the IA-CUC at the University of Delaware. Hydrogel biocompatibility studies used immunocompetent 3-month-old male Sprague-Dawley rats to ensure that hydrogel material was not immunogenic and biodegraded over time. Male athymic hooded rats (Harlan Laboratories) aged 3–4 months were used for all implantation studies involving human cells. Rats were anesthetized in a closed chamber, purged with 0.5–1.0 L/min oxygen supplemented with 3–5% isoflurane, and kept asleep using a nose cone at 0.5–1.0 L/min oxygen with 1–3% isoflurane. Backs were shaved and cleaned with alcohol wipes, and subsequent surgical procedures conducted under a laminar flow hood. A 1-cm incision was made on the back, and a small pocket was created for the implant. The 2.5D hydrogel was wrapped with a piece of electrospun gelatin membrane^{32,33} to avoid dispersion of cells cultured on it. Fabrication of electrospun gelatin scaffolds has previously been described.^{32,33} The 25% (w/w) gelatin (courtesy of Eastman Kodak Corporation) was dissolved in a solvent containing acetic acid (ACS reagent, 99.7% [w/v]; Sigma Aldrich), ethyl acetate (Fisher Scientific), and distilled water at a ratio of 60:10:30. The gelatin solution was incubated overnight at 37°C and stirred for 1 h before electrospinning. Electrospun scaffolds were generated using an electrospinning unit, consisting of a syringe pump (KD Scientific), a high-voltage power supply (Spellman), and a rotating mandrel collector. The syringe pump generates a constant flow from the needle at 0.5 mL/h flow-rate. Electrospun scaffolds were crosslinked with glutaraldehyde (Electron Microscopy Sciences) in the vapor phase for 19 h at a concentration of 25% (w/w). Electrospun scaffolds with a fiber diameter of $600 \pm 110 \text{ nm}$ were used.

The 3D hydrogel was not wrapped as the cells were fully encapsulated inside the hydrogel. Hydrogels were inserted in the incisional pocket and wounds closed with surgical clips. Animals recovered and resumed activity postsurgery. Rats were sacrificed at 11-day, 3-week, and 4-week time points and the implants removed for analysis. Three implants per time-point were used in this initial study.

Results

Acini-like spheroid formation in 3D HA-hydrogels

Cells seeded in 3D hydrogels self-assembled into organized acini-like spheroids within 3 days. Spheroids grew to a size of $\sim 50\mu\text{m}$ by day 12, and continued to merge and proliferate to form even larger structures ($60\text{--}200\mu\text{m}$) (Fig. 1C, D), with some even as large as $500\mu\text{m}$ by ~ 100 days. The progression of the growing acini-like structures is shown in Figure 1A–D. Tight junction proteins such as CL-1, ZO-1, and E-cadherin, and adherens junction proteins such as β -catenin hold these spheroids together (Fig. 1A–D). Spheroids larger than $40\mu\text{m}$ in diameter showed evidence of lumen formation via apoptosis of central cells (Fig. 1E). A representative brightfield image of an acinus-like structure is shown in Figure 1F.

Growth of acini-like structures in HA hydrogels

Self-assembled acini-like structures formed within 1–3 days of culture in both the 2.5D and the 3D hydrogel systems and continued proliferating (Fig. 2). Acini-like structures grew more quickly in 3D than in 2.5D. At a critical density, the acini-like structures in the 3D culture system began to merge faster and the number of spheroids plateaued (Fig. 2). Cells seeded at a higher density in the hydrogels merged quicker and formed larger spheroids. However, these spheroids often were less organized than those seeded at lower densities. Spheroids in 3D were stable over 100 days and structures as large as $500\mu\text{m}$ were observed. Structures in 2.5D rarely merged, were less organized, and grew much slower than those in 3D (Fig. 2).

Proliferation of acini-like structures in 3D was measured by quantifying their dsDNA over time as well as via staining with the proliferation marker, Ki67 (Fig. 2B–D). A linear rate of proliferation was observed among cells growing in 3D HA hydrogels (Fig. 2B). Acini-like structures cultured for over 48 days in 3D continued to proliferate (Fig. 2D). Ki67 staining was observed in a fraction of the cells located near the periphery of structures and at sites where they appear to be merging (Fig. 2C, D).

Profiling expression of neurotransmitter receptors in glandular tissue and cultured cells in 2D, 2.5D, and 3D

We profiled expression of the neurotransmitter binding receptors present on salivary acinar-like cells that activate pathways associated with salivary fluid secretion and protein synthesis. Salivary gland tissue sections, cells cultured in 2D, 2.5D, and those encapsulated in 3D were stained for neuroreceptors. Acinar-like cells cultured in 2D showed diffuse cytoplasmic expression of $\beta 1$ and $\beta 2$ adrenergic receptors, while the M3 muscarinic receptor was seen on the cell membranes of some acinar-like cells in 2D (Fig. 3A–C). Self-assembled salivary acinar-like cells cultured on 2.5D hydrogels expressed the $\beta 2$ and M3 receptors in the cytoplasm

and on the cell membranes (Fig. 3E, F). $\beta 1$ receptor expression was seen on the cell membrane of some acinar-like cells in 2.5D (Fig. 3D). Organized spheroid structures encapsulated in 3D expressed all three receptors on their basolateral membranes (Fig. 3G–I). Some M3 muscarinic receptor was expressed on the lateral membranes of cells (Fig. 3J). As expected, glandular tissue sections showed expression of $\beta 1$, $\beta 2$, and M3 muscarinic receptors on the cell membranes (Fig. 3J–L). As seen among 3D spheroids, expression of M3 muscarinic receptor was seen along lateral membranes of glandular cells in tissue (Fig. 3L). The encapsulated acini-like structures in 3D were more organized and better able to localize the receptor for stimulation by neurotransmitters.

Stimulating protein secretion by activating β adrenergic receptors

Functionality of salivary acinar-like cells was assessed by response to neurotransmitter agonists. Norepinephrine binding to the β adrenergic receptors activates the protein secretion pathway. In this study, the sympathomimetic β adrenergic agonists, norepinephrine and isoproterenol, were used to stimulate protein production and exocytosis. Salivary acinar-like cells cultured on/in hydrogels were treated with $50\mu\text{M}$ isoproterenol for 15 min to 1 h. After treatment, cells were immediately fixed and stained, and then analyzed for granule formation and α -amylase expression. Untreated acinar-like cells in 2.5D and 3D showed a basal level of secretory granules and α -amylase staining (Fig. 4A, D). Cells treated with $50\mu\text{M}$ isoproterenol for 15 min showed α -amylase near the membrane periphery, indicative of exocytosing vesicles (data not shown). Self-assembling structures with 1 h isoproterenol treatment showed the presence of granules and robust α -amylase staining in their secretory route in both 2.5D and 3D (Fig. 4B, E). Self-assembling acini-like structures treated with $50\mu\text{M}$ norepinephrine also displayed multiple secretory granules in both 2.5D and 3D (Fig. 4C, F). Exocytosing granules stained in red for α -amylase are seen budding out from the cell membrane in Figure 4C, E, and F. Quantification of granule production showed a 3.1-fold increase in visible granules at the 1 h time-point among the isoproterenol-treated spheroids and a 1.6-fold increase in granule production among the norepinephrine-treated spheroids, over control (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Upon stimulation, acini-like structures in 3D also secreted α -amylase into the hydrogel (Fig. 4E, F). α -Amylase staining was quantified by measuring the red, stained area (halo) around the structures (Fig. 4G). A 9.2-fold increase over control is seen in among the isoproterenol-treated samples and a 6.1-fold increase over control is seen among the norepinephrine-treated samples. Additionally, quantification of the α -amylase halo extension revealed a 4.6-fold increase in the isoproterenol-treated samples and a 2.4-fold increase in norepinephrine-treated samples, over control (Supplementary Fig. S2). Other quantification methods showed similar trends (Supplementary Figs. S3 and S4). This indicates that cells in 3D produce detectable levels of salivary proteins that can be secreted upon stimulation unlike those in 2.5D. Neurotransmitter treatment was repeated >3 times and representative images are shown in Figure 4. Over 50% of the cells in the treated samples displayed granules.

FIG. 1. Acini-like spheroids in 3D HA hydrogels. Spheroid structures express tight junction markers CL-1 (A), ZO-1 (B), E-cadherin (D), and adherens junction marker, β -catenin (C). Live/Dead staining shows Syto13-positive green cells and propidium iodide-positive red cells (E). A representative phase image of an acinus-like structure is seen in (F). Nuclei stain blue. An illustration of hydrogel dimensionality is seen in (G). HA, hyaluronic acid; 3D, three-dimensional.

FIG. 2. (A) Differences in growth of acini-like structures in 2.5D (red) and 3D (blue) HA hydrogels. (B) Quantification of dsDNA in acini-like structures growing in 3D HA hydrogels. Each point represents the average of $n=3$ measurements. Error bars are $\pm$ standard error. (C) and (D) show Ki67 staining (green) in acini-like structures at day 26 (C) and at day 48 (D). 2.5D, 2.5-dimensional.

FIG. 3. Salivary acinar-like cell assemblies express receptors for neurotransmitters. Confocal images show expression of β_1 adrenergic receptor (A, D, G, J), β_2 adrenergic receptor (B, E, H, K), and M3 muscarinic receptor (C, F, I, L) in 2D cultured cells (A–C), on 2.5D HA hydrogels (D–F), in 3D hydrogels (G–I), and in tissue (J–L). Nuclei stain red.

Stimulating fluid secretion by activation of M3 muscarinic receptors

To stimulate fluid secretion, salivary acinar-like cells were treated with acetylcholine. Addition of 50 μ M acetylcholine induced a robust calcium response in self-assembling acini-like structures cultured on 2.5D hydrogels (Fig. 5A). Acini-like structures on hydrogels were imaged without saline buffer to minimize movement of hydrogels in the liquid. The calcium response peak seen at ~ 5 min can be attributed to the diffusion time needed by the neurotransmitter to pass through the hydrogel to the cells. Treatment with 100 μ M acetylcholine produced calcium oscillations lasting over 20 min in multiple cells within a self-assembled acinus-like structure (Fig. 5B). Oscillations from different structures varied in intensity. Single cells that were not assembled into structures did not respond to the agonist. Experiments were performed three times with each agonist concentration, on separate hydrogel cultures. Data were recorded from 3 to 4 acini-like structures each time. Representative data sets are

shown in Figure 5A and B. A representative confocal image of stimulated cells in 2.5D is shown in Figure 5C. Negative controls performed with addition of saline buffer in place of the agonist failed to induce a calcium response. Positive controls used a hypotonic solution to induce a calcium response.

Treatment with 50 μ M acetylcholine induced calcium oscillations that lasted over 20 min in spheroid structures encapsulated in 3D hydrogels (Fig. 5D). Acetylcholine (100 μ M) induced similar oscillations among the 3D cultures (Fig. 5E). Compared to the response seen among 2.5D cultures, the increase in $[Ca^{2+}]$ was quicker in 3D, where cells responded to acetylcholine treatment within 30 s, while cells in 2.5D took nearly 300 s. The duration of the oscillations was significantly longer in the 3D cultures than in 2.5D, suggesting that the apical calcium channels might be open longer, allowing for enhanced fluid secretion (Table 1). A representative confocal image of responding cells in 3D is shown in Figure 5F. Thus, cell response to acetylcholine agonist in 3D was faster, more intense, and more prolonged than cell response in 2.5D.

FIG. 4. Salivary acinar-like cell assemblies in 2.5D and 3D HA hydrogels form secretory granules upon treatment with isoproterenol (ISO) and norepinephrine (NER). Images captured with the confocal microscope using brightfield and fluorescence. (A–C) show cells on 2.5D hydrogels, while (D–F) show cells encapsulated in 3D hydrogels. Control untreated cells are shown in (A, D). Cells treated with 50 μ M ISO for 60 min (B, E) show presence of granules (arrows). Cells treated with 50 μ M NER for 60 min (C, F) also show granule production. α -Amylase is stained red. α -Amylase staining around the structures in 3D is quantified in (G). Error bars=SEM, $n=3$, $*p<0.05$ compared to control. The image on the right shows a representative area measurement.

Structures are preserved in hydrogels when implanted *in vivo*

To evaluate the long-term survival of the acini-like structures on 2.5D and those encapsulated in 3D hydrogels *in vivo*, cell-seeded 2.5 and 3D scaffolds were implanted in an athymic rat model. To avoid dispersion of cells from the 2.5D hydrogel scaffold, the hydrogel with cells was wrapped in an electrospun gelatin membrane. The 3D cell-seeded scaffolds were implanted without wrapping. Implants were analyzed

at 11 days and 3 weeks. Some blood vessel infiltration (arrows) was seen within both implants in 2.5D and 3D (Fig. 6A, B, D, E). Despite the presence of the gelatin membrane, the cells in the 2.5D hydrogel scaffold lost their assembly in acini-like structures and dispersed as single cells in the rat tissue surrounding the implant (Fig. 6C). Encapsulated acini-like structures in the 3D scaffold remained intact in their spheroid structures within the hydrogel for over 3 weeks (Fig. 6F), survived implantation *in vivo*, and continued to produce α -amylase.

FIG. 5. Acetylcholine induces intracellular calcium release in self-assembling acini-like structures cultured on HA hydrogels. Intracellular calcium is released from the ER stores upon activation of M3 muscarinic receptors. Calcium curves generated from cells on 2.5D hydrogels (**A**), and oscillations from cells in 3D hydrogels (**D**), upon treatment with 50 μ M acetylcholine. Calcium oscillations generated upon treatment with 100 μ M acetylcholine in cells in 2.5D (**B**) and in 3D (**E**). Red arrow indicates the time-point at which acetylcholine was added. Representative images of structures in 2.5D (**C**) and in 3D (**F**) responding to acetylcholine are seen in a rainbow/heat map scale, where red indicates the highest response and blue indicates the lowest or no response.

Discussion

A biodegradable and human-compatible scaffold able to support growth and differentiation of human salivary gland cells into functional salivary units that secrete fluid and protein upon stimulation is a key step toward creation of an artificial salivary gland. Previously, we reported the isolation of human salivary acinar cells that self-assemble into acini-

like structures and express salivary biomarkers when cultured on ECM derived human-compatible biomimetic peptides.³⁰ To better mimic the *in vivo* environment, we developed an HA-based, 2.5D hydrogel culture system that can support the growth and partial differentiation of 3D acini-like structures *in vitro*, but cannot be used to encapsulate cells.⁶ We now report conversion of our 2.5D model to a fully 3D culture system that promotes organized growth and differentiation of

FIG. 6. Survival and retention of acini-like structures in 2.5D and 3D hydrogels. The 2.5D implants wrapped in gelatin membranes removed from athymic rats at day 11 (**A**) and at 3 weeks (**B**). The 3D implants removed from athymic rats at day 11 (**D**) and at 3 weeks (**E**). Dispersed single cells positive for α -amylase (red) in rat tissue stained with vimentin (green) are seen in (**C**). Intact spheroid structures (boxed) in the 3D hydrogel implant stained for α -amylase (red) and β -catenin (green) are seen in (**F**). Arrows point to blood vessels. White dashed lines mark the hydrogel-tissue interface.

TABLE 1. INTRACELLULAR CALCIUM RELEASE DATA FROM ACINI-LIKE STRUCTURES TREATED WITH ACETYLCHOLINE

Sample	Percent responders	Average peak height (intensity)	Average peak length (s)
2.5D 50 μ M ACh	33%	58 $\pm$ 11	42 $\pm$ 21
2.5D 100 μ M ACh	22%	10 $\pm$ 3	46 $\pm$ 9
3D 50 μ M ACh	43%	39 $\pm$ 6	164 $\pm$ 18
3D 100 μ M ACh	16%	14 $\pm$ 8	180 $\pm$ 34

ACh, acetylcholine; 2.5D, 2.5-dimensional; 3D, three-dimensional.

salivary acinar-like cells into functional acini-like structures and fosters their long-term survival in the 3D scaffold *in vivo* as well as *in vitro*. It should be noted that the 3D gels we used to encapsulate the cells are softer ($G' \sim 68$ Pa) than the 2.5D gels ($G' \sim 1490$ Pa) and they contain polyethylene glycol, but they allow full encapsulation of primary cells.

Groups that reported salivary acini-like structures or spheroids growing in 2D or 2.5D^{34–38} obtained less well-organized salivary structures compared to those seen in 3D cultures.^{9,39} Our results are consistent with this observation, and suggest that dimensionality determines the organized assembly of complex structures as much or more than gel composition. Additionally, most of these 3D salivary cultures used Matrigel™, collagen I, or other animal-derived products for their scaffolds.^{9,38–41} The human-compatible 3D culture system reported here supports the growth of organized spherical structures that merge and proliferate to form larger acini-like structures with a central lumen and can be maintained long-term *in vitro*. These stable spheroid structures expressed essential salivary biomarkers and neurotransmitter receptors. The presence of β adrenergic and M3 muscarinic receptors on acinar-like cells in 3D suggested that these cells can activate fluid and protein transport upon treatment with neurotransmitters.^{10–12}

Saliva contains an array of protein components that provide saliva with its anti-bacterial, anti-viral, anti-fungal, lubricative, digestive, and buffering qualities.^{3,42} α -Amylase, one of the most abundant proteins in saliva, is an essential enzyme that breaks down starch and initiates digestion. Acini-like structures in both 2.5D and 3D hydrogels increased production of α -amylase-positive granules upon stimulation with norepinephrine and isoproterenol, indicating a functional protein secretion pathway was present. A higher increase in α -amylase secretion was observed among structures treated with isoproterenol than those treated with norepinephrine as isoproterenol has a higher affinity for β adrenergic receptors and is 10 times more potent in inducing α -amylase release.⁴³ While a robust increase in the secretion of amylase and its accumulation in the hydrogel were promising, it also indicated a reverse polarity among many of the structures. Staining for MUC1 (a marker of polarity) revealed that only a few structures had luminal MUC1 (not shown). Lessons from studies with mammary gland acini suggest that cues from the ECM and the encasing myoepithelial cells are needed to reverse inside-out acini and attain correct polarity, a final step in functional assembly of the salivary gland.⁴⁴ Co-cultures with salivary myoepithelial cells may correct the acini polarity and ensure unidirectional secretion into the ductal lumen.^{44,45}

Fluid secretion is an important function of salivary acini where it aids essential oral functions, including mastication and speech.³ Several reports showed increases in Ca_i upon treatment with acetylcholine (or carbachol) in parotid or submandibular acini and dissociated cells.^{46–51} Here we demonstrated the induction of calcium oscillations upon neurotransmitter stimulation of 3D acini-like spheroids in a human-compatible hydrogel culture system. The increase in Ca_i and the recurring oscillations induced by acetylcholine among our 2.5D and 3D cultures indicates a functional fluid production pathway. Calcium oscillations have been reported by carbachol treatment of intact parotid acini, indicating that our 3D spheroids resemble glandular acini, functionally.^{46,47} Variability in intensity of the calcium response among cells in 2.5D reflects the lack of uniform organization among the structures in 2.5D. Spheroids in 3D were more organized and responded to stimulation better than the less-organized structures in 2.5D. Gap junctions among salivary acinar cells can induce synchronous calcium responses by cells within an acinus.⁴⁷ It is likely that the organized acini-like structures in 3D respond better and more consistently because they possess gap junctions indicating cellular connectivity.

Branching morphogenesis is a key step in the development of the salivary gland. Parasympathetic innervation occurs early in the developing salivary gland and an interaction of the nerves with the salivary gland parenchyma is critical for further gland development.⁵² It was reported recently that removal of the parasympathetic ganglion during early salivary gland development reduces progenitor cell populations, which could be rescued with carbachol, an acetylcholine analog.⁵³ Thus, treatment with muscarinic agonists can aid in branching morphogenesis and further development of salivary glands.

A major challenge in the field of tissue engineering is necrosis of implants *in vivo*. To ensure survival of our implant *in vivo*, we performed a series of animal studies with our 2.5D and 3D hydrogels. Implantation studies showed long-term survival and maintenance of spheroid structures in 3D but dispersal of cells in 2.5D. We believe that these cells in 3D can be maintained for a longer time in the presence of the protective hydrogel and more normal cell–cell interactions.

Although the 3D system reported here reflects an important advance because it produces protein and fluid upon stimulation, it still lacks essential machinery for full salivary restoration. Future studies can incorporate ductal and myoepithelial cells to form complete functional polarized salivary units with assembled ductal structures that can modify and transport salivary fluid. The studies reported here provide strong evidence that we can develop a functional, neurotransmitter responsive artificial gland that can be incorporated into the buccal mucosa or native salivary gland of patients to restore both fluid and salivary proteins present in saliva.

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Disclosure Statement

No competing financial interests exist.

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