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ORIGINAL RESEARCH

Porcine Esophageal Submucosal Gland Culture Model Shows Capacity for Proliferation and Differentiation

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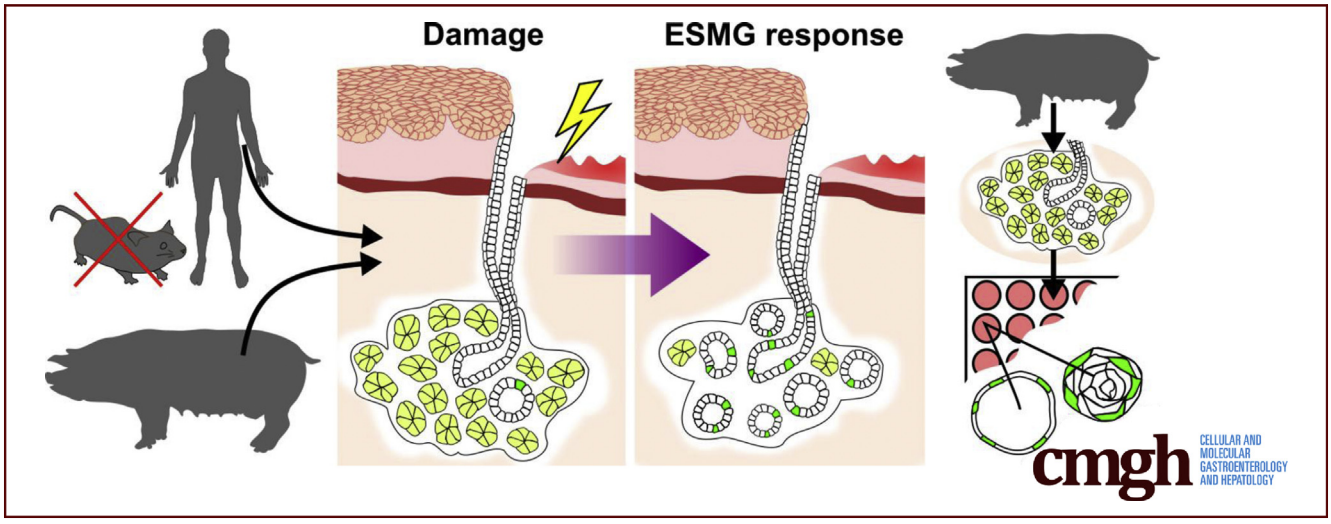

SUMMARY

We describe a novel porcine 3-dimensional culture model that reproduces esophageal submucosal gland proliferation in vivo associated with cancer and injury. Esophageal submucosal glands in culture form 2 different phenotypes of spheroids: one expressing markers of squamous epithelium and the other expressing markers of columnar epithelium.

BACKGROUND & AIMS: Although cells comprising esophageal submucosal glands (ESMGs) represent a potential progenitor cell niche, new models are needed to understand their capacity to proliferate and differentiate. By histologic appearance, ESMGs have been associated with both overlying normal squamous epithelium and columnar epithelium. Our aim was to assess ESMG proliferation and differentiation in a 3-dimensional culture model.

METHODS: We evaluated proliferation in human ESMGs from normal and diseased tissue by proliferating cell nuclear antigen immunohistochemistry. Next, we compared 5-ethynyl-2′-deoxyuridine labeling in porcine ESMGs in vivo before and after esophageal injury with a novel in vitro porcine organoid ESMG model. Microarray analysis of ESMGs in culture was compared with squamous epithelium and fresh ESMGs.

RESULTS: Marked proliferation was observed in human ESMGs of diseased tissue. This activated ESMG state was recapitulated after esophageal injury in an in vivo porcine model, ESMGs assumed a ductal appearance with increased proliferation compared with control. Isolated and cultured porcine ESMGs produced buds with actively cycling cells and passaged to form epidermal growth factor–dependent spheroids. These spheroids were highly proliferative and were passaged multiple times. Two phenotypes of spheroids were identified: solid squamous (P63+) and hollow/ductal (cytokeratin 7+). Microarray analysis showed spheroids to be distinct from parent ESMGs and enriched for columnar transcripts.

CONCLUSIONS: Our results suggest that the activated ESMG state, seen in both human disease and our porcine model, may provide a source of cells to repopulate damaged epithelium in a normal manner (squamous) or abnormally (columnar epithelium). This culture model will allow the evaluation of factors that drive ESMGs in the regeneration of injured epithelium. The raw microarray data have been uploaded to the National Center for Biotechnology Information Gene Expression Omnibus (accession number: GSE100543). *(Cell Mol Gastroenterol Hepatol 2017;4:385–404; http://dx.doi.org/10.1016/j.jcmgh.2017.07.005)*

Keywords: Esophagus; Barrett's Esophagus; 3D Culture; Acinar Ductal Metaplasia; Adult Stem Cell.

Esophageal submucosal glands (ESMGs) are composed of mucous-secreting clusters of cells located within the esophagus beneath the muscularis mucosa. The ESMGs serve a protective role in the esophagus by producing mucins, bicarbonate to neutralize acid, and growth factors, such as epidermal growth factor (EGF). The presence of ESMGs and ducts has been used to anatomically define the tubular esophagus because they are specific to the esophagus and absent from the stomach.[1] Ducts draining the ESMGs are lined by basaloid squamous epithelium and may contain a layer of columnar cells that is either simple or ciliated.[1] Although there is little proliferation of cells within healthy ESMGs in an uninjured esophagus,[2] evidence exists from other glandular gastrointestinal tissues that analogous glands harbor a reserve stem or progenitor cell compartment; this suggests that ESMGs also may be able to respond to esophageal injury.[3] Appropriate ESMG and duct model systems are lacking, however, and, as a result, little is known about the development of the ESMGs and their potential for proliferation and differentiation after injury to the esophageal epithelium.

In human disease, abnormal repair after esophageal injury may result in the development of an intestine-like columnar epithelium known as Barrett's esophagus (BE) rather than normal squamous epithelium.[4] BE is clinically important because of its association with esophageal adenocarcinoma (EAC), a particularly deadly cancer with an overall 5-year survival rate of less than 20%.[5,6] Despite this, the cell of origin for BE remains unknown. Histology shows a close association between ESMGs, their ducts, and overlying epithelium; ESMGs and ducts are present beneath both BE and squamous epithelium.[1] In esophageal resection specimens from human beings, Coad et al[7] described frequent evidence of glands and ducts beneath BE as well as direct histologic continuity of all examined squamous islands with an underlying gland duct. Other histologic studies have shown clusters of ESMGs beneath squamous islands within areas of BE.[8] ESMGs also were identified in patients with BE at the junction between the proximal squamous epithelium and the BE.[8] Importantly, studies of clonality in the esophagus found that a p16 mutation present in a squamous duct from an ESMG also was present in contiguous BE, whereas squamous islands in BE were contiguous with wild-type ESMG ducts.[9] In addition to the notable clonality assay linking ESMG ducts with columnar epithelium, using a different approach, culture of whole human biopsy samples of squamous epithelium and underlying ESMGs showed loss of squamous mucosa and fusion of ESMGs with the surface of the biopsy sample with generation of a single-cell columnar mucosa at 48 hours.[10] The in vivo clonality studies and these short-term whole-biopsy culture findings suggest a role of ESMGs in both repair of squamous epithelium as well as pathogenesis of BE.

Other evidence for a potential role for ESMGs in esophageal epithelial repair comes from an altered histologic appearance of ESMGs in association with esophageal ulcer and esophageal cancer. Specifically, our group has described acinar ductal metaplasia within ESMGs in association with both esophageal injury and esophageal cancer.[11] In acinar ductal metaplasia, rather than containing the mucin-producing acini that characterize normal ESMGs, groups of cells within ESMGs assume a dilated ductal appearance and express the ductal marker cytokeratin 7 (CK7). In other organs such as the pancreas, acinar ductal metaplasia is considered an early event in the progression to cancer.[12,13] However, the functional relationship between acinar ductal metaplasia in ESMGs and the development of BE and EAC is unknown. Similarly, little is known about potential stem cell populations within the protected niche of the ESMGs.

A major limitation to the study of ESMGs has been the lack of traditional rodent models because ESMGs are not found in the mouse esophagus.[14] As a consequence, what is known about ESMGs has resulted from human and atypical animal models. Human studies have shown that at baseline, there appears to be little proliferation of cells within healthy ESMGs. In human patients who were administered the thymidine analog bromodeoxyuridine an hour before undergoing esophagectomy, proliferating cells were identified in the squamous epithelium.[2] The proliferative response of the ESMGs and ducts in the context of esophageal injury has not yet been reported in human beings. However, there was no evidence of proliferation within ESMGs in the esophagus under normal conditions.[2] A similar study identified iododeoxyuridine-positive cells in the basal layer of the squamous epithelium and in the base to the midgland of BE.[15]

In an attempt to bridge the gap between various histologic observations of ESMGs and behavior in vitro, we developed a porcine 3-dimensional (3D) culture model of ESMGs that allows investigators to directly study the proliferative ability of ESMGs. We hypothesized that ESMGs contain reserve progenitor cells that become proliferative after damage and can generate both columnar and squamous epithelium. To this end, we first identified proliferation in human ESMGs in the context of acinar ductal metaplasia. Proliferation in the porcine 3D organoid model then was compared with proliferation in vivo after esophageal injury. Furthermore, in the in vitro system, we identified 2 phenotypes of outgrowths from ESMGs and evaluated these for similarities to the known esophageal epithelial types: normal squamous epithelium and columnar epithelium. ESMG spheroids were further characterized

Abbreviations used in this paper: **ANOVA, analysis of variance; BE, Barrett's esophagus; CK7, cytokeratin 7; DMSO, dimethyl sulfoxide; EAC, esophageal adenocarcinoma; EdU, 5-ethynyl-2′-deoxyuridine; EGF, epidermal growth factor; ESMG, esophageal submucosal gland; IHC, immunohistochemistry; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; RFA, radiofrequency ablation; 3D, 3-dimensional.**

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