Silk Fibroin Scaffolds Promote Formation of the *Ex Vivo* Niche for Salivary Gland Epithelial Cell Growth, Matrix Formation, and Retention of Differentiated Function

Bin-Xian Zhang, PhD,¹⁻³ Zhi-Liang Zhang, PhD,³ Alan L. Lin, PhD,³ Hanzhou Wang, MD, PhD,³ Marcello Pilia, PhD,⁴ Joo L. Ong, PhD,⁴ David D. Dean, PhD,³ Xiao-Dong Chen, MD, PhD,^{2,3} and Chih-Ko Yeh, BDS, PhD^{1,3}

Salivary gland hypofunction often results from a number of causes, including the use of various medications, radiation for head and neck tumors, autoimmune diseases, diabetes, and aging. Since treatments for this condition are lacking and adult salivary glands have little regenerative capacity, there is a need for cell-based therapies to restore salivary gland function. Development of these treatment strategies requires the establishment of a system that is capable of replicating the salivary gland cell “niche” to support the proliferation and differentiation of salivary gland progenitor cells. In this study, a culture system using three-dimensional silk fibroin scaffolds (SFS) and primary salivary gland epithelial cells (pSGECs) from rat submandibular (SM) gland and parotid gland (PG) was established and characterized. pSGECs grown on SFS, but not tissue culture plastic (TCP), formed aggregates of cells with morphological features resembling secretory acini. High levels of amylase were released into the media by both cell types after extended periods in culture on SFS. Remarkably, cultures of PG-derived cells on SFS, but not SM cells, responded to isoproterenol, a β -adrenergic receptor agonist, with increased enzyme release. This behavior mimics that of the salivary glands *in vivo*. Decellularized extracellular matrix (ECM) formed by pSGECs in culture on SFS contained type IV collagen, a major component of the basement membrane. These results demonstrate that pSGECs grown on SFS, but not TCP, retain important functional and structural features of differentiated salivary glands and produce an ECM that mimics the native salivary gland cell niche. These results demonstrate that SFS has potential as a scaffold for creating the salivary gland cell niche *in vitro* and may provide an approach for inducing multipotent stem cells to provide therapeutically meaningful numbers of salivary gland progenitor cells for regenerating these tissues in patients.

Introduction

SALIVARY GLAND HYPOFUNCTION is often associated with the use of xerostomic medications, radiotherapy for head and neck cancers, autoimmune diseases (e.g., Sjögren’s syndrome), and various systemic diseases such as diabetes mellitus and kidney disease.¹ In each case, the result is typically rampant and severe oral disease, such as caries, bad breath, and difficulty swallowing, accompanied with compromised quality of life. Unfortunately, adult salivary glands are highly differentiated tissues that display little regenerative capacity after physical (e.g., radiation) or pathological (e.g., Sjögren’s syndrome) insults. As a result, the development of strategies for preserving or regaining

secretory function is essential for managing patients with salivary diseases. Potential approaches for restoring salivary gland function include (1) inserting appropriate genes into residual salivary acinar or ductal cells, (2) replacing the salivary gland with functional artificial tissue, and (3) regenerating salivary gland tissue *in situ*.² The former can be achieved by gene transfer, but the latter two approaches require extensive knowledge of stem cells and tissue engineering.

Reconstruction of salivary glands is a complex process that involves cell–cell communication, cell–matrix interaction, and cell signal transduction within a three-dimensional (3D) structure.^{3,4} To accomplish these complex biological processes, several parallel lines of research have focused on

¹Geriatric Research, Education and Clinical Center and ²Research Service, Audie L. Murphy Division, South Texas Veterans Health Care System, San Antonio, Texas.

³Department of Comprehensive Dentistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas.

⁴Department of Biomedical Engineering, University of Texas at San Antonio, San Antonio, Texas.

identifying and isolating salivary gland stem cells/progenitor cells,^{5,6} elucidating pathways and factors associated with salivary gland development,⁴ and developing appropriate biomaterial scaffolds to support the proliferation and differentiation of salivary gland progenitors.^{7,8}

Extracellular matrix (ECM) is an important component of the cellular niche and supplies critical biochemical and physical signals to initiate or sustain cellular functions in the tissue.^{9–11} Within the rapidly developing field of tissue engineering, various biomaterial scaffolds have been developed for tissue regeneration or repair that mimic some of the critical properties of the ECM.¹² Examples of biomaterials that have been shown to support the proliferation and differentiation of salivary gland progenitors include chitosan, polyglycolic acid (PGA), poly-(L)-lactic acid (PLLA), poly(lactic-co-glycolic acid) (PLGA), and poly(ethylene glycol) terephthalate/poly(butylene) terephthalate (PEGT/PBT) block co-polymers.^{6,8,13} However, these polymeric scaffolds have the potential to induce inflammation *in vivo* due to the acidity of their degradation products.^{14,15} Another potential scaffold material, Matrigel, which contains basement membrane proteins secreted by EHS mouse sarcoma cells, has been used to grow primary salivary gland epithelial cells (pSGECs) in culture.¹⁶ Although varying levels of success have been achieved with this product, it is not consistent with our long-term goal to reconstitute the salivary gland niche (tissue-specific ECM) on a scaffold for controlling stem cell fate. In contrast, natural scaffold materials, such as silk, are desirable because of their wide range of elasticity (allowing tissue-specific scaffold formation) and pore sizes (allowing tissue specific nutrition and oxygen access), low bacterial adherence, ability to biodegrade, and low toxicity and immunogenicity.^{17,18}

In this study, we established a culture system using silk fibroin scaffolds (SFS) to characterize the behavior of pSGECs grown on this material versus tissue culture plastic (TCP) and the ECM they produce. The results show that the SFS culture system closely recapitulated the *in vivo* niche for these cells and promoted the growth of salivary acinar cells and the synthesis of salivary gland tissue-specific ECM, while TCP did not.

Materials and Methods

Preparation of the silk scaffolds

Three-dimensional SFS were prepared using a previously described technique.¹⁹ Briefly, *Bombyx mori* silk cocoons (Paradise Fibers, Spokane, WA) were boiled in aqueous 0.02 M Na₂CO₃ and 0.3% (w/v) ivory soap for 1 h to remove sericin from the silk fibroin. Cocoons were then thoroughly rinsed with deionized water to remove any traces of soap and impurities. The silk fibers were dissolved in 9.5 M LiBr solution for 30 min at 50°C, yielding a 10% weight/volume solution. Next, the liquid silk/LiBr solution was dialyzed against running deionized water for 3 days using 2 kDa molecular weight cut-off dialysis membranes (Thermo Scientific Pierce, Rockford, IL). The resulting aqueous solution was lyophilized for 48 h (LabConco, Kansas City, MO). Samples were then rehydrated in water to yield a 5% (w/v) solution, which was sonicated for 2 min. Fifty microliter aliquots of liquid silk were cast using Teflon® molds (5 mm diameter) to create thin films. The entire mold was placed in a –80°C freezer, and the silk was lyophilized a second time. After

lyophilization, the silk film was submerged in methanol for 10 min to change the structure of the protein from an α -helix to a β -sheet. This step produced films that were insoluble in cell culture media. After being submerged for 10 min, the methanol was removed and the films were washed repeatedly in distilled water. Before use, the silk films were sterilized for 12 h with ethylene oxide (AN74i Anprolene gas sterilizer; Andersen Sterilizers, Inc., Haw River, NC). Previous studies have shown that ethylene oxide sterilization did not alter the physical properties of the SFS.^{20,21}

Preparation of primary cells from parotid and submandibular glands

pSGECs from parotid gland (PG) and submandibular (SM) gland were prepared following the procedure previously described.²² Briefly, individual PG and SM glands from 3-month-old male Sprague–Dawley rats were dissected, finely minced, and then digested for 60 min, with vigorous agitation (300 rpm), at 37°C using purified collagenase (Catalog number LS005273 [code CLSPA], 96 U/mL; Worthington Biochemical Corp, Lakewood, NJ) and hyaluronidase (Type I-S, 0.19 mg/mL; Sigma Aldrich, St. Louis, MO) dissolved in Hank's balanced salt solution (HBSS), containing 33 mM HEPES buffer, pH 7.4. During digestion, the mixture was oxygenated every 10 min. When digestion was complete, the suspension was passed through a 100 μ m (for parotid) or 40 μ m (for SM gland) nylon cell strainer. Cells were collected by centrifugation at 100 *g* for 5 min, washed with HBSS, resuspended in 6 mL (PG) or 18 mL (SM gland) of DMEM/F12 medium (1:1 ratio) containing 1.1 μ M hydrocortisone, 15% fetal bovine serum (FBS; preselected for a high level of growth promoting activity for the cells from Becton Dickinson, Franklin Lakes, NJ), and antibiotic-antimycotic, and cultured in six-well plates to near confluence (6 mL/well for ~4 weeks). Previous studies have indicated that primary cells prepared by this method were 90% secretory (acinar-like) cells.²³ The cultured cells were harvested using trypsin/EDTA and then used for the experiments (see: "Cultures of salivary gland cells on 2D TCP or 3D SFS" below). All procedures using rats were approved by the IACUC of the Audie L. Murphy Hospital, South Texas Veterans Healthcare System (San Antonio, TX).

Cultures of salivary gland cells on 2D TCP or 3D SFS

All TCP and SFS culture surfaces were precoated with human plasma fibronectin (16.7 μ g/mL; EMD Millipore, Billerica, MA) dissolved in phosphate-buffered saline (PBS). One milliliter was added to each well of a six-well plate or onto SFS and incubated for 1 h at 37°C. After rinsing with PBS, the pSGECs (1 to 3 million cells per well or SFS), prepared as described earlier, were seeded onto the coated TCP wells or SFS and cultured in DMEM/F12 (1:1 ratio) media containing 1.1 μ M hydrocortisone, 15% FBS, and antibiotic-antimycotic at 37°C in a humidified 5% CO₂/95% air environment for 5 weeks. Media changes were performed every 3 days. During the last week in culture, ascorbic acid (50 μ M) was added to the media to promote ECM formation. The last medium change was collected for the analysis of amylase activity (see: "Measurement of α -amylase activity" below).

After culture, the 2D TCP and 3D SFS cultures were subjected to morphological, functional, and biochemical studies

FIG. 1. Attachment and proliferation of primary salivary gland epithelial cells (pSGECs) on tissue culture plastic (TCP) and silk fibroin scaffolds (SFS) were assessed using AlamarBlue. Rat submandibular (SM; *left panel*) gland and parotid gland (PG; *right panel*) primary epithelial cells were cultured till 12 days on SFS or TCP. The change in cell number was assessed with AlamarBlue at the indicated times. The data shown in the graphs are from one representative experiment (mean $\pm$ SD fluorescence intensity; $n=4$ wells).

as described next. Some of the TCP and SFS cultures were decellularized according to our previously published method⁹ for studying the role of the ECM in regulating stem cell self-renewal, differentiation, and other activities. In brief, the cultures were extensively washed with PBS and the cells were removed by incubation with 0.5% Triton X-100, containing 20 mM NH_4OH , in PBS for 5 min at room temperature. The ECM produced by the pSGECs on the SFS and TCP surfaces was evaluated using scanning electron microscopy (SEM).

Cell attachment and proliferation was determined with the AlamarBlue assay according to the manufacturer's instructions (Invitrogen, Grand Island, NY).^{24,25} Cell growth

was assessed every other day by incubation of the cultures with the AlamarBlue reagent (1:10 dilution) for 4 h at 37°C. After incubation, 100 μL of the culture media were transferred to a 96-well plate and fluorescence was measured using a Spectromax M2 microplate reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Histology and electron microscopy

SFS, after being cultured with PG or SM gland epithelial cells, were washed with PBS, fixed with 10% neutral-

FIG. 2. Histological staining of pSGECs grown on SFS. Rat SM gland (A–C) and PG (D–F) epithelial cells cultured on SFS were sectioned and stained with hematoxylin and eosin (H&E; A, D), periodic acid–Schiff (PAS; B, E), or Alcian blue (C, F) as described in the Materials and Methods section. The SFS without cells served as controls (G–I). Cell aggregates (solid black arrows in A, D) were observed in H&E stained sections from both SM and PG cultures. In addition, cells from both tissues were PAS positive (solid black arrows in B, E). Alcian blue staining, indicative of mucin production, was found in SM cultures (solid black arrows in C), but not in PG cultures (F). Solid arrow heads identify SFS in these micrographs. Color images available online at www.liebertpub.com/tea

buffered formalin (Sigma Aldrich) overnight, and then embedded in paraffin for light microscopy. Scaffolds were sectioned and stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS; detects polysaccharides and mucosubstances such as glycoproteins, glycolipids), or Alcian blue (detects mucins)²⁶ for viewing of the cells and their morphology and the SFS.

For electron microscopy, cultures on TCP and SFS were washed thrice with PBS, fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h, and then transferred to 0.1 M cacodylate buffer. Specimens were dehydrated using an ascending alcohol series (70% to 100% ethanol). After dehydration, the specimens were attached to a stub, sputter coated with gold–palladium, and viewed using an EVO-50EP SEM (Carl-Zeiss, Peabody, MA). For transmission electron microscopy (TEM), cell cultures were fixed as described earlier but embedded in Epon resin (Poly-science, Inc., Warrington, PA). Ultrathin sections were cut,

stained with uranyl acetate and lead citrate, and then examined using a Jeol 1230 TEM (Jeol USA., Peabody, MA).

Measurement of α -amylase activity

Alpha-amylase activity in the conditioned culture media was assessed using the EnzChek Ultra Amylase Assay Kit (Invitrogen) as an indicator of salivary gland cell secretory function. Amylase activity was analyzed under both “stimulated” and “unstimulated” conditions. To measure amylase secretion in unstimulated cells, conditioned media were collected at the last medium change after 5 weeks in culture. Protein concentration in the conditioned media was measured using the Bio-Rad protein assay with bovine serum albumin as a standard. Amylase activity, assessed by following the digestion of the DQTM starch substrate in the kit, was monitored as an increase in fluorescence at 515 nm after excitation at 495 nm with a SpectraMax M2 microplate reader (Molecular Devices).

FIG. 3. Scanning electron micrographs of pSGECs grown on TCP or SFS. SM gland epithelial cells were cultured on TCP (**A, C, E**) or SFS (**B, D, F, G**) for 5 weeks and then viewed by the scanning electron microscope (SEM) as described in the Materials and Methods section. With increasing magnification, different morphological features of the cells growing on the 2 culture surfaces could be clearly discerned (**A, C, E**: TCP; **B, D, F, G**: SFS). SM gland epithelial cells grown on SFS displayed secretory granule-like structures that could be clearly seen at high magnification (*black arrow* in **G**). The micrograph in (**H**) shows that PG epithelial cells grown on SFS at the same magnification as the SM cells (**F**) have remarkably similar surface morphologies.

To measure amylase secretion in response to β -adrenergic receptor stimulation, cell cultures were washed with PBS containing MgCl_2 (1 mM) and CaCl_2 (1 mM) (PBS solution) at room temperature. The cells were then incubated in PBS solution at 37°C for 30 min to assess the basal amylase secretion. Subsequently, cells were exposed to 10 μM isoproterenol at 37°C for 30 min in the PBS solution. Amylase activity and protein concentration were measured as described earlier.

Immunofluorescence of type IV collagen

The expression of type IV collagen in cultures grown on TCP or SFS was examined using a previously described immunofluorescence technique.²⁷ Briefly, cells were fixed for 30 min at room temperature with 4% paraformaldehyde and permeabilized with 40 $\mu\text{g}/\text{mL}$ digitonin dissolved in PBS. The permeabilized cells were incubated for 60 min with 10% FBS in PBS and subsequently incubated with or without rabbit polyclonal IgG anti-type IV collagen (1:50 dilution in PBS containing 2% FBS, 0.01% Triton X-100; Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. The cells were then washed with PBS containing 0.1% Tween 20 and incubated with Alexa 488-labeled goat anti-rabbit IgG (1:1000 dilution; Invitrogen) for 1 h at room temperature. Some specimens were also counterstained with DAPI. Labeled cells were viewed using an Olympus confocal laser scanning microscope, using excitation/emission wavelengths of 405 nm/450 nm for nuclei and 488 nm/554 nm for type IV collagen, and digital images were obtained.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation. Statistical analysis of the experimental data was performed using Student's *t*-test with significance at $p < 0.05$. Each experiment was repeated a minimum of thrice with an $n = 4$ for each treatment group.

Results

pSGECs attached and proliferated on both TCP and SFS

Cell attachment and proliferation were assessed during culture by use of the AlamarBlue assay.²³ The initial number of SM gland or PG epithelial cells attached to SFS was the same as that for TCP (Fig. 1). Further, the proliferative pattern displayed by PG epithelial cells when grown on both TCP and SFS was very similar over 12 days in culture (Fig. 1). In contrast, the proliferation of SM gland epithelial cells cultured on TCP plateaued around day 6, indicating that the cells had reached confluence, while cells cultured on SFS continued to proliferate during the entire culture period, suggesting that cell confluence was delayed (Fig. 1).

pSGECs cultured on SFS, but not TCP, maintained their secretory features in vitro

pSGECs, obtained from rat SM gland or PG, were cultured on either TCP or SFS in growth media for 4 weeks, followed by further incubation in media supplemented with

FIG. 4. Transmission electron micrographs of pSGECs grown on TCP or SFS. Cells were cultured on TCP (A, B) or SFS (C, D) for 5 weeks and then viewed by the transmission electron microscope (TEM) as described in the Materials and Methods section. The micrographs (A, B) on the left show SM gland epithelial cells grown on TCP; no secretory granule-like structures are observed in these cells. In contrast, SM gland epithelial cells cultured on SFS display prominent secretory granules (C, D).

ascorbic acid (50 μ M) for an additional 8 days to promote ECM synthesis. At the end of incubation, the cultures were harvested and processed for viewing by light microscopy, SEM, and TEM.

By use of bright field microscopy, pSGECs grown on SFS displayed features of salivary gland acinar cells (Fig. 2). Staining with H&E and PAS (indicative of polysaccharides and mucosubstances such as glycoproteins, glycolipids) revealed the presence of aggregated cells associated with the silk fibers. In the majority of the cells, the cytosol contained glycoprotein-rich secretory granules and nuclei located near the cell membrane. In sections stained with Alcian blue, mucin-like substances were found in SM gland epithelial cells but not PG epithelial cells.

SEM further revealed that SM gland epithelial cells grown on TCP were mainly round and flat (Fig. 3A, C), while those cultured on SFS formed 3D cell aggregates/clusters (Fig. 3B, D). At a higher magnification, cells maintained on TCP displayed numerous projections from the cell surface (Fig. 3E), whereas secretory granule-like structures were only observed on the surface of cells cultured on SFS (Fig. 3F, G). The diameter of these granule-like structures was $\sim 1 \mu$ m, which is consistent with the size of salivary gland secretory granules of acinar cells *in vivo*.²⁸ Similar granule-like structures were also observed in PG epithelial cells grown on SFS (Fig. 3H).

Using TEM, the ultrastructure of these secretory granule-like structures was further revealed in cross-section and it was found that they occupied the majority of the cytosol in cultures on SFS (Fig. 4C, D). In contrast, cells cultured on TCP contained very few secretory granules and the ones that were present appeared moderately to severely atrophic (Fig. 4A, B).

pSGECs cultured on SFS, but not TCP, maintained their secretory function in vitro

The secretory function of pSGECs cultured on TCP and SFS was first assessed by measuring amylase release into the culture media. There was a remarkable amount of enzyme produced by cultures of SM and PG epithelial cells grown on SFS, but not TCP (Fig. 5A). To further evaluate the secretory function of pSGECs grown on SFS, amylase release in response to β -adrenergic receptor stimulation, the receptor responsible for a major amount of salivary protein secretion,²⁹ was examined. When PG epithelial cells cultured on SFS were treated with isoproterenol (10^{-5} M for 30 min in PBS solution), amylase activity increased sharply over basal levels (5.3-fold; Fig. 5B), indicating responsiveness to this agonist. In contrast, amylase production by SM gland epithelial cells did not respond to isoproterenol stimulation, in agreement with a previous study.³⁰ Notably, these differences in measurable activity and response to agonist treatment were not due to differences in total protein production (Fig. 5C).

SFS facilitated the production of a tissue-specific ECM by pSGECs

To determine whether pSGECs cultured on SFS produced a tissue-specific ECM, we treated SM gland epithelial cells with ascorbic acid during the last 8 days of culture (i.e., 8 days postconfluence). At harvest, the ECM was prepared for

FIG. 5. Amylase and protein secretion by pSGECs grown on TCP and SFS. Rat SM gland and PG epithelial cells were cultured on TCP or SFS for 5 weeks in growth media as described in the Materials and Methods section. Amylase activity (A, B), released by the cells, was measured as described in the Materials and Methods section. Values shown in the graphs (A, B) represent the mean $\pm$ SD for amylase specific activity. (A) Enzyme activity was released into the media. (B) The enzyme activity was released in response to treatment with isoproterenol (10^{-5} M, 30 min at 37°C) (see Materials and Methods for details). Mouse saliva was used as a positive control for amylase activity. Representative data from one of two independent experiments are shown; each experiment was run in triplicate ($n=3$). The values in (C) represent total protein released by cells untreated or treated with isoproterenol (10^{-5} M, 30 min at 37°C) from a typical experiment (see Materials and Methods for details). * $p < 0.05$, TCP versus SFS (A) or Basal versus Iso treatment (B).

FIG. 6. Scanning- and transmission electron micrographs of extracellular matrix (ECM) produced by primary SM gland epithelial cells grown on TCP and SFS. The cells were cultured on TCP or SFS for 5 weeks, decellularized, and then prepared for viewing in the SEM and TEM as described in the Materials and Methods section. Differences in morphology were observed in the SEM with the two culture environments (A, B: TCP; C, D: SFS). Evidence of SFS remodeling could be observed when scaffolds before (E) and after culture with the cells (C) were compared. TEM revealed a fibrillar ECM that was deposited by the cells onto the SFS (F). White arrows (C, D, F) indicate the location of ECM produced by the SM cells.

viewing by SEM and TEM after removal of the cells. When SM gland cells were cultured on TCP, they produced a thin layer of ECM (Fig. 6A, B). In contrast, when the same cells were cultured on SFS, they were able to produce an abundant 3D ECM that covered the SFS (Fig. 6C, D). The fibrous nature of these proteins was clearly visible in the TEM (Fig. 6F). In addition, salivary cells remodel the SFS during culture. This can be seen by comparing the structure of the SFS after culture (Fig. 6C) with the original SFS not subjected to culture with the cells (Fig. 6E).^{18,31}

By use of phase-contrast and immunofluorescence microscopies, the presence of type IV collagen, a key basement membrane protein, was identified in cultures on SFS, but not TCP (Fig. 7). These results suggest that SFS promotes the formation of a 3D ECM by SM gland epithelial cells, resulting in an environment that maintains many of the differentiated features of pSGECs.

Discussion

In this study, we show that culture of pSGECs on SFS promoted the retention of the differentiated phenotype of salivary gland acinar cells, while culture on TCP did not. Cells on SFS formed aggregates or clusters and maintained the differentiation state as found in the native organ (salivary gland). Strikingly, pSGECs grown on SFS retained their secretory status as seen by the presence of secretory granule-like structures on the cell surface and in the cytosol.

In contrast, pSGECs grown on TCP were round, flat, and failed to demonstrate secretory function. The detection of mucins in SM gland epithelial cells, but not PG epithelial cells, further highlights the unique ability of SFS to promote maintenance of the differentiation state of pSGECs. In the resting (i.e., unstimulated) state, PG and SM gland epithelial cells cultured on SFS released amylase into the media in a sustained manner. Furthermore, PG cells on SFS remained sensitive to stimulation by isoproterenol, suggesting that functional β -adrenergic receptors were present on these cells. Interestingly, cells from the SM gland were insensitive to isoproterenol treatment (Fig. 5). These results are consistent with previous studies, indicating that isoproterenol has differential effects on amylase secretion in these two glands.³⁰

Silk is a natural product found in the *Bombyx mori* cocoon that predominantly consists of fibroin and sericin.¹⁷ Since sericins have been identified as allergens in humans, silk fibroin is typically used as a scaffold material only after sericin removal. Silk fibroin is an excellent biomaterial for tissue engineering, because it is elastic, porous (provides the cells with ready access to nutrients), biodegradable, and has low toxicity/immunogenicity and bacterial adherence.^{17,18} In this study, we demonstrated that fibronectin-coated 3D SFS supported the growth and differentiated secretory function of pSGECs for extended periods of time (~ 1 month). As a result, we believe that SFS provides an appropriate scaffold for growing highly differentiated salivary gland cells using

FIG. 7. Localization of type IV collagen produced by primary SM gland epithelial cells cultured on TCP and SFS. SM gland epithelial cells were grown for 5 weeks as described in the Materials and Methods section and then fixed for phase-contrast microscopy and localization of type IV collagen by confocal microscopy. Images in (A, C) are phase-contrast images of cells grown on TCP and SFS, respectively. (B) An immunofluorescent image of cells cultured on TCP and stained with antibody to type IV collagen; note the lack of staining under these culture conditions. (D–H) A confocal microscopy z-scan series (viewed in 3 μ m sections, *bottom to top*) of SM gland epithelial cell aggregates growing on and surrounding the SFS fiber (blue fluorescence). Most notably, these cells are producing high levels of type IV collagen (green fluorescence). Color images available online at www.liebertpub.com/tea

conventional culture media and conditions. Since it is well known that the physical and chemical properties of the ECM are critical for maintaining stem cell function,³² the results of this study suggest that further evaluation of SFS for maintaining acinar cell differentiated function and producing tissue specific ECM to replicate the stem cell/progenitor cell niche is warranted. If successful, this tissue engineering approach would provide a viable source of cells for regenerating salivary gland tissue.

One of the goals of this study was to establish a salivary gland tissue-specific 3D scaffold for salivary gland tissue engineering. The appropriate tissue-specific ECM (micro-environment or “niche”) is essential for multipotent stem cells to receive the proper chemical and physical cues, which govern their behavior both *in vivo* and *in vitro*, for tissue regeneration and repair.^{33,34} In this study, SFS not only maintained pSGECs in their differentiated state during long-term culture but also facilitated the synthesis of a 3D

ECM, based on microscopic evidence, by the cells. Using decellularization procedures, which minimally disturb the structure of the ECM,³⁵ SEM and TEM revealed that pSGECs were able to produce an extensive ECM on the surface of the SFS. While the amount of ECM produced by cells is visually different between the 2D and 3D cultures, the composition of the ECM under the two culture conditions was, based on immunofluorescent staining, different as well (Fig. 7). A detailed study of the differences between the two ECMs is the subject of ongoing investigation in our lab.

The basement membrane is critical for epithelial cell polarization and differentiation and has been demonstrated to play a key role in salivary gland development.³⁶ In this study, immunofluorescent staining demonstrated that type IV collagen, a major component of the basement membrane, was indeed present in the ECM made by the pSGECs and that it was localized to areas surrounding the 3D aggregates, which were not found in cultures on TCP. Based on these results, it is likely that SFS facilitates the synthesis of a 3D basement membrane structure by the pSGECs that resembles the acini in native salivary gland.²⁸ This study demonstrates that pSGECs have the potential to synthesize a native salivary gland 3D microenvironment that may be valuable for directing multipotent stem cells into functional salivary gland epithelial cells *in vitro* and/or *in vivo* for future studies and, perhaps, therapeutic purposes.

Adult salivary glands are known to contain both progenitor and stem cells that can be directed to differentiate into salivary tissue or other organs depending on the tissue-specific microenvironment or niche which is composed predominantly of ECM proteins and growth factors.³⁷ Whether pSGECs, maintained on SFS in this study, represent salivary gland stem cell or progenitor cell populations will require additional studies. Currently, 3D matrices used for the expansion and differentiation of salivary gland cells *in vitro* are impractical for producing cells for therapeutic applications, because their components are either complex and tumor derived (e.g., Matrigel) or only have one or two basement membrane components.¹⁶ In contrast, the 3D ECM produced by normal pSGECs cultured on SFS in this study may be useful in establishing a salivary gland basement membrane that can be used for expansion and differentiation of sufficient numbers of cells. In our view, further characterization and optimization of the properties of this ECM to enhance salivary gland cell growth is warranted.

Conclusions

Our findings indicate that the 3D SFS culture system provides the appropriate conditions for synthesis of a tissue-specific ECM by pSGECs that replicates the microenvironment (“niche”) of salivary gland cells and promotes the growth and secretory function of these cells. Importantly, the organization and relationship of the cells and ECM on the scaffolds was similar to that observed in native salivary gland tissues.²⁸ When compared with 2D culture systems, SFS is superior in its ability to mimic the *in vivo* environment and may be valuable for studying the behavior of pSGECs and their response to various treatments, including the testing of new radio- or chemo-therapeutics. The establishment of this 3D model will allow us to proceed with developing the concept of a tissue-specific microenviron-

ment (“niche”) and use it to prepare salivary gland epithelial cells (or their progenitors) for transplantation to directly repair damaged salivary gland tissue *in vivo* or to prepare cell-seeded tissue engineering scaffolds for salivary gland regeneration. In addition, the fundamental principles described here have potential to lead to new strategies for regenerating other functional tissues as well.

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Disclosure Statement

Dr. Chen is a Board member and shareholder in Stem-BioSys, Inc. (San Antonio, TX). Other authors have no other financial or competing interests to declare.

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Address correspondence to:

Chih-Ko Yeh, BDS, PhD

Geriatric Research, Education and Clinical Center (182)

Audie L. Murphy Division

South Texas Veterans Health Care System

7400 Merton Minter Boulevard

San Antonio, TX 78229-4404

E-mail: yeh@uthscsa.edu

Xiao-Dong Chen, MD, PhD

Department of Comprehensive Dentistry

University of Texas Health Science Center at San Antonio

7703 Floyd Curl Drive

San Antonio, TX 78229-3900

E-mail: chenx4@uthscsa.edu

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