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Bottom-up assembly of salivary gland microtissues for assessing myoepithelial cell function

Tugba Ozdemir [a], Padma Pradeepa Srinivasan [b, c], Daniel R. Zakheim [b], Daniel A. Harrington [d, g], Robert L. Witt [b, f], Mary C. Farach-Carson [b, d, g], Xinqiao Jia [a, b, e, *], Swati Pradhan-Bhatt [b, c, e, **]

[a] Department of Materials Sciences and Engineering, University of Delaware, Newark, DE, USA
[b] Department of Biological Sciences, University of Delaware, Newark, DE, USA
[c] Center for Translational Cancer Research, Helen F. Graham Cancer Center & Research Institute, Newark, DE, USA
[d] BioSciences, Rice University, Houston, TX, USA
[e] Department of Biomedical Engineering, University of Delaware, Newark, DE, USA
[f] Otolaryngology – Head & Neck Surgery, Thomas Jefferson University, Philadelphia, PA, USA
[g] Diagnostic and Biomedical Sciences, University of Texas Health Science Center at Houston School of Dentistry, Houston, TX, USA

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ABSTRACT

Myoepithelial cells are flat, stellate cells present in exocrine tissues including the salivary glands. While myoepithelial cells have been studied extensively in mammary and lacrimal gland tissues, less is known of the function of myoepithelial cells derived from human salivary glands. Several groups have isolated tumorigenic myoepithelial cells from cancer specimens, however, only one report has demonstrated isolation of normal human salivary myoepithelial cells needed for use in salivary gland tissue engineering applications. Establishing a functional organoid model consisting of myoepithelial and secretory acinar cells is therefore necessary for understanding the coordinated action of these two cell types in unidirectional fluid secretion. Here, we developed a bottom-up approach for generating salivary gland microtissues using primary human salivary myoepithelial cells (hSMECs) and stem/progenitor cells (hS/PCs) isolated from normal salivary gland tissues. Phenotypic characterization of isolated hSMECs confirmed that a myoepithelial cell phenotype consistent with that from other exocrine tissues was maintained over multiple passages of culture. Additionally, hSMECs secreted basement membrane proteins, expressed adrenergic and cholinergic neurotransmitter receptors, and released intracellular calcium [Ca^{2+}_i] in response to parasympathetic agonists. In a collagen I contractility assay, activation of contractile machinery was observed in isolated hSMECs treated with parasympathetic agonists. Recombination of hSMECs with assembled hS/PC spheroids in a microwell system was used to create microtissues resembling secretory complexes of the salivary gland. We conclude that the engineered salivary gland microtissue complexes provide a physiologically relevant model for both mechanistic studies and as a building block for the successful engineering of the salivary gland for restoration of salivary function in patients suffering from hyposalivation.

1. Introduction

Radiation-induced hyposalivation is characterized by severe dry mouth/xerostomia, dysphagia, dysphonia, increased incidence of oral infections and an overall reduced quality of life [1]. While current treatment modalities are palliative and short-lived [2], restoration of salivary gland function using autologous cell-derived therapies can effectively restore quality of life for head and neck cancer survivors who have undergone radiation therapy. Our long-

* Corresponding author. Department of Materials Science and Engineering, University of Delaware, Newark, DE 19716, USA.
** Corresponding author. Center for Translational Cancer Research, Suite 4300, Helen F. Graham Cancer Center & Research Institute, Christiana Care Health Systems, Newark, DE 19713, USA.
E-mail addresses: xjia@udel.edu (X. Jia), swati@udel.edu (S. Pradhan-Bhatt).

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term goal is to engineer a functional, three-dimensional salivary gland for autologous implantation in patients suffering from xerostomia [3].

The salivary secretory complex is composed of two major cell types: acinar and myoepithelial cells, whose products are carried through an elaborate ductal network to the oral cavity. Although they are the least studied salivary cell type, myoepithelial cells are essential for glandular function and secretion in exocrine tissues. Situated between the acinar epithelium and the basement membrane, human salivary gland myoepithelial cells (hSMECs) are characterized by their dual epithelial and smooth muscle-associated expression profile [4]. This unique proteome comprises smooth muscle cytoskeletal elements and contractile machinery (α-smooth muscle actin: α-SMA; smooth muscle myosin heavy chain: SMMHC) as well as epithelial cytokeratins (e.g. KRT14 and KRT5) and adhesion molecules (EpCAM). Myoepithelial cells form desmosomal adhesions [5], cadherins [6] and gap junctions [7] with the gland's secretory acinar cells and mediate attachment of the acinar assembly to the basement membrane through hemidesmosomes and specific integrin isoforms. The myoepithelium also aids in the synthesis and deposition of the basement membrane, upon which epithelial cells anchor and polarize. In tissue, myoepithelial cells perform diverse functional roles, best studied in mammary glands where robust contraction of the mammary alveolus in response to oxytocin has been documented [8]. Myoepithelial cells also play an integral role in establishment and maintenance of luminal cell apicobasal polarity [9]. Abundant cell-cell and cell-matrix adhesions in the myoepithelium support their role in maintaining proper tissue architecture during fluid production and secretion [10].

To build a salivary gland tissue model from the bottom up, it is necessary to isolate and characterize the constituent cell populations. We have previously isolated and expanded hS/PCs [11], confirmed their expression of stem/progenitor markers [12], encapsulated them as dispersed single cells in hyaluronic acid (HA)-based hydrogels [13], investigated the role of hydrogel properties on the formation of hS/PC spheroid [14], and differentiated them to acinar cells using neurotransmitters [12]. Separately, consistent isolation, immunophenotypic characterization and functional analyses of hSMECs are essential for the generation of *in vitro* models with lobular cells. With the two types of cells in hand, a flexible, high-throughput 3D culture platform is desirable for the organization of the respective cell types in a well-defined spatial manner [15]. Finally, the complex assemblies should be encapsulated in a permissive and instructive hydrogel matrix that approximates the properties of the connective tissue mesenchyme surrounding the salivary gland. In such heterocellular organoid models, hSMECs should enable the exchange of paracrine and juxtacrine signals, contributing to the proper function of the salivary gland [16]. In this regard, HA-based hydrogels are particularly attractive as they can be tailored to represent the biochemical and physical characteristic of the desired tissue [3,17].

In this study, we first describe the successful isolation of hSMECs from human salivary tissue. We showed the characteristic resemblance of isolated cells with myoepithelial cells in the native salivary gland tissue and analyzed their contractile function *in vitro*. Using a hydrogel replicated microwell plate, we assembled 3D tissue-mimicking modules consisting of hS/PC spheroids with hSMECs wrapped around them. The modular co-assemblies were further encapsulated in an HA-based hydrogel to probe the function of hSMECs in a physiological relevant *in vitro* model. Our study showed, for the first time, the successful *in vitro* assembly of a salivary gland microtissue model that is reminiscent of the cellular organization of native salivary acini, providing a reliable platform to investigate myoepithelial cell biology *in vitro*.

2. Materials and methods

2.1. Isolation and culture of hSMECs

Healthy human salivary gland tissues for hSMEC isolation were procured from consented patients undergoing surgery for head and neck tumors following a protocol approved by Institutional Review Board (IRB) at both Christiana Care Health Systems and the University of Delaware. Any remnant connective tissue pieces were removed during the disinfecting and mincing stage. Thus, the resected parotid salivary gland tissue was predominantly epithelial in nature, largely free of stromal contaminants. A protocol for isolating hSMECs was adapted from work by Ohtomo et al. for the isolation of rat lacrimal myoepithelial cells and optimized for hSMECs [18]. Tissues were washed for 5 min in 1% (v/v) Betadine solution in DMEM/F12 (Life Technologies, Frederick, MD) and rinsed in DMEM/F12 for 2 min. The tissue was minced to a slurry, incubated at 37 °C in 0.1% (w/v) collagenase I (Thermo Fisher, Barnsted, NH) in Hank's Balanced Salt Solution (HBSS) for 20 min and then centrifuged at 1400 rpm for 3 min. The digestion process was repeated on the minced tissue, and the collected cells were added to cells from the first digest. The supernatant was strained through a 70 μm cell strainer, spun down at 1700 rpm for 4 min, and the pellet was re-suspended in 7 ml hSMEC media [RPMI media with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin] and plated into a T-25 cell culture flask. Cells were grown under standard 37 °C, 5% CO_2, 95% relative humidity conditions, and complete hSMEC media was replenished every 2 days. Following the repeat digest, only a few cells were observed in culture (Fig. S1A). As the media was replenished, floating cells were discarded and attached cells proliferated. Upon reaching 60% confluence after 30 days of incubation (Fig. S1B), hSMECs were trypsinized using 0.05% (w/v) trypsin-EDTA (Thermo Fisher) and neutralized with complete hSMEC media containing 10% FBS. Passages 2 through 9 were used for throughout the study.

2.2. Isolation and culture of hS/PCs

Human salivary gland stem/progenitor cells (hS/PCs) were separately isolated following our previously published protocol [12]. Briefly, human parotid gland tissue was disinfected using 1% (v/v) Betadine solution in DMEM/F12, followed by mincing into a slurry in complete HepatoSTIM media (Corning Inc. Corning, NY). After reaching 70–80% confluence, cells were trypsinized using 0.05% trypsin/EDTA, neutralized by equal volume of Soybean Trypsin Inhibitor (ATCC, Manassas, VA) and subcultured into a new flask in complete HepatoSTIM media. Passages 2 through 15 were used for this study.

2.3. Quantitative PCR (qPCR)

RNA was isolated from cultured hSMECs and human foreskin fibroblasts (hFF, ATCC, Manassas, VA) using the RNAeasy® mini kit (Qiagen, Valencia, CA) and DNAse treatment (Turbo DNAfree, Ambion Inc. Austin, TX). The mRNA concentration was measured using a spectrophotometer, and 1 μg mRNA was used for cDNA synthesis (cDNA synthesis kit, BioRad, Hercules, CA). The qPCR reaction was set up by mixing 12.5 μl of 2× SYBR Green mix, 5 μl forward and reverse primers, 0.5 μl of cDNA and 7.5 μl of water for each sample. Primer sequences for the genes used in this study are listed in Table 1. PCR reactions were run in two biological repeats and in triplicates, using the ABI 7300 PCR system (Life Technologies). The obtained C_T values were normalized to the housekeeping gene GAPDH using ΔC_T method [19,20].