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REVIEW

Esophageal 3D Culture Systems as Modeling Tools in Esophageal Epithelial Pathobiology and Personalized Medicine

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SUMMARY

Pathology of the esophageal epithelium leads to perturbations of the normal proliferation-differentation gradient. Three-dimensional culture of esophageal epithelial tissue provides a unique platform to study diseases of the esophageal epithelium. Herein, we examine various types of three-dimensional esophageal culture and discuss the future of these applications for translational research and personalized medicine.

The stratified squamous epithelium of the esophagus shows a proliferative basal layer of keratinocytes that undergo terminal differentiation in overlying suprabasal layers. Esophageal pathologies, including eosinophilic esophagitis, gastroesophageal reflux disease, Barrett's esophagus, squamous cell carcinoma, and adenocarcinoma, cause perturbations in the esophageal epithelial proliferation-differentiation gradient. Three-dimensional (3D) culture platforms mimicking in vivo esophageal epithelial tissue architecture ex vivo have emerged as powerful experimental tools for the investigation of esophageal biology in the context of homeostasis and pathology. Herein, we describe types of 3D culture that are used to model the esophagus, including organotypic, organoid, and spheroid culture systems. We discuss the development and optimization of various esophageal 3D culture models; highlight the applications, strengths, and limitations of each method; and summarize how these models have been used to evaluate the esophagus under homeostatic conditions as well as under the duress of inflammation and precancerous/cancerous conditions. Finally, we present future perspectives regarding the use of esophageal 3D models in basic science research as well as translational studies with the potential for personalized medicine. *(Cell Mol Gastroenterol Hepatol 2018;5:461–478; https://doi.org/10.1016/j.jcmgh.2018.01.011)*

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The esophageal mucosa comprises stratified squamous epithelium in which esophageal epithelial cells (keratinocytes) show a proliferation-differentiation gradient and provide a barrier against the chemical and biological milieu of luminal contents. Disruption of this differentiation gradient or barrier function is linked to multiple human pathologies. Eosinophilic esophagitis (EoE), gastroesophageal reflux disease (GERD), and intestinal metaplasia (Barrett's esophagus [BE]) are benign esophageal conditions featuring aberrant epithelial cell proliferation and differentiation. In addition, adenocarcinoma (EADC) and squamous cell carcinoma (ESCC) represent the 2 primary types of malignancies arising within the esophageal epithelium and progressing via dissemination and invasion toward the underlying subepithelial stromal compartment. Three-dimensional (3D) cell culture model systems have been used as near-physiological experimental platforms to study esophageal biology under homeostatic and pathologic conditions. These 3D platforms include organotypic 3D culture (OTC) and the more recently developed 3D organoid system. In this review, we highlight the historical background of these technologies while also discussing differences among 3D culture model systems as well as applications and current limitations. Finally, we address potential future directions for these 3D model systems as they relate to esophageal epithelial biology, tumor biology, and translation in personalized medicine.

Esophageal Stratified Squamous Epithelium: Structure and Physiological Function

As a hollow muscular organ, the esophagus serves the passage of ingested food and liquid from the oral cavity to

Abbreviations used in this paper: BE, Barrett's esophagus; COX, cyclooxygenase; CSC, cancer stem cell; EADC, esophageal adenocarcinoma; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EoE, eosinophilic esophagitis; ESCC, esophageal squamous cell carcinoma; FEF3, primary human fetal esophageal fibroblast; GERD, gastroesophageal reflux disease; OTC, organotypic 3-dimensional culture; STAT3, signal transducer and activator of transcription-3; 3D, 3-dimensional.

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the stomach. Its luminal surface is lined by the mucosa, comprising stratified squamous epithelium and the underlying lamina propria and muscularis mucosa. The esophageal epithelium consists of proliferative basal keratinocytes and suprabasal keratinocytes, the latter undergoing postmitotic terminal differentiation, passive migration toward the luminal surface, and, ultimately, desquamation into the lumen. Through this dynamic process, a proliferation-differentiation gradient is generated while epithelial renewal occurs over a period of 2 weeks.[1] Molecular markers defining basal keratinocytes include cytokeratins K5 and K14,[2,3] transcription factors p63,[4] and Sry-related HMG box (SOX2),[5] and cell surface molecules such as neurotrophin receptor p75NTR,[6] integrin β1 (CD29), integrin α6 (CD49f), and transferrin receptor (CD71).[7] Suprabasal keratinocytes are defined by differentiation markers such as cytokeratins K4 and K13, involucrin, and filaggrin,[8–10] coupled with down-regulation of basal keratinocyte markers.

Doupe et al[1] proposed that the esophageal epithelium is maintained by a single population of basal keratinocytes that give rise stochastically to proliferating and differentiating daughters with equal probability; however, functional cell heterogeneity has been postulated among basal esophageal keratinocytes. A minor subset of basal keratinocytes divide slowly or rarely and may have properties of quiescent stem cells.[11] Such a cell population may provide an explanation as to how premalignant keratinocytes accumulate genetic alterations over years without being lost through epithelial renewal. Multiple cell surface and functional markers have been suggested to identify unique subsets of basal keratinocyte stem/progenitor cells, including neurotrophin receptor $p75^{NTR}$,[6] integrins (β4, α6),[7,12] and *ABCG2* gene product.[11] Esophageal keratinocytes expressing these molecular markers have shown colony formation and self-renewal capabilities while also generating terminally differentiated progenitor cells.

Species differences exist between rodents and human beings with regard to anatomic esophageal structure. Foremost, the rodent esophagus lacks esophageal glands and papillae, both of which are present in the human esophagus. In addition, the rodent esophagus shows more explicit keratinization in the superficial cell layers, also known as *stratum corneum of the squamous epithelium*, as compared with its human counterpart. The rodent stomach consists of 2 compartments: the forestomach and distal stomach, featuring squamous epithelium and columnar epithelium, respectively, and some regard the forestomach as the counterpart of the human lower esophagus. This is important to note because Barrett's esophagus (ie, intestinal metaplasia of the esophagus) is a human mucosal lesion involving the esophagogastric junction and has been modeled at the squamocolumnar junction within the murine stomach.[13,14]

One essential physiological function of the esophageal mucosa is to serve a barrier against thermal, physical, or chemical agents, and factors related to luminal contents, including microorganisms, food antigens, gastroduodenal acids, and alcohol, all of which may contribute to the pathogenesis of esophageal diseases. Unlike the stomach, duodenum, and intestine, the luminal surface of the esophagus is not densely covered by mucus layers. Given the lack of the stratum corneum in the human esophagus and the lack of esophageal glands in rodents, the epithelial barrier function of the esophagus is attributed mainly to intercellular junctional complexes including tight junctions, adherens junctions, and desmosomes formed by cell–cell adhesion molecules such as E-cadherin, p120 catenin, and claudins. The dysfunction of these adhesion molecules has been implicated in esophageal disease conditions.[15–18]

Organ Culture and Multiple 3D Culture Models: What Are the Differences?

Throughout a long history of cell culture, various forms of 3D culture methodologies have been developed along with unique scaffolds, matrices, and cell culture media. In the esophagus, 3D culture systems have provided unique platforms to study multiple biological processes, including epithelial cell proliferation, differentiation, motility, stress response, and both homotypic and heterotypic cell–cell communications. Cellular interactions involve a variety of cell types (eg, fibroblasts, endothelial cells, and inflammatory cells) in the esophageal tissue microenvironment under homeostatic and pathologic conditions (eg, inflammatory milieu), and are mediated via cell surface molecules (eg, integrins and receptors such as Notch) as well as extracellular matrix proteins (eg, matrix metalloproteinases), as discussed in this review. The ability to experimentally manipulate 3D cultures has greatly enhanced our understanding of the molecular mechanisms and signaling pathways underlying esophageal physiology and pathophysiology.

Organ (explant) culture was a major tool for in vitro live esophageal tissue analyses before primary esophageal epithelial cell culture[19] and esophageal cancer cell lines[20] became available in late 1970s and early 1980s, respectively. The foremost advantage of organ culture is the maintenance of natural tissue architecture in situ. Organ culture may be used to study cross-talk between epithelial cells and nonepithelial cells in a live tissue-like context. Given the potential importance of a variety of cell types (ie, epithelial cells, fibroblasts, nerve cells, immune cells, and endothelial cells) present in the tissue microenvironment, organ culture indeed may be more physiologically relevant than other 3D culture systems because co-culturing multiple cell types remains difficult.

Early organ culture studies have shown that esophageal explants from animals and human beings remained viable for 3–14 days ex vivo[21] and provided substantial insight into esophageal physiological functions, including secretory and absorptive activities by basal and differentiating prickle cells,[22] as well as endocytosis mediated by prickle cells and terminally differentiated superficial cells.[23] In fetal human esophagi, organ culture detected not only epithelial cell proliferation[24] but also replacement of columnar ciliated epithelium with stratified squamous epithelium,[25] recapitulating the epithelial changes occurring during esophageal development.

Organ culture also has been used to study esophageal pathologies. Production of esophagitis-relevant cytokines was shown in patient-derived squamous epithelial explants.[26] Retinoic acid induced BE-like glandular differentiation in explants derived from squamous esophageal epithelium.[27] Explanted patient-derived mucosal biopsy specimens of Barrett's esophagus showed increased proliferation and cyclooxygenase (COX)-2 expression in response to bile salt exposure.[28,29] Likewise, acid pulse induced a hyperproliferative response in Barrett's esophagus explants[30] via altered Na^+/H^+ exchange.[31] Radiation sensitivity was tested in tumor explants from ESCC and EADC.[32]

Although the use of chemically defined medium containing insulin and hydrocortisone enabled the maintenance of live human esophageal tissues in organ culture for up to 6 months,[33] most experiments in organ culture are performed for short time periods (24–72 h) only. This is in part because the proliferation kinetics of esophageal keratinocytes in organ culture are not necessarily reflective of those occurring in vivo.[34] In addition, monolayer outgrowth of keratinocytes has been observed in explant culture.[35] Organ culture is inevitably more complicated than the 3D culture systems discussed later. Other limitations of this method include the difficulty of genetic manipulation of human tissues and the inability to passage organ cultures. Esophageal explants have yet to be derived from genetically engineered mouse models. To this end, Cre-mediated gene deletion can be performed ex vivo in organ culture with tissues isolated from transgenic mice carrying tamoxifen-inducible cell type–specific Cre and a floxed gene of interest.

Unlike organ culture, most 3D culture systems involve dissociation of originating tissue samples or preparation of cells grown in monolayer cultures before 3D reconstitution in vitro. Such 3D culture systems can be largely classified into 2 categories. One category is represented by OTC in which esophageal epithelial cells are grown over a collagen matrix containing fibroblasts that mimics the subepithelial lamina propria. The second category features spherical 3D structures of esophageal epithelial cells typically generated under submerged conditions. This includes multicellular spheroid culture, sphere formation assays, and 3D organoids.

OTC, also known as raft culture, is a form of tissue engineering with recapitulation of esophageal physiology and pathology (Figure 1). OTC was first established in the field of skin biology in the 1980s when the dermal equivalent comprising contracted type I collagen and fibroblasts[36] was submerged in liquid medium and used as a raft-like platform to grow multilayered epidermal keratinocytes at the air–liquid interface, mimicking skin architecture as found in vivo.[37–40] The air–liquid interface triggers epithelial stratification and cornification by inducing terminal differentiation in the superficial cell layer with induction of a variety of squamous cell differentiation markers including cytokeratins, transglutaminase, involucrin, and filaggrin.[41] Although normal keratinocytes generate well-organized epidermal architecture, keratinocytes transformed by oncogenic viruses such as human papilloma virus form disorganized epithelia displaying aberrant proliferation, differentiation, and invasion into the dermal compartment.[42–46]

We adopted this system for normal and genetically engineered esophageal keratinocytes, optimizing culture conditions and evaluating the influence of a variety of sources of fibroblasts,[47,48] including the skin and the esophagus of adults, children, and embryos as well. Fibroblasts evaluated in OTC include those isolated from the skin and the esophagus of adults, children, and embryos, as well as cancer-associated fibroblasts.[49–51] Although multiple esophageal cell lines were used successfully in OTC[52] (Table 1), telomerase-immortalized normal human esophageal keratinocytes (EPC2-hTERT) and primary human fetal esophageal fibroblasts (FEF3) have served as standard and quality-control cells. EPC2-hTERT cells show complete stratified epithelia in OTC.[48] Of note, commonly used HET1A, an oncogenic simian-virus-40 T-antigen-immortalized human esophageal epithelial cell line, did not recapitulate normal squamous esophageal epithelium in OTC.[49] With detailed protocols published by Kalabis et al,[51] we have interrogated gene and molecular functions as well as cell–cell and cell–extracellular matrix interactions in broad contexts related to the esophageal physiological and pathologic microenvironment,[41,47–49,52–60] as detailed later in this article.

In vivo transplant culture has served as another platform to reconstitute esophageal epithelium in 3D.[61] According to this method, which may not be categorized as cell culture in a strict sense, epithelial cells and fibroblasts are injected into the devitalized and denuded trachea tube of rats, which then is further transplanted into immunodeficient mice. Esophageal keratinocytes are allowed to grow for 4 weeks inside the xenografted trachea and the resulting epithelial structure may be analyzed morphologically. Wang et al[62,63] used this system successfully to characterize the contribution of Hedgehog signaling to esophageal development and metaplasia with esophageal keratinocytes isolated from genetically engineered mice.

Multicellular spheroid culture emerged in the early 1970s, stemming from dissociation-aggregation experiments in which dissociated tissue-derived cells undergo aggregation and self-organization under free-floating conditions with gentle stirring via spinner flasks or roller bottles. This technique was applied to a variety of cell types with primary culture and established cell lines recapitulating histologic tissue architecture of the originating organ.[64–66] This platform has been used to study cross-talk between tumor cells and other cell types (eg, immune cells and endothelial cells) in co-culture experiments.[67–69] Although multicellular spheroid culture was performed with nontransformed bovine esophageal epithelial cells as well as ESCC cell lines (Table 1), these studies were limited to morphologic comparison or analysis of cell viability in resulting spheroids.[70,71]

In the history of 3D culture, the importance of extracellular matrix components for self-organization of functional epithelial cells has been recognized by studies using mammary epithelial cells grown under floating conditions in type I collagen or Matrigel (Corning, Tewksbury, MA) rather

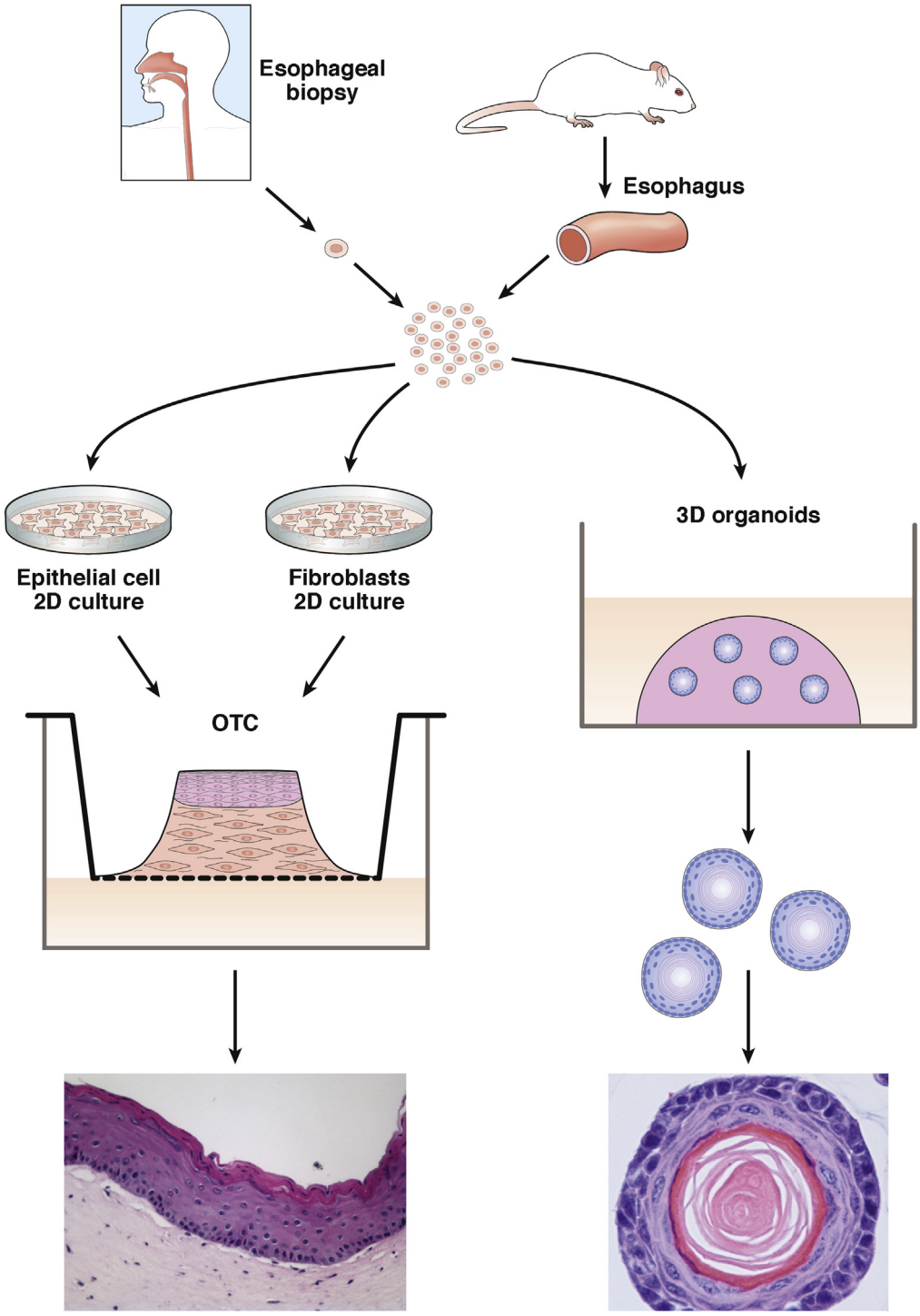

Figure 1. Recapitulation of stratified squamous epithelium in OTC and 3D esophageal organoids. In both 3D culture systems, esophageal epithelial cells (keratinocytes) and fibroblasts are dissociated from tissues. In OTC, primary culture or cell lines are prepared in monolayer 2-dimensional culture before 3D reconstruction in the Transwell insert with a bottom filter that permits medium in the bottom chamber feeds the entire system. The trapezoid-shaped collagen matrix containing fibroblasts support keratinocyte growth. Note that keratinocytes are incorporated sequentially, but not simultaneously, into the OTC system because fibroblasts are first grown in the collagen matrix. The air–liquid interface triggers epithelial stratification. In 3D esophageal organoids, keratinocytes are mixed into Matrigel at a low density and allowed to grow as single-cell–derived spherical 3D structures with a proliferation-differentiation gradient. After enzymatic digestion of Matrigel, the 3D structures are recovered and subjected to analyses or can be continued in the subsequent passages. Note the cornification seen in the outmost superficial cell layer in OTC while cornification occurs in the inmost core in 3D organoids.

than on a plastic surface. Under such conditions, mammary epithelial cells showed not only polarity but also luminal formation with milk protein secretion, which occurred more efficiently in the presence of Matrigel compared with type I collagen.[72] Andl et al[73] embedded esophageal epithelial cell aggregates into either type I collagen or Matrigel (Table 1) to observe that cell migration and invasion from the resulting spheroids were coupled with loss of E-cadherin–mediated cell adhesion. They further identified activin in the tumor microenvironment as a regulator of spheroid growth and invasion.[74]

Sphere formation assays gained popularity in the 2000s as an excellent tool to characterize stem cells from multiple tissue types,[75] including tumors (see Weiswald et al[76] for an excellent review regarding tumor spheres and other spherical cancer models). Sphere formation assays were first used to show proliferation, self-renewal, and multipotent capabilities of neural stem cells in serum-free

Table 1. Published Esophageal Epithelial 3D Culture Studies

3D culture system	Esophageal epithelial tissue/cell[a]	Cell designation	Research focus	Reference
OTC	Tissue/normal: m	FACS-purified keratinocytes	Stem/progenitor cells	110
	Normal: h	Primary keratinocytes	Epithelial reconstitution	53,54
	Immortalized cell line: h	HET-1A	Epithelial reconstitution	111
	Immortalized cell line: h	EoE2-T	Epithelial reconstitution	112
	Immortalized cell line: h	EPC1-hTERT EPC2-hTERT and derivatives	Notch in squamous-cell differentiation and Notch	41
	Nontransformed and transformed		AKT in squamous-cell differentiation	56
			EGFR in basal cell hyperplasia	54
			EGFR, cyclin D1, p53, and Notch in malignant transformation	55,61
			EGFR and p53 in ESCC cell invasion	55,113
			EGFR and p120 in ESCC invasion	114
			PI3K and p120 in ESCC invasion	115
			Periostin and ESCC invasion	58
			STAT1 and ESCC invasion	116
			Wnt in ESCC invasion	61,117
			Notch in ESCC invasion	60,61
			IGFBP3 in ESCC invasion	59
			Activin A in ESCC invasion	118
			CD44 and ESCC invasion	108
			Autophagy and EMT in ESCC	119
			c-Met and p53 in ESCC invasion	120
			Wnt and COX2 in columnar-cell differentiation	121
			Cdx1, c-Myc, and Notch in columnar-cell differentiation	57,62
			Modeling esophagitis	122
			EMT in EoE	123,124
			Epithelial barrier functions in EoE	17,125,126
	ESCC cell line: h	TE9, TE10, KYSE70	ESCC invasion	52
		OE21	ESCC invasion	111
		TE12	p38 MAPK in ESCC invasion	127
		TE7, TE12	Fibroblast HGF in ESCC invasion	49
	Barrett's esophagus cell line: h	CP-A, CP-B, CP-C, CP-D	Modeling Barrett's esophagus, all-*trans* retinoic acid in columnar differentiation	128
	EADC cell line: h	MFD-1, OE19, OE33, FLO-1	Modeling invasive EADC, cancer-associated fibroblasts and periostin in EADC cell invasion	50,51,111
Organoid	Normal, disease tissues: m	FACS-purified keratinocytes	Stem/progenitor cells	12,107
		Bulk or primary keratinocytes	Basal cell hyperplasia and autophagy	129
			Alcohol and autophagy	134
			EMT and Notch in ESCC	108
	Normal, GERD, EoE biopsy specimens: h	Bulk keratinocytes	Common culture conditions for murine and human organoids	130
	Immortalized cell line: h	EPC2-hTERT and derivatives	Basal cell hyperplasia and Notch in squamous-cell differentiation	130
	Barret's esophagus biopsy specimens: h	Barrett's esophagus	Human Barrett's esophagus 3D organoids	96

Table 1. Continued

3D culture system	Esophageal epithelial tissue/cell[a]	Cell designation	Research focus	Reference
Sphere	ESCC cell line: h	KYSE70, KYSE140, KYSE150, KYSE520, TE1	ESCC CSC	82,83,87
		CE81T, CE146T	ICAM1 in ESCC CSC	80
		TE4, TE8	JARID1B in ESCC CSC	88
		YES-2, Eca109	ALDH and ESCC CSC	84,85,91
		KYSE270, T.Tn	miR-377 in ESCC CSC	93
		Eca109, Eca9706	miR-181b and STAT3 in ESCC CSC	92
		KATO-TN	YAP1 in ESCC	86
	EADC cell line: h	OE33, JH-EsoAd1	EADC CSC	81
		JH-EsoAd1	YAP1 in EADC CSC	86
		JH-EsoAd1	ALDH in EADC CSC	89
		OE33, OE19, FLO1, JH-EsoAd1	Notch in EADC CSC	90
Organotypic sphere culture	Tissue/normal: m, h	Primary keratinocytes	Stem/progenitor cells	109
Multicellular spheroid/ aggregate culture	Normal (bovine)	Primary keratinocytes	Morphologic comparison with spheroids formed with other cell types and detection of cell death in the inner cell mass within the resulting spheroid structures	72
	ESCC cell line (h)	OSC-1, OSC-2	Confirmation of spheroid formation	73
	Immortalized cell line (h)	EPC2-hTERT and derivatives	E-cadherin and cell adhesion	75

NOTE. In OTC, primary human FEF3 and other fibroblasts (eg, cancer-associated fibroblasts) were used to form subepithelial collagen matrix. Extensive lists of fibroblast cell lines and epithelial cell lines validated in 3D OTC are available in Kalabis et al.[52]

FACS, fluorescence-activated cell sorter; h, human; HGF, hepatocyte growth factor; ICAM1, intercellular adhesion molecule 1; m, murine; MAPK, mitogen-activated protein kinase.

[a]All of esophageal origin.

medium supplemented with epidermal growth factor (EGF) and differentiation-inducing other growth factors.[77] To form 3D spherical structures, a single cell or a small number ($<10^1$–10^2) of cells are inoculated per well and allowed to proliferate under free-floating conditions. This permits cell fate determination by tracking cell lineage within resulting spherical 3D structures. Unlike multicellular spheroid culture, cell aggregation is deliberately avoided at the onset of sphere formation assays to ensure a proliferative expansion of single-cell derivatives. In cancer research, sphere formation was used to explore cancer stem cells (CSCs) or tumor-initiating cells in conjunction with a variety of putative CSC markers and evaluation of therapeutic sensitivity for such cell populations.

As summarized in Table 1, sphere formation assays have been used to characterize EADC and ESCC CSCs expressing a variety of markers, including CD54 (intercellular adhesion molecule 1), CD49f, CD44, CD271, CD90, and aldehyde dehydrogenase, either alone or in combination.[78–84] Increased sphere formation corroborated CSC attributes such as chemoresistance, invasiveness, and tumorigenicity, and expression of stemness markers (*SOX2*, *ALDH1A1*, and *KLF4*) induced by transforming growth factor-β1.[85] Sphere formation assays were used to document the requirement of H3K4 demethylase Jumonji/Arid1b (Jarid1b) in the maintenance of ESCC CSCs[86] and the role of Hippo coactivator yes-associated protein 1-mediated transcriptional regulation of SOX9 in EADC CSCs.[84]

Sphere formation also has been used to test pharmacologic therapeutic effects upon putative esophageal CSCs. Metformin appeared to inhibit sphere formation by Aldehyde dehydrogenase (ALDH)1^+ EADC CSCs by targeting phosphatidylinositol 3-kinase/AKT and mammalian target of rapamycin.[87] Pharmacologic inhibition of Notch signaling by γ-secretase inhibitors impaired tumor initiation as well as sphere formation by EADC CSCs, and Notch appeared to regulate genes such as SOX2, which is essential in stemness.[88] The plant-derived agent curcumin also was found to decrease the sphere formation capability of ESCC cell lines.[89]

In sphere formation assays, microRNA miR-181b was implicated in signal transducer and activator of transcription-3 (STAT3)-mediated transcriptional regulation of CSCs,[90] whereas miR-377–mediated regulation of CD133+ ESCC CSCs has been shown.[91] It must be noted that sphere formation assays in the studies described earlier were performed with cell lines, but not cancer cells isolated from primary tumors. In 1 study, sphere formation assays failed to detect ESCC CSCs expressing CD44 despite the high tumor-initiating capability of these cells being validated in mice.[92] In addition, high densities (10^3–10^4 cells/well) of cells were seeded to form spheres in many of the described studies,[78,82–85,87–89] while several studies failed to provide information as to how spheres were generated,[81,86,91] precluding determination of whether resulting spherical 3D structures represented cell aggregates or single-cell–derivative products. Because this represents a deviation from the original neurosphere assay principle,[75] data obtained in these studies must be interpreted carefully.

The 3D organoid system has emerged in the past several years as a robust tool in basic research with the potential for personalized medicine. 3D organoids have been defined as a miniature organ-like 3D structure derived from single cells or a small number of cell clusters containing stem/progenitor cells grown in basement membrane (ie, Matrigel) under submerged conditions (Figure 1). Overcoming the difficulty to culture intestinal cell types, Sato et al[93,94] were successfully able to grow stem and progenitor cells from either isolated intestinal crypts or single-cell suspensions prepared from the small or large intestine into lobulated 3D structures containing crypt and villus compartments, the latter containing terminally differentiated secretory and absorptive cell lineages. By passaging dissociated primary structures to generate secondary 3D organoids, this system has been used to validate the self-renewal activities of putative stem cells. Because this can be performed using live tissue pieces from biopsy specimens or even frozen tissues, this novel cell culture method has been transformative with great potential to advance personalized medicine, for example, by testing the chemotherapeutic sensitivity of colorectal cancer–derived 3D organoids from individual patients.[95] Intestinal organoids have been coupled successfully with genetically engineered mouse models of colorectal cancer,[96] facilitating functional studies into the biology of this organ in the context of health and disease. Application of the 3D organoid system has been extended to a variety of cell types from digestive (intestines, stomach, liver, pancreas) and nonintestinal organs (eg, brain, lung, breast, kidney, prostate, and ovary) from both human and murine tissue sources as well as embryonic or induced pluripotent stem cells.[97,98] Besides colorectal cancer,[95,99,100] patient-derived tumor organoids have been generated successfully from pancreas,[101,102] prostate,[103] breast, and uterine[104] cancer cells in primary and metastatic lesions and blood samples, with a goal of translation into personalized therapy.

3D organoids have been cultured in Dulbecco's modified Eagle medium:nutrient mixture F12-based serum-free medium supplemented with transferrin, selenium, ethanolamine, insulin, antioxidants, and vitamins.[94] This medium is supplemented with pharmacologic agents, such as transforming growth factor-β kinase/activin receptor-like kinase inhibitor A83-01, the p38 mitogen-activated protein kinase inhibitor SB202190 and the rho-associated kinase inhibitor Y-27632 to facilitate the establishment of organoids. Besides Matrigel, addition of growth factors and hormones, including noggin, EGF, Wnt3A, R-spondin, and gastrin, to 3D organoid media provides essential niche factors present in the tissue microenvironment in situ. Unique niche factors may influence organoid formation differentially from different cell types as pioneered by Sato et al[94] in intestinal cell types. For example, Wnt3A is essential for stem cell maintenance in colonic, but not small intestinal, 3D organoids, in mice in which withdrawal of Wnt3A facilitates differentiation in murine colonic organoids.[94] Optimization of 3D organoid culture medium has broadened the cell types of human and rodent origin that can be grown successfully and passaged as 3D organoids.[95]

3D organoids featuring the stratified squamous epithelium of the esophagus were first established from murine esophageal mucosa by DeWard et al[12] to investigate esophageal stem and progenitor cells. This group used medium similar to that used for human intestinal 3D organoids.[94] We have used simplified medium components to generate single-cell–derived murine esophageal 3D organoids for multiple passages,[12,105] indicating that certain agents and factors are dispensable for murine esophageal 3D organoid culture. The conditions that we have optimized were permissive for generation of murine 3D organoids from normal esophageal epithelium as well as chemical carcinogen-induced precancerous and ESCC lesions.[106]

Jeong et al[107] showed successful generation of clonally formed spherical 3D structures from both murine and human esophageal mucosa in a method referred to as "3D organotypic sphere culture." Although esophageal keratinocytes were suspended in Matrigel, this method used 2 different types of keratinocyte serum-free media to maintain undifferentiated progenitor cells and induce terminal differentiation. In addition, the esophageal keratinocyte-containing Matrigel compartment was placed atop a cell culture insert and exposed to the air–liquid interface, although the contribution of this exposure to sphere formation and/or differentiation was not discussed.[107]

Among the earlier-described esophageal 3D cell culture systems, OTC and 3D esophageal organoids have been used most extensively to model epithelial physiological as well as pathologic conditions (Table 1). As a form of tissue engineering, OTC has been most broadly used in studies focusing on epithelial cell behaviors, including epithelial-stromal interaction after pharmacologic and genetic manipulations of either epithelial or stromal cells[17,41,47–60,73,106,108–125] (see Kalabis et al[52] for detailed protocols and resources available). OTC has been better characterized using human esophageal cells as compared with cells of rodent origin, although this method is possible with the latter.[108] Although OTC requires preparation of monolayer cell culture before epithelial and subepithelial tissue reconstitution in vitro, the 3D organoids system is initiated directly after dissociation of live tissues and has been applied to both murine and human esophageal cells (Figure 1). In the following section, we review several normal and disease conditions in which OTC or 3D organoids have served as modeling tools.

Esophageal Normal Stem/Progenitor Cell Proliferation and Differentiation in 3D

Because the esophageal 3D organoid system features single-cell–derived clonal expansion, establishment of a squamous cell differentiation gradient, and the maintenance of 3D structure upon serial passaging, this experimental platform has been used to test the self-renewal capability of putative esophageal stem cells. DeWard et al[12] have shown that esophageal basal keratinocytes defined by Sox2 expression comprise stem cells and transit-amplifying cells defined by distinct levels of cell surface CD73, α6 integrin (CD49f), and β4 integrin expression with differential 3D organoid formation capabilities. Jeong et al[105] showed that undifferentiated esophageal keratinocytes defined by positive/high CD49f and low CD24 expression show high 3D sphere formation capability where the transcription factor p63 appeared to regulate self-renewal and gene expression of basal cell markers. Giroux et al[104] identified a long-lived stem/progenitor cell population characterized by expression of keratin 15 in murine esophageal epithelium, which showed self-renewal, proliferation, and differentiation capabilities in 3D organoid assays. As has been postulated as a weakness in conventional neurosphere assays,[75] organoid formation involves cell proliferation, and, thus, may not necessarily determine quiescent stem cells. In addition, the frequency of stem cells detected by these assays may not necessarily be representative of that found in originating tissues, potentially owing to loss of subsets of cells during tissue dissociation and cell isolation. The proliferation-differentiation gradient has been analyzed in esophageal stem cell–derived 3D organoids using molecular markers of basal keratinocytes and differentiated suprabasal keratinocytes in the earlier-described studies.[12,105,107,126] Because the most proliferative and undifferentiated basal-like (or basaloid) keratinocytes are present in the outmost cell layer of 3D organoid structures and less proliferative differentiated cells represent the inner cell mass, one caveat of this model system is that the former does not migrate toward the center of the 3D structure to become the latter, unlike generation of the differentiation gradient in vivo or in OTC. Namely, the proliferative stem/progenitor cells form first the inner cell mass, which undergoes terminal differentiation because organoids grow in an outward fashion, and, thus, the outmost cell layers represent the cells that were generated last.

OTC also was used to show that putative esophageal stem cells are capable of reconstituting stratified squamous epithelia in vitro[107]; however, a limitation of the use of OTC for the assessment of esophageal stem cells is that this technique fails to generate single-cell–derived 3D structures. Esophageal epithelial cell proliferation and differentiation have been characterized extensively along with their regulatory signaling pathways in OTC using either primary human esophageal keratinocytes or telomerase-immortalized cell lines.[41,47,48] Esophageal keratinocytes engineered to overexpress epidermal growth factor receptor (EGFR) showed hyperproliferation in an EGFR tyrosine kinase activity–dependent manner in OTC.[47] This study showed that EGFR overexpression induces translocation of p120 catenin to the cell membrane, which mediates epithelial cell–cell adhesion.[47] Among downstream effectors of EGFR is the phosphatidylinositol 3-kinase/AKT signaling pathway.[127] In OTC, EGFR overexpression led to AKT activation via phosphatidylinositol 3-kinase. Although inducible activation of AKT increased cell size concurrent with a decreased level of keratohyalin granules, a marker of terminal differentiation, AKT activation did not increase esophageal cell proliferation in OTC.[54] EGFR signaling regulates the cell cycle by inducing cyclin D1, a key G1 cyclin.[128] Cyclin D1 was found to be responsible for basal keratinocyte proliferation because tetracycline-inducible cyclin D1 overexpression resulted in basal cell hyperplasia

in OTC.[58] The transcription factor KLF4 facilitates esophageal differentiation via activation of noncanonical Wnt5A signaling.[114]

Notch signaling plays a critical role in esophageal epithelial cell fate decisions. In studies using OTC coupled with RNA interference, genetic or pharmacologic pan-Notch inhibition showed that activated Notch signaling drives terminal differentiation by transcriptionally activating early differentiation markers such as involucrin and cytokeratin K13 in a Notch3-dependent manner.[41,59] These observations in OTC were recapitulated in genetically engineered mice with esophageal epithelium-targeted pan-Notch inhibition showing Notch3 down-regulation and impaired terminal differentiation.[41] In our recent attempt to dissect Notch signaling in normal human esophageal 3D organoids, a similar approach confirmed the role of Notch signaling in basal keratinocyte exit toward terminal differentiation.[130] Thus, the OTC and 3D organoid models may complement studies in genetically engineered mouse models.

Impaired Epithelial Homeostasis and Barrier Defect Modeled in 3D

A variety of stressors in the tissue microenvironment influence esophageal homeostasis. Little has been explored as to how esophageal epithelial cells respond to these stressors in 3D culture. In esophageal 3D organoids, we recently investigated epithelial response to oxidative stress induced by alcohol and its toxic metabolite acetaldehyde or inflammatory cytokines. These stressors were found to induce autophagy, a homeostatic cytoprotective mechanism to decrease oxidative stress, recapitulating epithelial changes observed in mice subjected to excessive alcohol drinking in the presence of dysfunctional Aldh2, a mitochondrial acetaldehyde metabolizing enzyme[129] or EoE-like esophageal inflammation.[126]

An epithelial barrier defect has been implicated in the pathogenesis of esophageal diseases including GERD and EoE. Squamous epithelial stratification is less mature and suboptimal in OTC lacking the subepithelial matrix compartment containing fibroblasts,[53] underscoring the cross-talk between stromal fibroblasts and epithelial cells in epithelial homeostasis.[121] Interestingly, OTC-like air–liquid interface was used in the absence of fibroblasts to study the role of desmoglein-1, calpain 14, and LRRC31 in epithelial barrier functions.[17,122,123] In these studies, genetic modulations of these molecules resulted in impaired epithelial stratification as corroborated by altered transepithelial resistance or dextran flux,[17,122,123] however, the absence of fibroblasts in the OTC-like system may have potentially exaggerated the observed barrier defects because the reconstituted epithelia contained much fewer regular basal cells than the typical OTC with fibroblasts.

Modeling Inflammatory Disease Conditions in 3D

Inflammation is pertinent to multiple esophageal pathologies including GERD, EoE, radiation-induced esophagitis, as well as preneoplastic and neoplastic conditions; however, modeling inflammation in 3D culture systems remains largely unexplored. Laczko et al[119] created an OTC esophagitis model by incorporating human peripheral blood mononuclear cells and exogenous cytokines into OTC, recapitulating the T-helper cell type 1 acute inflammatory response found in human GERD. Indeed, esophageal epithelial cells showed aberrant proliferation, differentiation, oxidative stress, DNA damage, and apoptosis in response to peripheral blood mononuclear cells and stimulation by cytokines in OTC.[119]

EoE is characterized by long-term inflammation mediated by eosinophils and other immune cell types that lead to substantial tissue remodeling affecting both epithelial and subepithelial stromal compartments. In EoE, esophageal epithelial cells show basal cell hyperplasia, which features not only expansion of undifferentiated basal keratinocytes but morphologic and functional changes such as epithelial-mesenchymal transition (EMT),[120,121,131] contributing to the inflammatory milieu as well as decreased epithelial barrier functions. In the stromal compartment of EoE, activation of myofibroblasts leads to fibrostenotic disease, the most serious functional consequence in EoE inflammation. These alterations in keratinocytes and fibroblasts have been hypothesized to create a unique epithelial-stromal cross-talk mediated by EoE-relevant cytokines. We have used OTC to investigate the role of transforming growth factor-β1, tumor necrosis factor-α, and interleukin-1β as essential mediators in EMT and myofibroblast activation in the context of EoE to corroborate their molecular profiling of endoscopic biopsy specimens from EoE patients.[120,121] Cheng et al[109] tested the effect of esophageal fibroblasts from EoE patient biopsy specimens upon epithelial stratification in OTC.

The role of cytokines in EoE has been evaluated in a 3D organoid system in which transforming growth factor-β1, tumor necrosis factor-α, and interleukin-13 stimulated the expansion of basal cells in single-cell–derived murine esophageal 3D organoids, recapitulating reactive epithelial changes and basal cell hyperplasia as found in human EoE biopsy specimens.[126] We envision the field moving toward optimized 3D organoid culture to allow expansion and passaging of human and murine organoids from single cells. Such protocols will allow for generation of organoids from patients with normal mucosa, GERD, and EoE, as well as correlation with human pathology found on biopsy specimens. Our group recently optimized 3D organoid culture conditions to allow expansion and multiple passages of both murine and human esophageal epithelial single-cell–derived organoids in a common and simplified culture medium (Kasagi et al, CMGH in press). In this study, human esophageal organoids were generated from biopsy specimens of patients with normal mucosa, GERD, and EoE. These 3D organoids showed hyperplasia in the presence of tumor necrosis factor-α or interleukin-13, indicating that reactive epithelial changes in situ may indeed occur in response to the inflammatory milieu.

Modeling Neoplastic and Preneoplastic Conditions in 3D

ESCC and EADC, 2 major esophageal cancers, and their precursor lesions have been modeled in 3D OTC while 3D organoid models of esophageal cancers are emerging (Table 1). Both OTC and 3D organoids permit morphologic assessment of a broad range of cancer cell characteristics, including proliferation (Ki67),[59] DNA damage (γH2AX),[129] epithelial-mesenchymal transition (E-cadherin and vimentin),[58,59] and CSC markers (eg, CD44),[116] in conjunction with genetic or pharmacologic modifications of cancer cells and fibroblasts. In OTC, pharmacologic treatment is not selective for either cancer cells or fibroblasts, although genetic modifications (eg, RNA interference or tetracycline- or tamoxifen-inducible systems) can be performed in a cell type–specific manner.[49,53,54,59] Because cancer cells invade collectively or individually, cell invasion is evaluated quantitatively by measuring the area of invasive tumor islands within the stromal compartment.[49,53] Laser capture microdissection can be used to analyze molecular changes in invasive cells.[56,57] Moreover, conditioned media can be used for protein analyses to determine growth factors and cytokines that may influence cancer cell invasion and other activities.[49]

ESCC and Squamous Dysplasia

ESCC develops from squamous dysplasia as a common histologic precursor lesion.[132,133] Studies on ESCC using 3D organoids are emerging, in which our group has established protocols to generate esophageal 3D organoids from murine ESCC and precancerous lesions.[105] By using cell-lineage traceable transgenic mice, we have documented that ESCC cells arise from basal keratinocytes. Moreover, 3D organoids from conditional *Notch1* knockout mice showed the role of Notch1 in EMT in premalignant and advanced ESCC lesions.[105] In human ESCC patients, 3D organoids from diagnostic biopsy specimens may be used for molecular profiling as well as prediction of chemotherapy and radiation sensitivity, thus having a translational potential for personalized medicine.

OTC has served as a robust platform to study malignant transformation of esophageal keratinocytes and invasive disease progression of ESCC (Table 1). Multiple ESCC cell lines show invasive growth in OTC.[49,52,108,124] Common genetic lesions in ESCC include inactivation of the p53, p120 catenin, and $p16^{INK4A}$ tumor-suppressor genes, as well as overexpression of the cyclin D1 and EGFR oncogenes.[134] Our genetically engineered mouse models of ESCC, which include targeting of EGFR and cyclin D1 to esophageal epithelium coupled with or without chemically induced carcinogenesis, show esophageal epithelial hyperplasia[47] and dysplasia,[134,135] respectively. ESCC develops in cyclin D1 transgenic mice upon p53 loss.[136] These in vivo approaches now have been coupled with ex vivo 3D organoids and complemented with OTC in vitro.

EPC2-hTERT derivatives show hyperplasia (eg, EPC2-hTERT-EGFR,[47] EPC2-hTERT-cyclin D1[59]), dysplasia (eg, EPC2-hTERT-$p53^{R175H53,59}$), and tumorigenicity, as well as the invasive growth properties of ESCC (eg, EPC2-hTERT-EGFR-$p53^{R175H53,59}$ and EPC2T[58,105,116]) in OTC, depending on their genotypes. In particular, concurrent EGFR overexpression and p53 mutation appeared to be necessary for malignant transformation and invasive growth of human esophageal keratinocytes.[53,136] Growing in a concentric manner, invasive tumor cell nests within the stromal compartment of OTC often show central cornification reminiscent of keratin pearl,[53,56,58,59] a hallmark of well-differentiated ESCC. Similar keratinization is seen in ESCC 3D organoids.[105]

Laser capture microdissection in OTC and gene expression profiling showed unique molecular signatures at the onset of invasive growth of tumorigenic cells, but not premalignant nontumorigenic cells.[56–58,137] Critical molecules identified and studied in OTC related to early neoplastic changes and ESCC cell invasion include markers for EMT, matrix metalloproteinase-9,[53] periostin,[56,113] p120 catenin,[111] c-Met,[49] IGFBP3,[57] STAT1,[113] Wnt10A,[114] Notch,[59,138] and activin A.[115] In addition, OTC showed cancer cell heterogeneity with distinct growth and invasive characteristics defined by distinct CD44 isoforms, EMT, and antioxidant capacity.[116,138] Moreover, fibroblasts were found to support ESCC cell invasion depending on fibroblast AKT activity[53] as well as hepatocyte growth factor, the ligand for c-Met.[49] Treatment of ESCC cells in OTC with bortezomib, a 26S proteasome inhibitor, induced apoptotic cancer cell death via concurrent activation of the p38 mitogen-activated protein kinase pathway.[124]

Barrett's Esophagus and EADC

Barrett's esophagus, intestinal metaplasia in the esophagus, has been linked to EADC development.[139] Replacing normal squamous epithelium with columnar epithelium with mucin-filled goblet cells proposed theories regarding the BE cell origin include BE stem/progenitor cells residing at the squamous columnar junction, residual embryonic stem cells, transdifferentiation of esophageal keratinocytes, and esophageal glands.[14]

Recent studies using 3D culture systems have provided additional insights. Yamamoto et al[140] induced goblet-like cells via the air–liquid interface with BE biopsy-derived colony-forming cells. Sato et al[94] generated human BE tissue–derived organoids under conditions including pharmacologic Notch inhibition to promote secretory cell lineage differentiation whereas addition of fibroblast growth factor-10 permitted long-term passage of BE-derived organoids. Lee et al[141] showed that cholecystokinin-2–receptor expressing cardia progenitor cells may give rise to intestinal metaplasia and dysplasia in response to hypergastrinemia in murine cell–lineage tracing experiments coupled with organoid formation assays. Jiang et al[142] identified unique p63-positive transitional basal cells expressing cytokeratins K5 and K7 as a putative BE cell of origin along with functional validation in 3D organoid assays. In addition, von Furstenberg et al[143] used a porcine model of epithelial injury and human tissues to identify 2 distinct esophageal submucosal gland–derived cells expressing p63 or K7 that

give rise to squamous and ductal 3D spheroid structures, respectively.

BE-derived cell lines have been characterized in OTC where all-*trans*-retinoic acid was found to influence the phenotypic switch from squamous-like multilayered epithelial cells to columnar epithelial cells.[125] In addition, inflammatory mediators such as interleukin-1β and COX-2 have been implicated in BE development. COX-2 overexpression resulted in the development of intestinal mucin-filled epithelia.[118] OTC also has been used to study potential transdifferentiation of cell types. By using esophageal keratinocytes in OTC, Stairs et al[55] found that transdifferentiation may be regulated by Cdx1 homeodomain transcription factor and the c-Myc pathway, which cooperate to promote mucin production and changes in keratin expression, recapitulating gene expression profiles in BE. Moreover, genetic or pharmacologic inhibition of Notch appeared to facilitate transdifferentiation of esophageal keratinocytes toward columnar-like cell phenotypes in OTC with concurrent expression of Cdx1 and c-Myc.[60] Wang et al[62] have used the in vivo transplant culture system to show that hedgehog signaling promotes esophageal embryogenesis and BE development in the esophageal squamous epithelial cells in response to acid-mediated injury in which hedgehog activates transcription factor Forkhead Box A2 to induce intestinal mucin MUC2 and MUC2 processing protein AGR2. Aberrant hedgehog activation in keratinocytes leads to hyperproliferation in OTC,[144] warranting investigating the role of hedgehog in esophageal OTC in the presence of exposure to acids.

To date, the 3D organoid has not been used to characterize EADC cells; however, OTC has been used to document mucin production and the invasiveness of several EADC cell lines (OE19, FLO-1, and MDF-1),[50,51,108] in which the role of cancer-associated fibroblasts expressing periostin via the phosphatidylinositol 3-kinase was evaluated. Cancer-associated fibroblasts promote invasion via fibroblast-derived periostin.[51] OTC served as a testing platform for molecularly targeted therapeutics including EGFR,[110] mutant p53,[117] and PIK3CA.[112]

Conclusions and Future Perspectives

Esophageal 3D culture systems including OTC and the organoid system have provided substantial molecular and mechanistic insights into a number of physiological and pathologic conditions. Addition of immune cells and potentially other nonepithelial components in epithelial-fibroblast co-culture–based OTC may open new avenues of research and preclinical drug testing. Single-cell–derived 3D esophageal organoids may provide sustainable resources for functional and mechanistic investigations into esophageal physiological and pathologic conditions with applications for both basic research and personalized medicine; however, further optimization may be required to permit 3D organoid growth from esophagi with normal and differential disease conditions (eg, ESCC and EADC). The necessity of differential culture conditions for different species, in particular human and murine esophageal 3D organoids, remains to be elucidated. It also needs to be determined how faithfully organoids mimic the genetics, epigenetics, and biology of the originating tissues after extended ex vivo culture. Developing an organoid bank of patient-derived esophageal 3D organoids may facilitate discovery and validation of novel translational applications in the setting of precision medicine, including testing chemotherapy and radiation therapy in a moderate-to-high throughput manner for neoplastic organoids. Unlike neoplastic conditions, reactive epithelial changes in conditions such as GERD and EoE may be normalized ex vivo. Given disease susceptibility, polymorphic loci such as *Aldh2* for esophageal carcinogenesis and *Tslp* for EoE, 3D esophageal organoids may be used to determine individual esophageal epithelial sensitivity to environmental toxic agents such as alcohol metabolites, tobacco smoke constituents, as well as disease-specific inflammatory cytokines. The influence of other environmental factors such as microbiota (bacteria, fungi, and viruses) and their interactions with antibiotics and probiotics upon epithelial biology may be tested using 3D esophageal organoids.

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Conflicts of interest
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