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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

The Esophageal Organoid System Reveals Functional Interplay Between Notch and Cytokines in Reactive Epithelial Changes

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SUMMARY

We optimized culture conditions for 3-dimensional mouse and human esophageal organoids and used this experimental platform with potential applications toward personalized medicine to identify disruption of notch3-mediated squamous cell differentiation as a mechanism contributing to reactive epithelial changes under inflammatory conditions.

BACKGROUND & AIMS: Aberrations in the esophageal proliferation-differentiation gradient are histologic hallmarks in eosinophilic esophagitis (EoE) and gastroesophageal reflux disease. A reliable protocol to grow 3-dimensional (3D) esophageal organoids is needed to study esophageal epithelial homeostasis under physiological and pathologic conditions.

METHODS: We modified keratinocyte-serum free medium to grow 3D organoids from endoscopic esophageal biopsies, immortalized human esophageal epithelial cells, and murine esophagi. Morphologic and functional characterization of 3D organoids was performed following genetic and pharmacologic modifications or exposure to EoE-relevant cytokines. The Notch pathway was evaluated by transfection assays and by gene expression analyses in vitro and in biopsies.

RESULTS: Both murine and human esophageal 3D organoids displayed an explicit proliferation-differentiation gradient. Notch inhibition accumulated undifferentiated basal keratinocytes with deregulated squamous cell differentiation in organoids. EoE patient-derived 3D organoids displayed normal epithelial structure ex vivo in the absence of the EoE inflammatory milieu. Stimulation of esophageal 3D organoids with EoE-relevant cytokines resulted in a phenocopy of Notch inhibition in organoid 3D structures with recapitulation of reactive epithelial changes in EoE biopsies, where Notch3 expression was significantly decreased in EoE compared with control subjects.

CONCLUSIONS: Esophageal 3D organoids serve as a novel platform to investigate regulatory mechanisms in squamous epithelial homeostasis in the context of EoE and other diseases. Notch-mediated squamous cell differentiation is suppressed by cytokines known to be involved in EoE, suggesting that this may contribute to epithelial phenotypes associated with disease. Genetic and pharmacologic manipulations establish proof of concept for the utility of organoids for future studies and personalized medicine in EoE and other esophageal diseases. *(Cell Mol Gastroenterol Hepatol 2018;■:■–■; https://doi.org/10.1016/j.jcmgh.2017.12.013)*

Keywords: Three-Dimensional; Keratinocytes; Eosinophilic Esophagitis; Squamous Cell Differentiation.

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Abbreviations used in this paper: aDMEM/F12, advanced Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; BCH, basal cell hyperplasia; DAPI, 4′,6-Diamidino-2-Phenylindole, Dihydrochloride; DNMAML1, dominant negative MAML1; DOX, doxycycline; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; EoE, eosinophilic esophagitis; GERD, gastroesophageal reflux disease; GFP, green fluorescent protein; GSI, γ-secretase inhibitor; H&E, hematoxylin and eosin; IF, immunofluorescence; IHC, immunohistochemistry; IL, interleukin; IVL, Involucrin; KSFM, keratinocyte SFM; KSFMC, KSFM containing 0.6 mM Ca^{2+}; MAML1, Mastermind-like protein1; OFR, organoid formation rate; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; 3D, 3-dimensional; TNF-α, tumor necrosis factor-α; Tslp, thymic stromal lymphopoietin.

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Stratified squamous epithelia comprises basal and suprabasal cell layers, displaying an explicit differentiation gradient. The basal cell layer contains undifferentiated and proliferative basal cells (keratinocytes) that exit cell division cycles and undergo terminal differentiation within the suprabasal cell layers to migrate and desquamate into the lumen, permitting epithelial renewal. Basal cells express defining molecular markers, such as β1-integrin (CD29).[1] The basal cell layer harbors stem cells with long-term self-renewal capacity.[2,3] Cell-cell junctions in suprabasal cell layers provide epithelial barrier function. The differentiation gradient is disrupted under esophageal disease conditions, both benign and malignant. Esophageal epithelial homeostasis is influenced by a variety of agents and factors including chemical carcinogens, radiation, acids, growth factors, and inflammatory cytokines. These factors activate multiple signaling pathways, including epidermal growth factor (EGF) receptor, bone morphogenetic protein, Wnt, and Notch, which regulate esophageal epithelial renewal, proliferation, differentiation, transdifferentiation, senescence, apoptosis, or survival.[4-10]

The Notch pathway is critical in squamous epithelial homeostasis.[11] Notch signaling is activated via cell-cell contact, permitting cell surface ligand-receptor interaction. Activation of Notch receptor leads to a series of proteolytic cleavages and nuclear translocation of its intracellular domain, which physically associates with the common downstream effector transcription factor CSL/RBPJ along with the coactivator Mastermind-like protein 1 (MAML1). Notch-activated CSL-mediated transcriptional targets include Involucrin (IVL), a squamous-cell differentiation marker. Of 4 mammalian paralogs of Notch receptor, Notch1 is the master regulator of squamous cell differentiation. Loss of Notch signaling in the epidermis results in noncell autonomous and cell autonomous effects, inducing basal cell hyperplasia (BCH; expansion of basal keratinocytes without postmitotic terminal differentiation),[12] deregulated squamous cell differentiation and hyperkeratosis, and dermal inflammation and eosinophilic infiltrates.[13] In murine esophageal keratinocytes, genetic or pharmacologic pan-Notch signaling inhibition via dominant negative MAML1 (DNMAML1) or γ-secretase inhibitors (GSI), impairs squamous cell differentiation with a concurrent downregulation of IVL and other differentiation-related genes *in vitro* and *in vivo*.[8]

Many esophageal diseases, such as gastroesophageal reflux disease (GERD) and eosinophilic esophagitis (EoE), involve esophageal epithelial pathologies. For example, common histopathologic manifestations of EoE and GERD include esophageal BCH and spongiosis (dilated intracellular spaces). Featuring eosinophilic inflammation and lamia propria fibrosis,[14] EoE is an emerging food allergen–induced cytokine-mediated inflammatory disorder, affecting children and adults. Long-term disease status leads to irreversible fibrotic esophageal stenosis. Genome-wide association studies and gene expression profiling of endoscopic esophageal biopsies from patients with EoE have implicated several epithelial cell–associated molecules, such as thymic stromal lymphopoietin (Tslp) and Desmoglein-1, the latter a regulator of esophageal epithelial barrier function.[15,16] Transcriptome analysis has revealed differentiation as the most affected biologic process in EoE.[17] Additionally, EoE-relevant inflammatory cytokines, such as tumor necrosis factor (TNF)-α and transforming growth factor-β, alter epithelial cell characteristics by inducing epithelial-mesenchymal transition (EMT).[18,19] Esophageal inflammation associated with GERD also involves TNF-α and

Table 1. Patients Used to Generate Biopsy-Derived Esophageal 3D Organoids

#	Age	Sex	Diagnosis	Passage
1	15	M	Normal	n.d.
2	5	M	Inactive	n.d.
3	8	M	Inactive	n.d.
4	14	M	Inactive	n.d.
5	14	F	Normal	n.d.
6	11	F	GERD	n.d.
7	11	M	GERD	n.d.
8	13	M	GERD	n.d.
9	8	M	Active	n.d.
10	14	F	Active	n.d.
11	16	M	PPI-REE	2[a]
12	6	M	Inactive	3[b]
13	8	F	Inactive	n.d.
14	11	M	Inactive	3[b]
15	5	M	Active	4[b]
16	18	F	Active	5[b]
17	16	F	Normal	3[b]
18	9	M	Active	6[a]
19	10	F	Normal	n.d.
20	8	F	Normal	n.d.
21	6	M	Inactive	4[b]
22	5	M	PPI-REE	5[b]
23	7	F	Active	4[b]
24	14	M	Inactive	7[a]
25	8	M	Normal	4[b]
26	11	F	Active	3[a]
27	10	M	Inactive	3[b]
28	6	M	Inactive	n.d.
29	8	M	Inactive	n.d.
30	10	M	Active	4[a]
31	18	M	Active	n.d.
32	18	M	Inactive	n.d.
33	18	F	Normal	4[a]
34	12	F	Normal	n.d.

NOTE. Normal, no pathologic diagnosis; inactive, <15 eos/hpf but with previous diagnosis of EoE; GERD, 1–5 eos/hpf; active, >15 eos/hpf.
n.d., not determined; PPI-REE, proton pump inhibitor–responsive esophageal eosinophilia.
[a]3D organoid culture was not passaged after this passage number.
[b]3D organoids failed to grow after this passage number.