

**Using *In Vitro* Modeling of Esophageal Epithelium to Investigate
Biological Determinants of Racial Disparities in Gastric Reflux Response**

By

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Disclaimer

We must be careful about how we, as the biomedical community, use race, as a socially constructed categorization, in researching population differences on a genomic level. Furthermore, the categories that we classify as distinct races are designated by socially constructed boundaries along the continuous spectrum of variation between populations, and there exist no essentialist biological features that necessitate membership to any given racial group. Here I am using self-identified race as a proxy for geographic origin of ancestral lineage in hopes that this will help identify genetic features that are more prevalent in certain populations for further investigation, purely as a starting point in elucidating patterns. However, identification of differences in polymorphic distribution between populations should not then be used as justification for differential treatment of all members of a socially constructed category. Instead studies of this nature should be used to inform our understanding of the biological systems at play to more effectively tailor treatments at the individual level based on the genotypic and phenotypic profile of each patient independent of their racial identification. I hope that this research can contribute to the full picture of how racial disparities have emerged in our healthcare system, and make more apparent the biologically based strategies we can utilize alongside sociological research to combat inequalities and provide optimal care for every individual patient, especially those who have been previously harmed and disregarded in medicine.

For further information on the academic debate regarding racial classification I recommend the Stanford Encyclopedia of Philosophy's page on Race, as it addresses biological, sociological, and philosophical perspectives on the issue. <https://plato.stanford.edu/entries/race/>

Abstract

Purpose

Gastroesophageal reflux disease (GERD) is an extremely common condition in North America, with an incidence estimated to be as high as 18.1-27.8%. Chronic reflux is harmful to the exposed epithelial tissue of the lower esophagus, with the associated damage linked to increased risk of developing the metaplastic condition known as Barrett's esophagus (BE), which can progress into esophageal adenocarcinoma (EAC) through a process of progressive dysplasia. Strikingly, European Americans (EA) develop EAC at four times the rate of African Americans (AA). While social determinants of health play prominent roles in health disparities, it is likely that biological determinants also contribute to this marked difference in disease incidence. Understanding the role of biologic variability in this racial disparity is important to more effectively treat the heterogeneous population of GERD and EAC patients.

Experimental design

To investigate potential contributing factors, we optimized an *in vitro* model for culturing tissue from patient-derived esophageal epithelium. Single cell RNA sequencing and immunofluorescence were used to characterize molecularly distinct populations of cells within the native esophageal epithelium. Using these measures, cell populations in the cultured system were compared to cell populations *in vivo*. Cultured tissues from racially diverse patients were treated with bile acid and HCl to mimic reflux and assayed for changes in cell morphology as determined by an automated imaging protocol, DNA damage as measured by γ H2AX foci, proliferative response based on cell density, and expression of ECAD and TP63 as assayed by fluorescence intensity using immunofluorescence.

Results

The native esophagus was characterized into four distinct cellular populations. They were denoted as basal (COL17A1/CAV1/CAV2 positive), supra-basal (LY6D/ KRT4 positive), proliferative (KI67/TP63 positive), or differentiated (CRNN positive) identity. Tissue samples cultured *in vitro* maintained these cell population markers, with low density cultures enriched for the proliferative populations of the basal and supra-basal cell types. However, when cultured at high density the tissue formed polarized epithelium with differentiating KRT4 positive squamous cells. An automated high content morphology screening revealed a greater than ten-fold difference in average IC₅₀ values for BA treatment between EA (130.4 μ M) and AA (1551.7 μ M) samples ($P=0.0083$). Treatment with BA and HCl also produced significantly more DNA damage in EA samples, with an average of 7.45% of AA cells showing γ H2AX foci, whereas EA samples on average presented with 19.51% damaged cells ($P=0.008$). Lastly, EA cells treated with acid conditions induced a 1.61-fold increase in cell density four days post treatment, which differed significantly from the 0.65-fold change for the AA cells tested ($P=0.0012$). Immunofluorescent assays for ECAD and TP63 expression did not yield a significant difference between reflux treated EA and AA samples.

Conclusion

In summary, The optimized *in vitro* culture system is capable of expanding patient derived esophageal epithelium and modeling the stratified polarity of native tissue. Preliminary assays on the effects of reflux on esophageal cultures revealed different responses in EA samples compared to the AA samples, indicating a difference in tissue susceptibility. While polymorphisms of the

glutathione s-transferase protein GSTT2 have previously been proposed as a potential mediator of this differential response to reactive oxygen species, GSTT2 polymorphisms present in the EA versus AA samples examined did not provide substantial predictive power in explaining response differences. This work highlights a potential role of biological determinants in the differential manifestations of GERD and EAC in racially distinct populations. Further studies can potentially validate biological differences in response to reflux between AA and EA populations and determine the specific contributions of GSTT2 to disease incidence.

Introduction

The role of the esophagus in the transport of food to the digestive system and proximity to corrosive gastric contents endows significant risk of injury to the esophageal epithelium. The distal esophagus is protected from gastric contents by the lower esophageal sphincter at the gastroesophageal junction. This sphincter does not create a perfect barrier, however, and gastric contents can reflux back into the lower esophagus. Chronic seepage of gastric contents into the lower esophagus is called gastroesophageal reflux disease (GERD). Risk factors for GERD include genetic predispositions but also lifestyle variables such as diet, smoking, and physical activity (Jarosz and Taraszewska, 2014; Yamasaki et al., 2018). GERD rates have been rising in North America, likely due to the increasing prevalence of these behavioral risk factors, with current incidence estimated at 18.1-27.8% (El-Serag, 2014).

Chronic exposure to gastric contents can lead to inflammation and tissue damage (Dvorak et al., 2007). As such, severe GERD has been shown to cause erosive esophagitis that increases the risk of developing the metaplastic condition known as Barrett's esophagus (BE), which can progress into esophageal adenocarcinoma (EAC) through a series of dysplasia (Ronkainen et al., 2005, Reid et al., 2010). BE is a metaplastic disorder characterized by the emergence of intestine-like tissue in the lower esophagus (Barrett, 1950). Both GERD and BE have been associated with progression to EAC, with BE in the context of GERD serving as the most important etiological factor for EAC (Anderson et al., 2007; Langergren et al., 1999; Whiteman et al., 2008; Shaheen et al., 2002).

Coinciding with the rising prevalence of GERD, EAC rates have increased more than four-fold since the 1970s (Holmes and Vaughan, 2007; Brown and Devesa, 2002; Hur et al., 2013; Thrift and Whiteman, 2012). EAC and esophageal squamous cell carcinoma (ESCC) comprise the two main classes of esophageal cancer. As ESCC is typically found in the upper esophagus, is not associated with GERD or BE, and has decreased in prevalence in recent decades it will not be further addressed here (Holmes and Vaughan, 2007; Jain and Dhingra, 2017). EAC is typically found in the lower esophagus, between the junction of the stomach and esophagus, and is characterized by glandular formations and mucosal irregularities, with BE foci commonly found around the periphery of the tumor (Jain and Dhingra, 2017). EAC is the sixth most common cause of cancer related deaths worldwide, with five-year survival rates of 45% for localized disease and as low as 5% for metastatic disease (Hur et al., 2013). Because this disease is so aggressive and its prevalence is increasing at an alarming rate it is essential to understand the mechanisms by which EAC develops in these reflux conditions and to identify preventative measures to protect at-risk patients.

Strikingly, self-identified African American populations develop EAC at one quarter the rate of self-identified European Americans (Pickens and Orringer, 2003; Thrift and Whiteman, 2012; Holmes and Vaughan, 2007; Thrift and El-Serag, 2016). The primary EAC risk factors of GERD, obesity, and smoking do not differ significantly between the two racial groups, and do not mitigate the racial disparity in EAC rates (El-Serag and Sonnenberg, 1997; El-Serag et al., 2014; Spechler et al., 2002). Furthermore, African American GERD patients present with erosive esophagitis at roughly half the rate of European American GERD patients, suggesting a difference in tissue response to reflux between the racial groups (El-Serag et al., 2004).

The discussion of disparities in healthcare has become increasingly prominent in recent decades. There is overwhelming evidence that healthcare disparities exist dependently on racial, ethnic, cultural, gender, socioeconomic, and geographic identities (Nelson, 2000; Fiscella et al, 2002). Race and ethnicity have been at the forefront of these discussions given their impact, with systemic discrimination playing a role in health outcomes. The factors that produce and perpetuate these health disparities are extremely complex. The past and present systemic discrimination of racial and ethnic groups heavily contribute to the gap in health outcomes (Williams and Sternthal, 2010). While this systematic persecution and oppression negatively impact mental and physical health in and of themselves (Huang et al., 2006), their consequences, a few of which include lower socioeconomic status and poorer geographic access to healthcare providers, further contribute to reduced rates of diagnostic and therapeutic procedures (Williams, 1996; Baicker et al, 2006; Newacheck et al, 1996; Escarce and Puffer, 1997). However, even when income, age, and education are controlled for, prevalence and mortality of prominent diseases such as diabetes, cancer, heart disease and maternal mortality rates are still significantly higher among racial/ethnic minority groups (Nelson, 2000; Fiscella et al, 2002; Anachebe, 2006; An et al., 2012).

Inequalities and bias still exist within our healthcare systems that can impact the quality of care that minority groups receive, further exacerbating the disparities in disease prevalence and outcome (Egede, 2006; Nelson, 2000; Fiscella et al, 2002). For instance, black individuals with severe pain are prescribed pain relief medication less frequently than other racial groups (Hoffman et al., 2016). As such, it is clear that access to healthcare is not the only issue. These

biases and discrimination have generated mistrust of the healthcare system, along with other negative psychological and sociological associations with healthcare, that have reduced the willingness of marginalized groups historically harmed by medical professionals to seek out medical consultation and treatment (Dovidio et al., 2008; Trivedi and Ayanian, 2006; Williams and Sternthal, 2010). These factors are all extremely important and must be addressed in order to achieve equitable care for all patients.

Another feature that complicates the issue of racial disparities in the manifestation and outcome of disease is the possible existence of biological determinants of these differing physiological responses between populations with different geographic origins. It is becoming increasingly clear that the prevalence of genetic polymorphisms in patient populations can underlie differences in the magnitude of risk or response to disease and treatments (Mattei et al., 2009). This has led researchers to investigate whether polymorphic distributions between groups with different geographic origins, which socially constructed categories of race can aid in inferring, can explain disparities in patient incidence and outcomes, especially in diseases that are heavily dictated by genetic factors. For instance, increased incidence of childhood nephrotic syndrome in African American populations has been closely linked to greater proportions of high-risk alleles of *APOLI* (Varner et al., 2018).

Racial disparities in cancer have been studied extensively (Ellis et al., 2018; Deshmukh et al., 2017). For example, young African American women have a higher incidence of breast cancer than young white women, with increased rates of developing triple negative breast cancer and higher mortality rates (Wheeler et al., 2013; Carey et al., 2006; O'Brien et al., 2010). Tumors of African American patients differ in relevant histological features from tumors of white

patients and are more likely to possess P53 mutations, HRAS1 proto-oncogene overexpression, and dysregulation of cell cycle proteins (Porter et al., 2004; Bowen et al., 2006; Chen et al., 1994). Similar trends have been observed in breast cancer samples of patients in West Africa, with genome-wide association studies identifying enrichment of several high-risk polymorphisms in individuals of African descent (Chen et al., 2013; Newman, 2017).

It should be noted that even if biological determinants contribute to observed racial disparities, they alone can not account for the extent of inequalities present in patient outcomes, and as such should not undermine the importance of the previously discussed social factors. Survival differences still remain when breast cancer subtype and stage are matched between racial groups, and truly equitable care for all patients can not be achieved without considering all aspects of inequality (O'Brien et al., 2010; Masi and Olopade, 2005). Biological determinants should be used to inform drug development and individualized treatment plans for patients (Ortega and Meyers, 2015) alongside continued improvements to the social inequalities that impact health.

Given the known role of genetics in cancer etiology, disease kinetics, and response to therapy, it is probable that biological determinants contribute to the prominent racial disparity among patients. To disentangle the role of biological determinants from other social influences on health, the physiological systems must be observed under controlled experimental conditions. *In vitro* model systems provide a promising means of accomplishing this, as they allow for the analysis of differences between cells and tissue removed from many of the potential confounding environmental factors present in clinical studies.

During my time in the Spence Lab I have worked closely with Daysha Ferrer-Torres, PhD, to optimize an *in vitro* model system for culturing racially diverse patient esophagus samples. We have used this model to examine physiological differences between AA and EA tissue in response to reflux, and have begun investigating the role of the glutathione pathway in mediating this disparity.

Chapter 1 - Modeling Esophagus *In Vitro*

In order to investigate the biological mechanisms of disease and racial disparities in the esophagus, I worked alongside Dr. Ferrer-Torres to establish an *in vitro* model system for culturing racially diverse patient biopsies. Biopsies were dissociated and cultured in the presence of irradiated 3T3-J2 fibroblast feeder cells and a combination of growth factors. We designed culture conditions based on previous discoveries regarding the expansion and maintenance of patient-derived epithelial tissue (Rheinwald and Green, 1975a, 1975b; Liu et al., 2012, 2017; Mou et al., 2016).

Irradiated 3T3 fibroblasts have been prominent feeder cells used for decades to provide extracellular matrix for culturing epithelial tissue (Rheinwald and Green, 1975a, 1975b). The cells constitutively express hyaluronic acid and are suspected to provide juxtacrine and paracrine signals to cultured cells that mimic native epithelium-mesenchyme interactions (Llames et al., 2015). The use of hydrocortisone in combination with 3T3s has also been widely used to promote proliferation and expansion of cultured epithelial cells (Rheinwald and Green, 1975a, 1975b).

Liu and colleagues have previously demonstrated that treatment of biopsied keratinocytes with rho kinase inhibitor (Y-27632) in the presence of 3T3 feeders allowed for the conditional reprogramming (CR) of the keratinocytes into proliferative stem-like cells (Liu et al., 2012, 2017). Unlike iPS cells these CR cells resemble the characteristics of their native basal epithelial cells and maintain an intrinsic capacity to return to a differentiated state of their native tissue. Increases in both BMP and TGF- β signaling are associated with transition from basal

proliferative cells to apical differentiated cells in multiple epithelial tissues. Work by Mou and colleagues revealed that dual SMAD inhibition of both BMP and TGF- β signaling promoted the maintenance and enrichment of proliferative epithelial basal cells in culture (Mou et al., 2016). We hypothesized that the combination of rho kinase inhibition and dual smad inhibition could function synergistically to conditionally reprogram basal-like cell types and subsequently maintain them in culture to efficiently expand patient epithelial tissue.

The establishment of a Wnt gradient is essential to the patterning of the endoderm, with high levels of Wnt signaling required for the formation of the anterior foregut that subsequently gives rise to the mature esophagus (Stevens et al., 2017). Increased Wnt signaling is also observed in the basal proliferative cells of the mature gastrointestinal tract, esophageal epithelium, and other epithelial tissues, with multiple studies defining a critical role of this pathway in tissue homeostasis (Flanagan et al., 2018; Ali et al., 2009; Kretzschmar and Clevers, 2017). This role remains relevant in culture, as the activation of Wnt signaling via the small molecule inhibitor of GSK-3, CHIR99021 (Chiron), induces epithelium proliferation in a variety of *in vitro* model systems (Mou et al., 2016). Lastly, Epidermal Growth Factor (EGF) has also been shown to play a role in proliferation of esophageal epithelium, functioning in general tissue maintenance and wound healing (Jankowski et al., 1992; Qureshi et al., 1997; Takaoka et al., 2010).

Based on these previous findings we optimized a conditioned media containing hydrocortisone, Y-27632 (a rho kinase inhibitor), EGF, Noggin (a natural BMP inhibitor), A-8301 (a synthetic TGF- β inhibitor), and CHIR99021, called HYENAC. During the early

stages of optimization we cultured several different combinations of these growth factors with and without Calf Bovine Serum (CBS), as well as in the presence of collagen, matrigel, 3T3 feeders, or no extracellular matrix. These experiments ultimately demonstrated that HYENAC+CBS with 3T3 feeders was the most effective culturing condition (data from less effective conditions not shown) to rapidly expand tissue, maintain viability for multiple passages, and mimic native tissue.

Results

Characterization of the Native Esophagus

In order to verify the accuracy of our *in vitro* model we first had to identify prominent markers of the native esophageal epithelium. To accomplish this, single-cell RNA sequencing was performed on two patient biopsies. Louvian clustering was used to cluster the data based on their transcriptomic signature (see methods for further details). Seven distinct cell clusters (C0-6) were identified (Figure 1A). The two patient biopsy samples showed similar cluster proportions, with both samples significantly enriched for epithelial cell types (patient 1: C0 41.89%, C1 19.12%, C2 6.47%, C3 13.76%, C4 16.54%, C5 1.41%, C6 0.81%, epithelial cells 81.27%; patient 2: C0 34.21%, C1 23.42%, C2 20.80%, C3 11.28%, C4 7.37%, C5 2.02%, C6 0.90%, epithelial cells 89.71%) (Figure 1B, C). *CDH1* defined clusters 0, 1, 2 and 3 as e-cadherin expressing epithelial cells, separated from the *VIM* expressing cells of the lamina propria (LP) (Figure 1C). Feature plots verified the specificity of the top genes for each cluster and highlighted that the different clusters are molecularly distinct (Figure 1D) For additional resolution, the *CDH1* positive cells were isolated and re-clustered into four groups (Figure 2A). This re-clustering produced four distinct clusters that resembled the epithelial clusters from the

original sample set and maintained similar cluster proportions (Figure 2B). Within each cluster the highest expressing genes were screened for their specificity between clusters as well as the availability of immunofluorescent antibodies, leading to the selection of cluster specific markers (Figure 2C). Immunofluorescence was then used to verify the robust and specific expression of these markers at the protein level in biopsy-resection tissue sections (Figure 2D), and the localization of the cluster markers was used to spatially profile the molecularly distinct zones of the esophageal epithelium (Figure 2E). Additional staining was performed to visualize the transitions between the histologic zones and evaluate the accuracy of TP63 as a marker for the basal and suprabasal proliferative population (Figure 3).

In summary, these analyses define molecularly distinct cellular subtypes within the epithelium of the esophagus. The basal layer of cells is defined by highly localized expression of COL17A1 and Caveolin 1/2. Above the basal layer the suprabasal transitional zone is characterized by the expression of two genes LY6D and KRT4. LY6D expression is present immediately above the basal layer of cells. KRT4 is expressed in a gradient beginning part way through the suprabasal zone and intensifying in higher cell layers. The proliferative population is interspersed within these first two zones, more predominantly among the suprabasal zone, and is characterized by the proliferation markers KI67, TP63, TOP2A, and CDK1. Lastly, the luminal zone contains fully differentiated cells expressing CRNN and CNFN.

Recapitulating Native Esophagus In Vitro

For tissue culture, patient biopsies (Figure 4A) were finely minced and plated with 3T3 feeder cells in HYENAC+CBS media on the day of collection. The pieces of biopsy tissue

attached to the plates and expanded in monolayer colonies that eventually reached confluency (Figure 4B). To investigate whether *in vitro* cultures retained native cell types, scRNAseq was performed after three passages (roughly 30-40 days). Louvain clustering identified five clusters, with clusters 1 and 2 expressing characteristic basal markers, clusters 0 and 3 expressing suprabasal markers, and cluster 4 expressing proliferative markers as determined by the *in vivo* expression analysis (Figure 4C, 5A). The expression of *CDH1* mRNA in 96.6% of sequenced cells (Figure 4D) and prominence of e-cadherin protein in immunofluorescent staining (Figure 4E) verified that cultures were supporting epithelial cells. Quantification of ECAD⁺ cells by immunofluorescence showed that for the seven cell lines tested all had roughly 50-80% of cells with visible ECAD expression, and that this expression persisted across passages (Figure 4F).

Expression of markers from each of the four previously defined zones was apparent in the *in vitro* scRNAseq as seen in feature plots (Figure 5A). Specifically, as in the previously reported culturing protocols where an enrichment of basal stem-like cell types was observed, CAV1 and COL17A1 were expressed in a large proportion of the total sample. These basal cells comprised 50.56% of the total cultured cells (C1 25.56% and C2 25.00%) as opposed to only 25.57% in the biopsies (Figure 4C, 2B). Conversely, the expression of LY6D and KRT4 (marking supra-basal transitional cells) as well as CRNN⁺ (marking luminal cells) were diminished. These findings were validated with immunofluorescent staining of cultured samples in which COL17A1 was expressed in the majority of cells (Figure 5B). The expression of TP63 and KI67 has similarly been used to classify proliferative cells in the basal and suprabasal region. Across four cell lines the proportion TP63/KI67 double positive cells was significantly higher in

the cultured cells at 43.83% compared to the *in vivo* epithelial tissue at 12.21% ($P=0.004$) (Figure 5C, D).

This asymmetric cell type distribution is mitigated when cells are allowed to continue culturing at confluency. After culturing for five days, high density cells, plated at 500,000 cells/well, possessed only 57% of cells expressing both TP63 and KI67, a significantly reduced proportion compared to cells plated at 125,000 cells/well for which every cell was TP63+/KI67+ ($P<0.001$) (Figure 6A, B). This is supported at the mRNA level, for which we see a decrease in KI67 and an increase in CRNN and KRT4 expression in high density cell cultures compared to low density (Figure 6C). The tissue patterning of these high-density cultures also mimics the polarity of native tissue stratification. Three-dimensional immunofluorescent imaging revealed that the basal/suprabasal TP63+ cells localized to the bottom of the tissue whereas the KRT4+ luminal cells localize to the top of the tissue and begin showing stratified squamous-like morphology (Figure 6D). Comparison of DAPI normalized fluorescence intensity of KRT4 between high- and low-density cells further validated the significant increase in KRT4 expression when cells are cultured at confluency (low vs high HT-261 15.58 vs 92.94; HT-253 4.63 vs 112.07; HT-269 1.59 vs 3.06; $P<0.01$ for all 3 samples)(Figure 6E).

All together these analyses demonstrate that our *in vitro* culturing conditions are capable of expanding patient derived esophageal epithelium for multiple passages. The native esophageal epithelium is characterized by basal expression of COL17A1 and CAV1/2, followed immediately by suprabasal expression of LY6D and more apically by KRT4 that extends into the luminal zone which is characterized by CRNN expressing squamous epithelial cells. Proliferative populations

expressing KI67 and TP63 are scattered throughout the basal and suprabasal regions. The *in vitro* culturing conditions maintain expression of each of these regional markers, but enriches for basal proliferative cell types expressing COL17A1, CAV1/2, KI67, and TP63. These basal proliferative cells maintain their differentiation potential as seen by their ability to produce KRT4 positive stratified squamous tissue when allowed to reach confluency. As such, this *in vitro* model is a promising analogue to the epithelium of the esophagus that can mimic native tissue. This model provides a powerful tool for examining tissue physiology and disease in the controlled laboratory setting and can allow for the evaluation of biological differences between cell populations independently of environmental factors.

Chapter 1 Figures

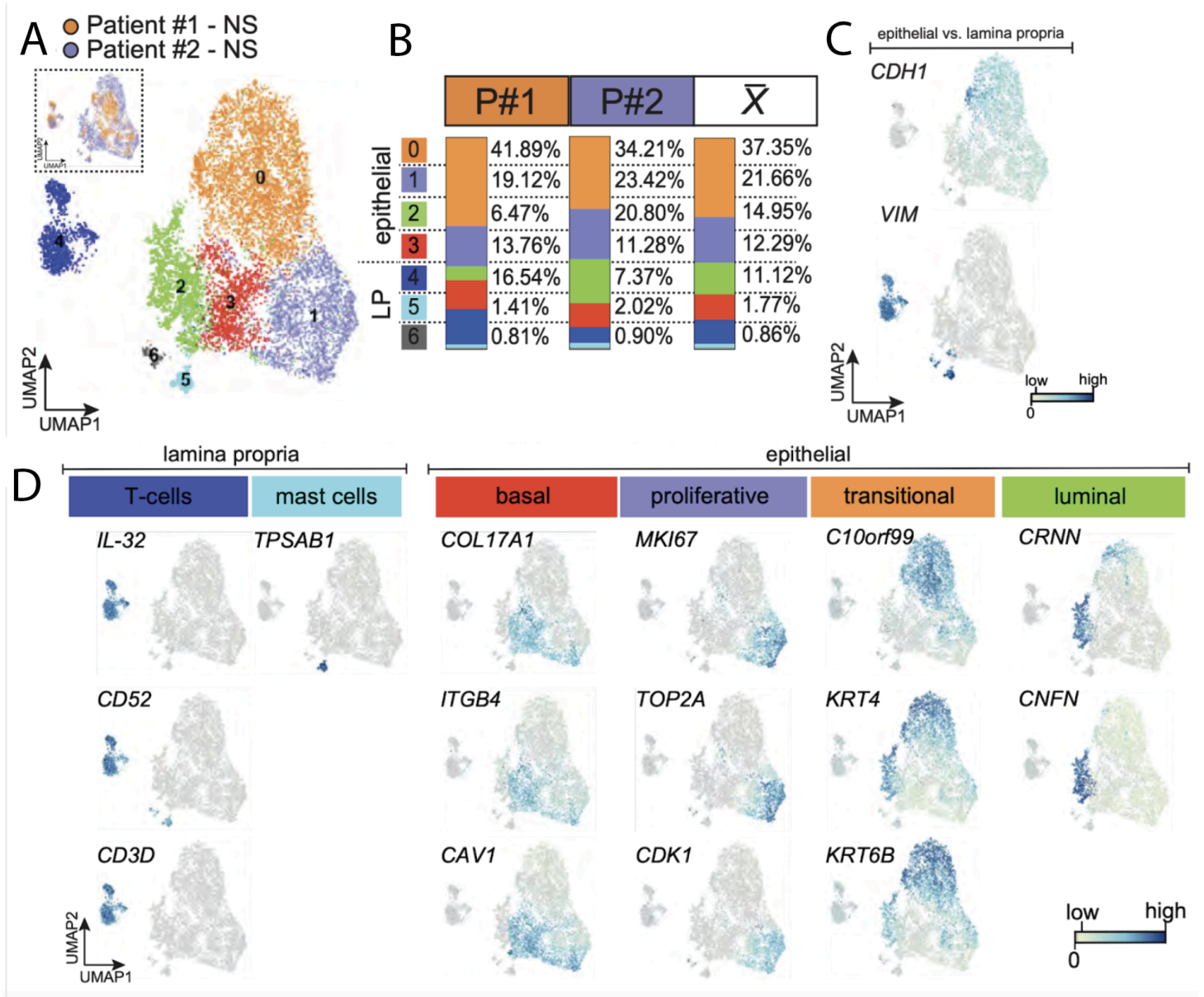


Figure 1. Single cell RNA sequencing of *in vivo* patient biopsies

(A) Two patient biopsies were collected to yield 9,039 cells for sequencing. A total of 3,897 genes were analyzed and cluster identities were determined based on the top 200 genes expressed in each cluster. This analysis produced seven molecularly distinct clusters. (B) Cluster proportions between the two samples were similar and largely enriched for epithelial cell types. (C) Epithelial cells were identified based on *CDH1* expression (clusters 0, 1, 2, and 3), whereas lamina propria (LP) cell types were identified based on *VIM* expression (clusters 4, 5, and 6). (D) Feature plots showing gene expression levels for several defining genes within each cluster.

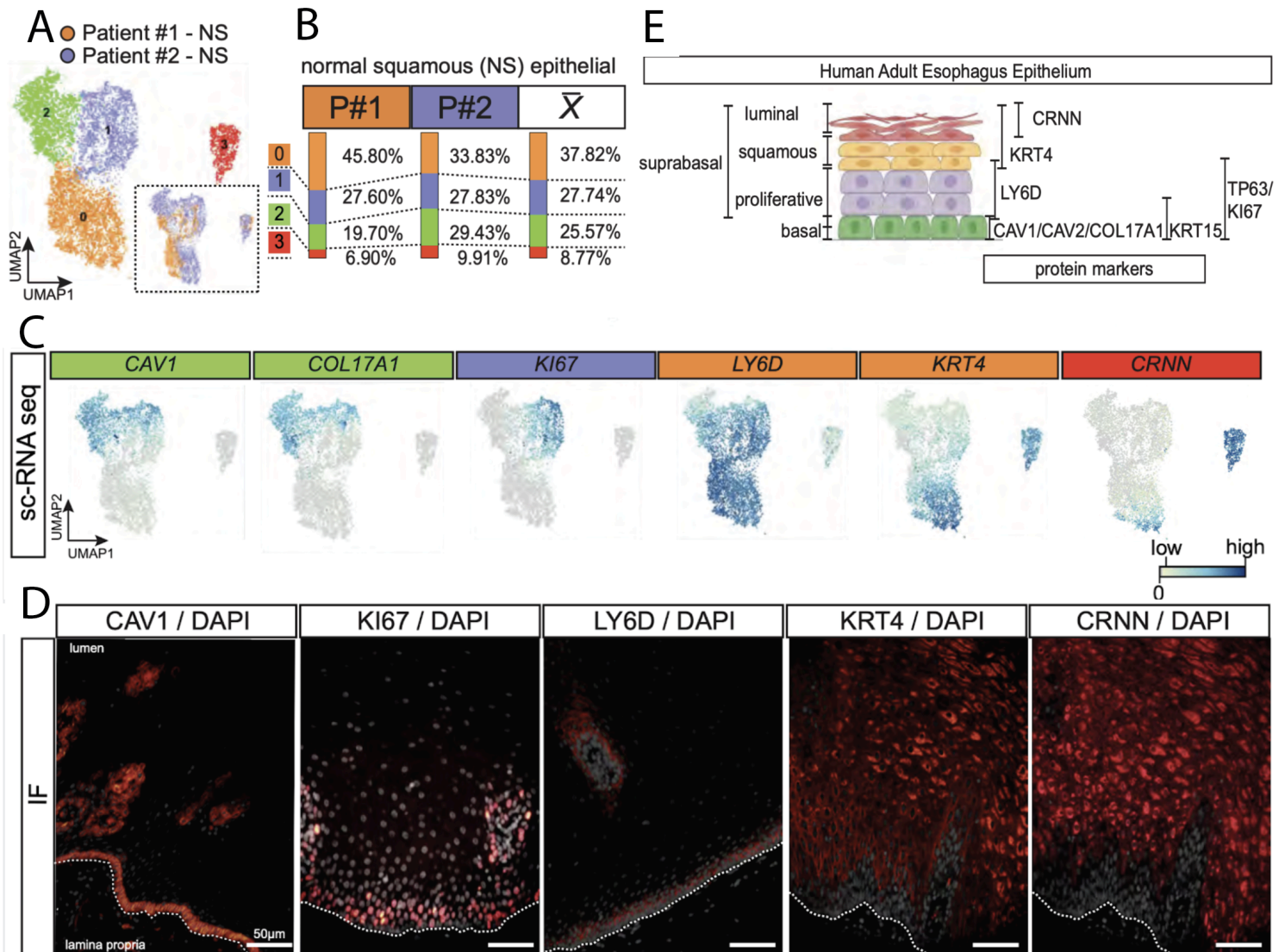


Figure 2. Characterizing zones of the esophageal epithelium

(A) From the samples analyzed in figure 1, epithelial clusters 0, 1, 2, and 3 were isolated and examined separately. This population contained 7796 cells and detectable expression of 2651 genes. Re-clustering of this sample identified four molecularly distinct populations. (B) Cluster proportion remained similar between the two patient samples. (C) Feature plots showing the expression pattern for the top candidate genes within each cluster: CAV1/COL17A1 for Cluster 2, KI67 for Cluster 1, LY6D and KRT4 for Cluster 0, and CRNN for Cluster 3. (D) Immunofluorescent validation for spatial patterning of candidate genes and cluster localization within the esophagus epithelium. CAV1 is expressed at the basal most layer of cells, KI67 expression is scattered throughout the basal and suprabasal zones, LY6D expression begins in the first suprabasal layer while KRT4 begins higher in the suprabasal zone and demarks transition to the early differentiation zone, and CRNN is expressed in the luminal cell layers identifying differentiated cell fates. (E) Schematic representation of the expression zones throughout the layers of the esophagus epithelium.

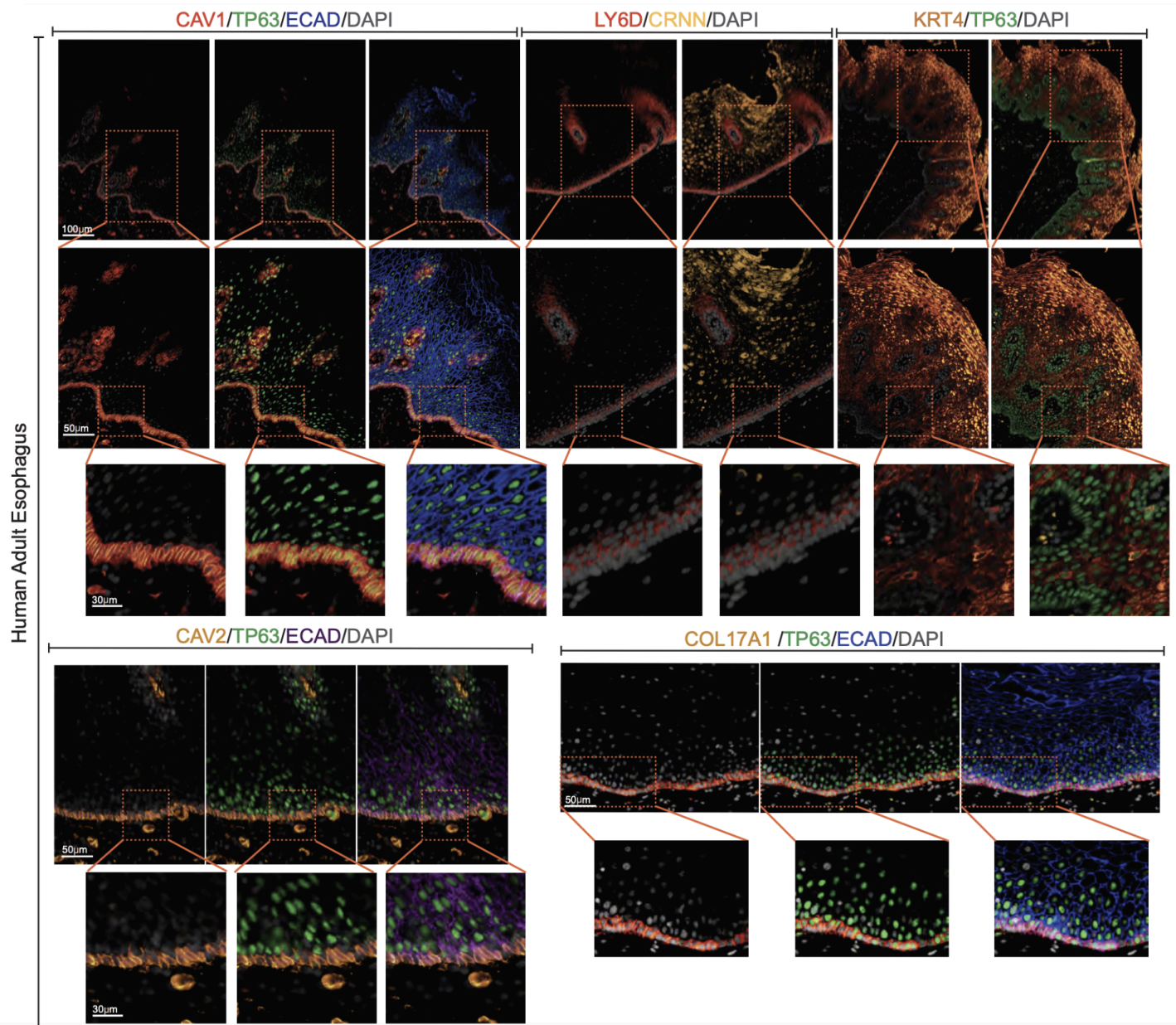


Figure 3. Immunofluorescent visualization of esophagus epithelium zones

Further immunofluorescent validation was performed for the cluster markers evaluated in figure 2 alongside additional marker candidates. CAV2 protein expression showed similar localization to CAV1 and COL17A1 in the restricted basal-most cell layer. LY6D, KRT4, and CRNN showed an overlapping spatial pattern, with LYD6 and KRT4 overlapping in the suprabasal zone and KRT4 and CRNN overlapping in the luminal zone. TP63 was expressed in the basal and suprabasal zones, but also extended up into the KRT4+ transitional zones.

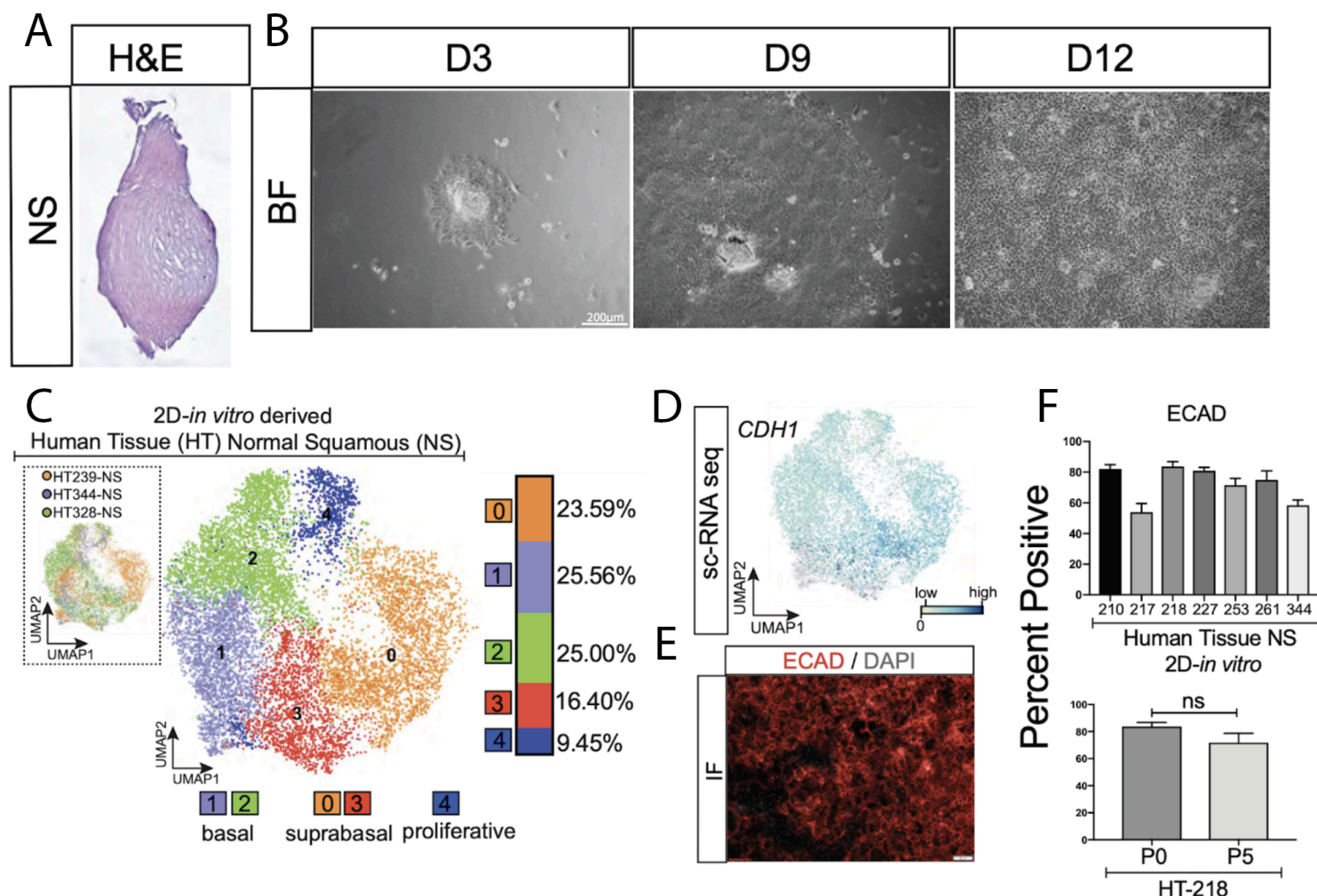


Figure 4. Expanding patient epithelium in vitro

(A) Hematoxylin and Eosin staining of a representative esophagus epithelium biopsy. (B) Bright field images of cultured tissues after biopsy processing (passage number zero) taken 3, 9, and 12 days after being transferred to 6-well plates. (C) Three cultured samples were analyzed by single cell RNA sequencing, with a total of 10550 cells and 4269 genes used for clustering analysis. Cells were grouped into five clusters, with clusters 1 and 2 expressing characteristic basal markers, clusters 0 and 3 expressing suprabasal markers, and cluster 4 expressing proliferative markers. (D) Feature plot showing expression of epithelial marker *CDH1* throughout all of the clusters. (E) Immunofluorescence of ECAD protein expression validating enrichment of epithelial cells in the expansive culturing of patient biopsies. (F) Quantification of ECAD positive cells in seven patient cultures, for which six possessed greater than 50% ECAD positive cells. Furthermore, HT-218 was used to validate that the proportion of ECAD positive cells does not vary significantly between passages.

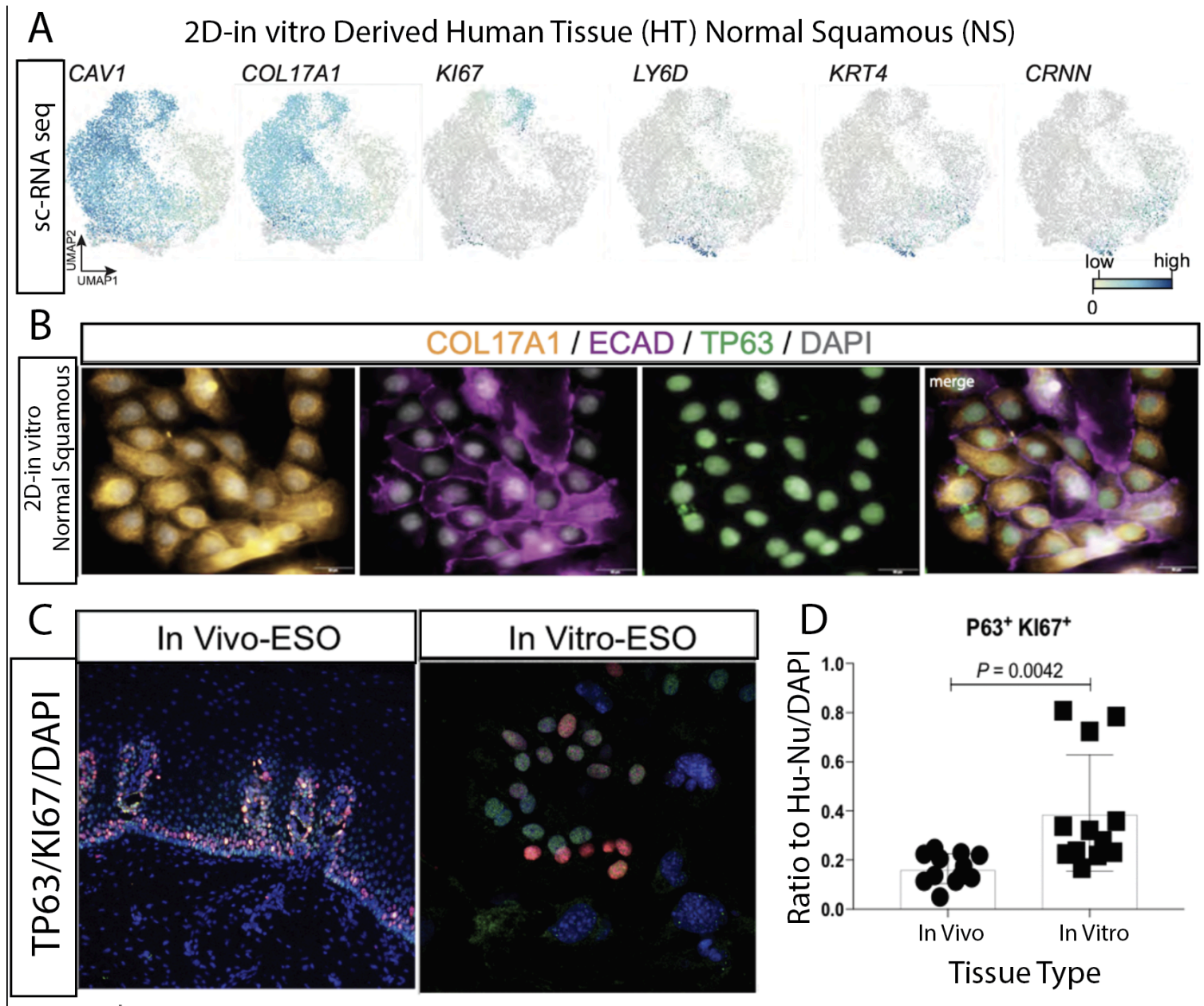


Figure 5. Single cell RNA sequencing clusters of in vivo cultures

(A) Feature plots showing the expression of the zonal markers for the in vitro single cell sequencing samples. The basal markers CAV1 and COL17A1 are expressed throughout the majority of the cells, whereas the transitional and luminal markers LY6D, KRT4, and CRNN are restricted to a smaller subset of the population. (B) Immunofluorescent validation of basal COL17A1 and TP63 enrichment for in vitro cultured cells. (C) Immunofluorescent imaging of enrichment of KI67 and TP63 as markers of proliferative cell populations. (D) Quantification of this immunofluorescent imaging to validate that KI67/TP63 double positive cells are significantly enriched in the in vitro samples.

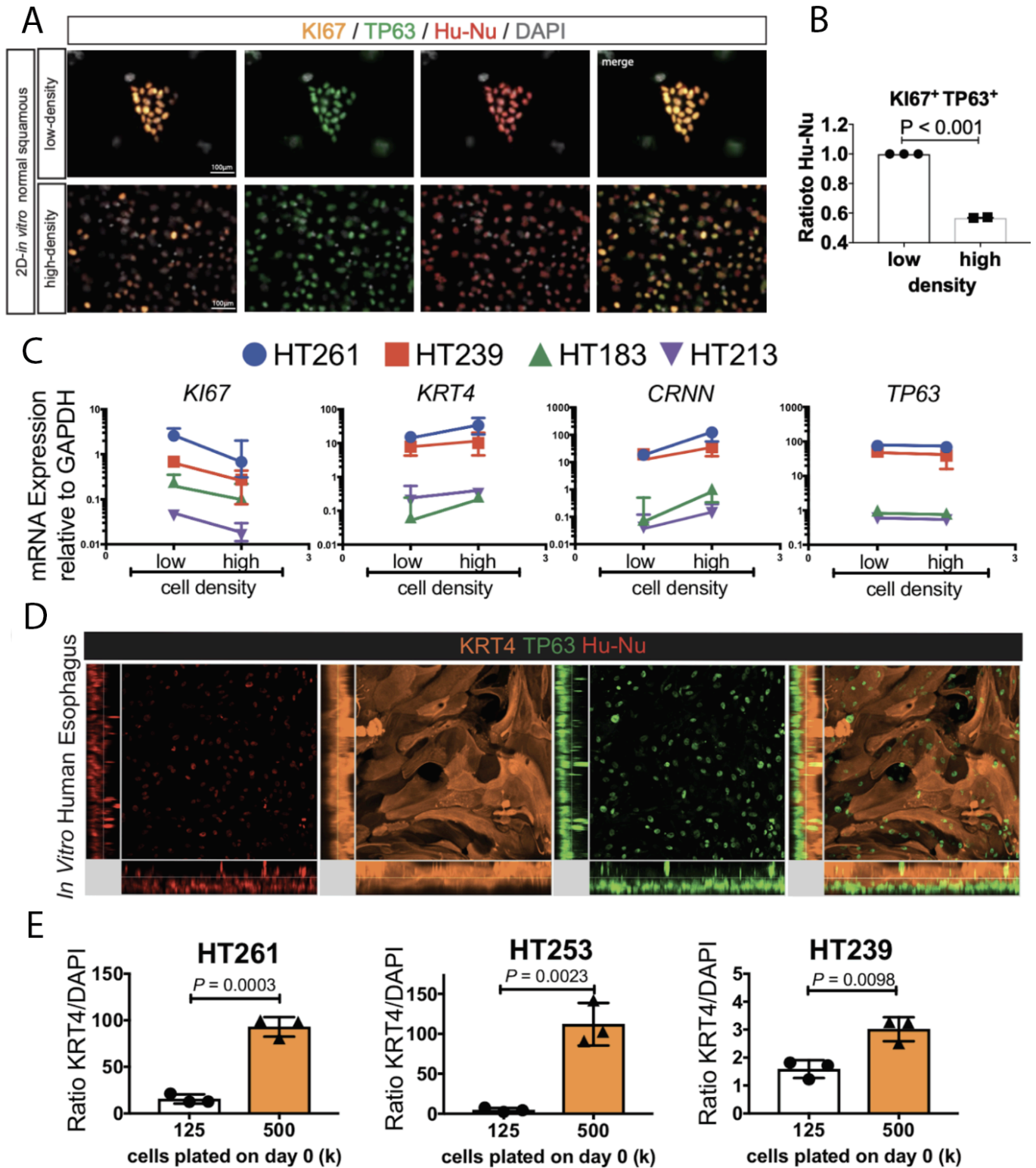


Figure 6. Differentiation potential of in vitro cultures

(A) Immunofluorescent imaging of KI67 and TP63 as proliferative markers. (B) At low cell densities 100% of cells are positive for KI67 and TP63, whereas when cells reach high densities the proportion of proliferative cells declines significantly. (C) Quantitative RT-PCR of low versus high density cells validated that KI67 expression decreases, and is replaced by increased expression of the transitional and differentiated markers KRT4 and CRNN. (D) Immunofluorescent staining with 3D Z-stack imaging shows that these high density cultures create zonal stratification with differentiating KRT4+ luminal cells localizing to the apical surface of the culture while the basal/suprabasal TP63+ cells localize to the basal surface of the culture. (E) The enrichment of KRT4 expression was validated at the protein level by quantifying KRT4 pixel saturation relative to DAPI pixel saturation, verifying a significant increase in KRT4 protein expression across three patient samples when cells are cultured at high density.

Chapter 2 - Differential Reflux Response

Using our *in vitro* model for culturing patient biopsies we next wanted to explore whether biological determinants at the cellular level could be contributing to differing physiological responses among GERD patients. Patients with severe GERD have a 15-fold increase in incidence of BE and an 8-fold increase in incidence of EAC, while patients with BE have a 10- to 55-fold increased risk of developing EAC (Thrift, 2018). This connection is likely due to the damaging effects that gastric reflux can have on cells. Gastric reflux exposes the epithelium of the esophagus to harmful bile acids (BA) and low pH. Exposure to BA and low pH have both been shown to cause cell acidification and production of nitric oxide that results in DNA damage and ultimately gene mutation (Goldman et al., 2010; Jolly et al., 2009; Dvorak et al., 2007). This ability to damage DNA helps explain why the presence of higher BA concentrations and longer exposure to acidic pH were correlated with the onset of erosive esophagitis and Barrett's esophagus (Stein et al., 1992; Nehra et al., 1999). Glyco-conjugated bile acids in particular have been associated with metaplasia and Barrett's-like progression in esophagus (Straub et al., 2019). Exposure to BA and low pH HCl are therefore used as analogues to the harmful effects of gastric reflux. Given the lower rates of esophagitis and EAC among AA GERD patients, it is possible that this damage mechanism is mitigated in the AA tissues. Therefore, we hypothesized that AA samples would exhibit less cellular damage compared to the EA samples when exposed to these reflux conditions.

Results

High Content Assay For Bile Acid Damage

We collaborated with Charles Zhang from the Sexton Lab to perform a multifactorial analysis of cell morphology in response to bile acid (BA) treatment. Three EA and three AA cell lines were treated with a dosage gradient from 0-3000 μM BA for 48 hours in order to test whether there was an observable difference in response. Cells were plated on a 384 well plate after which they were stained with the Hoechst 33342 Cell Mask Deep Red for visualization of nuclei and cell boundary segmentation alongside NF κ B to visualize cell stress. An automated protocol was used to image and analyze the samples based on 1,400 unique cellular features. These data were used to generate treatment curves of cellular response and produce IC_{50} values for each cell line (EA: HT-180 128.8 μM , HT-239 144.4 μM , HT-253 118.1 μM ; AA: HT-183 1230 μM , HT-218 1282 μM , HT-261 2143 μM) (Figure 7A). Strikingly, the observed average IC_{50} value for the EA samples was 130.4 μM , which is greater than a tenfold decrease from the average AA IC_{50} value of 1551.7 μM ($P=0.008$) (Figure 7B). This suggests that the EA cells are significantly more susceptible to cellular damage in response to BA treatment, whereas the AA cells are more highly protected. However, additional replicates are needed to make large scale conclusions about EA and AA populations.

DNA Damage

Bile acids and low pH have been shown to induce DNA damage in esophagus epithelial cells, suggesting a mechanism by which reflux leads to metaplasia and cancer (Goldman et al., 2010; Jolly et al., 2009; Dvorak et al., 2007). To examine if a potential difference in susceptibility to this DNA damage exists, AA and EA samples were cultured and exposed to a

combination of 300 μ M Bile Acid and HCl at pH of 4 for one hour. The presence of γ H2AX was used as a marker for DNA damage, as the phosphorylation of H2AX to form γ H2AX is an early cellular response to DNA double strand breaks (Mah et al., 2010). Exposure to the reflux conditions induced DNA damage, as measured by the ratio of nuclei with at least 5 γ H2AX foci. The two AA samples tested, HT-183 and HT-261, possessed 6.72% and 8.17% of cells positive for DNA damage, respectively. By contrast, the two EA samples treated, HT-180 and HT-253, presented greater than a two-fold increase with 18.67% and 20.36% of cells positive for DNA damage post treatment, respectively (Figure 8). This significant difference in DNA damage between the EA and AA cells ($P=0.008$) indicates that the EA samples were more susceptible to the damaging effects of the treatment while that AA samples were more resistant.

Proliferation Rates

When epithelial tissue is damaged increased proliferation is an integral response to compensate for lost cells (Lehrer et al., 1998). This holds true for the esophageal epithelium, which shows a significant increase in proliferation in response to bile acids and low pH (Calabrese et al., 2009; Souza, 2011; Mastracci et al., 2007). As reported in Ferrer-Torres et al. 2019, Dr. Ferrer-Torres demonstrated that the epithelial tissue of patients with chronic GERD express the proliferation marker KI67 at significantly higher levels than non-GERD patients ($P=0.003$) (Figure 9B). These proliferative signatures have further been associated with progression to Barrett's metaplasia (Souza, 2011). Tomasetti and Vogelstein have proposed that variations in rates of cancer and mutation driven disease are intimately linked to the proliferation rates of tissues, in which case an increase in proliferation among GERD patients could be serving to exacerbate risk of disease progression (Tomasetti and Vogelstein, 2015).

To investigate whether this proliferative response differed between our EA and AA cell lines we treated confluent cultures with HCl at pH of 4 for 10 minutes, let them incubate for 24 hours, and then split samples into 500 thousand cells per well (Figure 9A). The cell density was then measured after four days to compare proliferation. The EA cell lines tested showed a significant increase in cell count after treatment compared to control conditions, with HT-227 showing a 1.56-fold increase and HT-180 showing a 1.67-fold increase (Figure 9C). Alternatively, the AA cell line tested did not show a statistically significant change in cell count compared to the control conditions ($P=0.085$), and actually had lower cell density after treatment with a 0.65-fold change (Figure 9A). When plotted together, we see that the AA cells showed a significantly different change in cell density compared to the EA cells ($P=0.0012$) (Figure 9C). This suggests that the AA cell line responded differently to the treatment. This could be due to increased resistance to the acid treatment or a reduced propensity to respond to damage by increasing proliferation. Alternatively, the slight decrease in cell density following treatment could be explained by increased susceptibility and therefore increased cell death that masks any enhanced proliferative response. This distinction could be better assessed in the future with larger sample sizes, use of additional techniques for assaying proliferation, and additional measures of cell viability and apoptosis.

ECAD and TP63 Expression

As a final potential measure of cellular response to reflux we investigated e-cadherin and TP63 expression via immunofluorescence. A common feature of epithelial damage in GERD patients is increased tissue permeability due to reduced integrity of intercellular junctions (Tobey et al., 2014). This phenotype is predominantly caused by proteolytic cleavage of surface

e-cadherin upon exposure to gastric acid, leading to an overall decrease in surface e-cadherin available for cell-cell adhesion (Jovov et al., 2011). An additional protein differentially expressed in response to reflux is TP63. The treatment of esophageal epithelial cells with bile salts and acids has been shown to decrease TP63 expression (Roman et al., 2007). Furthermore, downregulation of TP63 has been associated with Barrett's-like metaplasia in mice and poorer prognosis in human esophageal cancers, although this point has been debated due to conflicting observations of TP63 enrichment in other studies (Fang et al., 2021; Takahashi et al., 2006).

To examine whether these responses differed in our patient models, *in vitro* samples from two AA and two EA patients were treated with either HCl at pH of 4, 300 μ M Bile Acid, or a combination of both for one hour. Immunofluorescent images were captured at constant exposures. Protein expression was represented as the pixel density of the fluorescence channel for which each protein was captured (488nm for TP63 and 647nm for ECAD), and data points were normalized to DAPI levels per image. To evaluate changes in expression these pixel saturations were then normalized to the untreated control levels per each patient and compared between EA and AA patients. This analysis did not yield any statistically significant differences between the EA and AA samples ($P>0.05$), although a mild trend of increased response was observed in the EA samples (Figure 10 A, B). Due to the high variability within samples and small sample size it is difficult to make any definitive conclusions from these data. It would be valuable to repeat this experiment with a larger number of patients, more replicates per patient, and validation with immunoblot analysis to measure protein levels.

Chapter 2 Figures

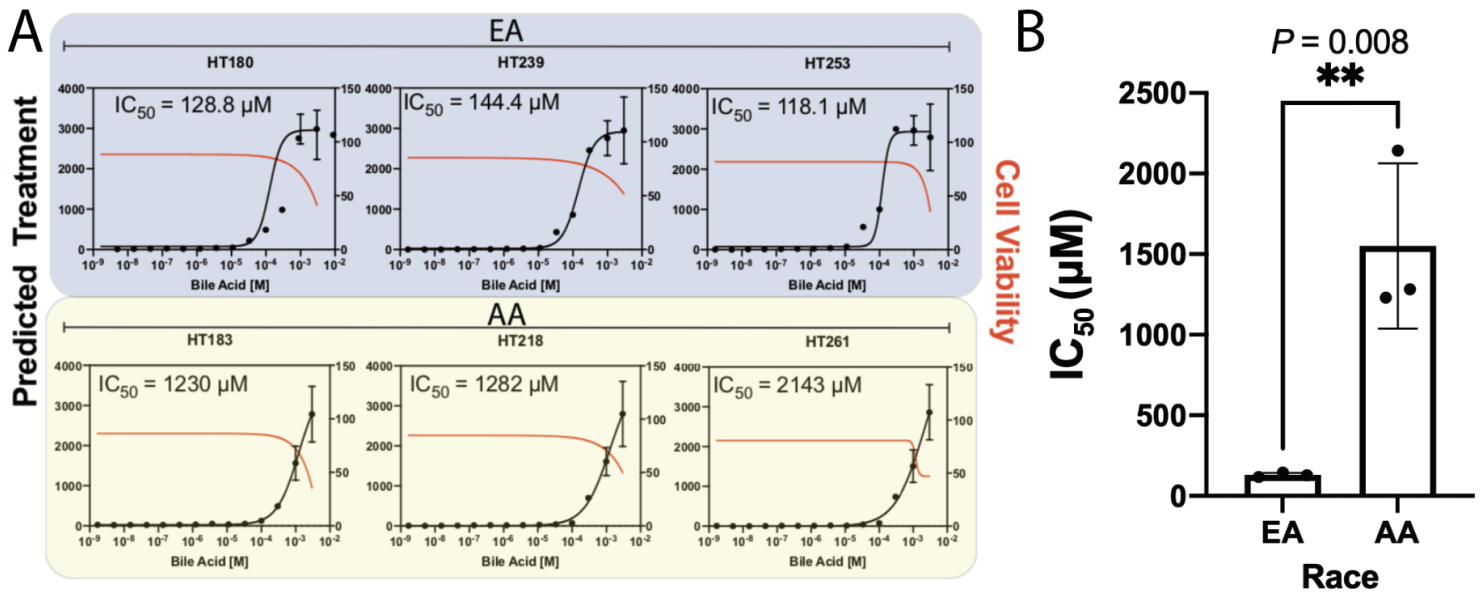


Figure 7. High content morphology screen for bile acid response

(A) Cells from six patient samples were treated with a dosage curve (0-3000 μM) of bile acid for 48 hours. Samples were then analyzed by an automated process that imaged the cells and measured 1400 morphological features at the individual cell level. Ensemble learning with a random forest model was used to calculate predicted exposure level of bile acid per-cell. The predicted treatment curves were used to estimate IC_{50} values for each patient. (B) IC_{50} values were plotted based on racial groups showing a significant difference between groups.

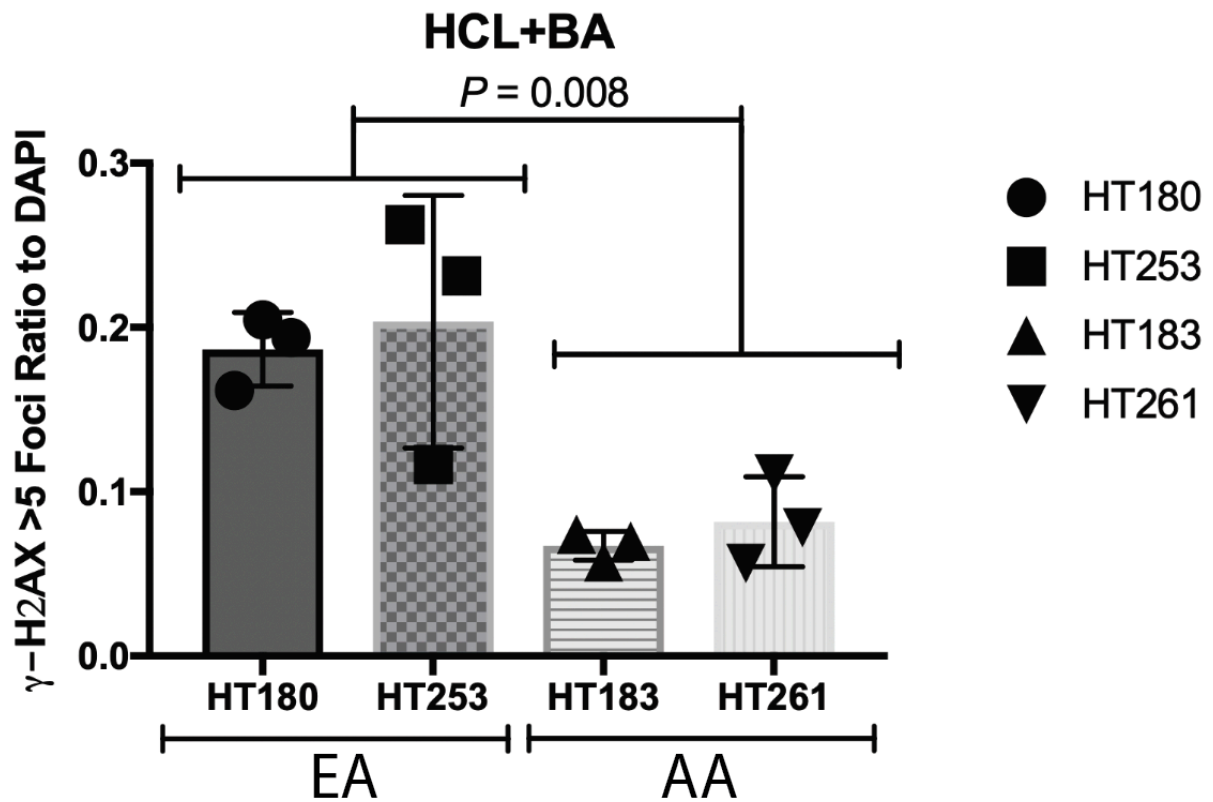


Figure 8. DNA damage in response to reflux exposure

Four patient samples were cultured in vitro and exposed to reflux analog (300 μ M Bile Acid and HCl at pH of 4) for one hour. Samples were treated in triplicate and immunofluorescence was used to visualize γ H2AX foci as a measure of DNA damage. The number of total cells was counted using DAPI, and the proportion of cells possessing at least five γ H2AX foci was plotted. Samples were grouped based on race, and the EA group showed a significantly higher proportion of positive cells than the AA group.

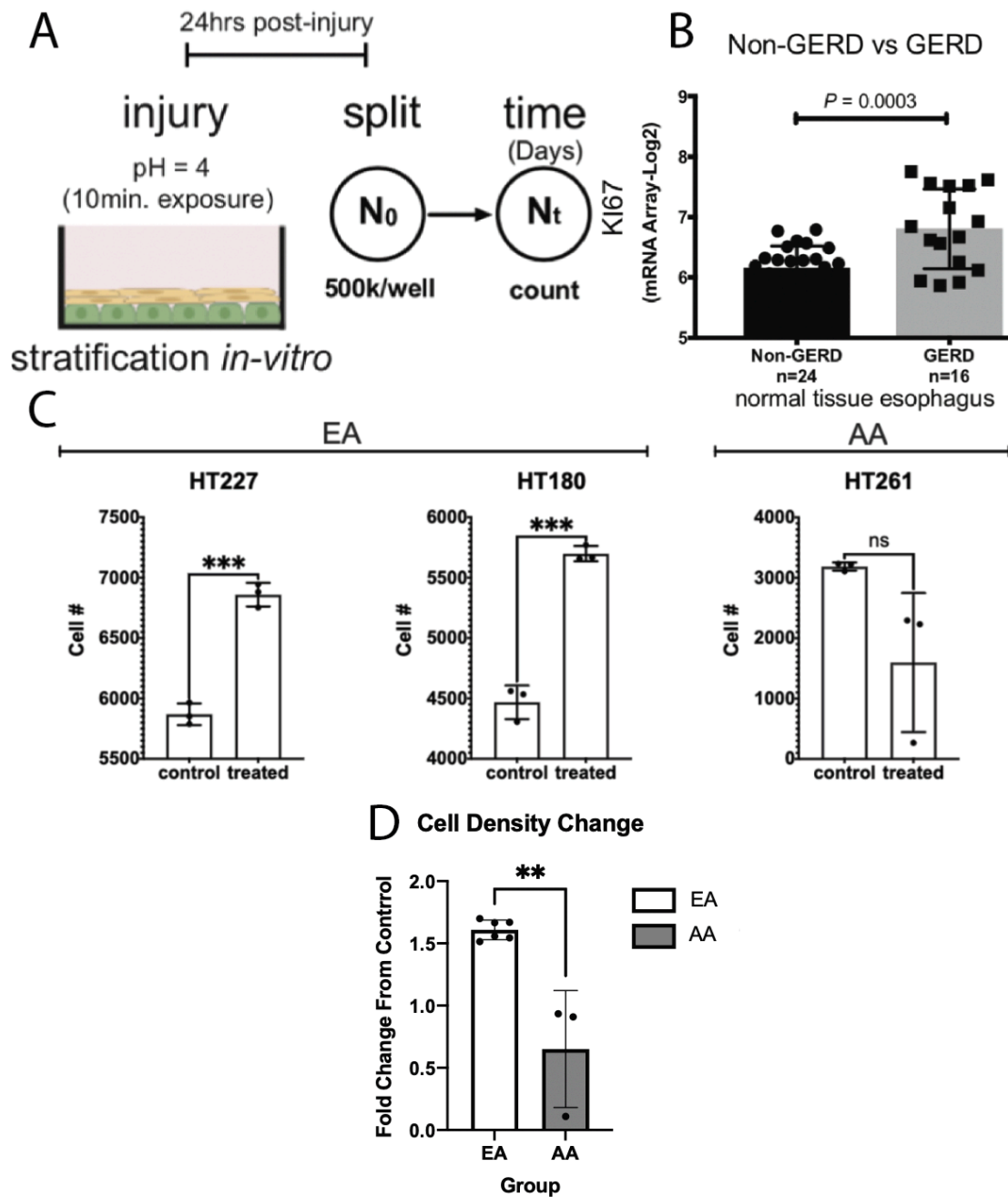


Figure 9. Cell proliferation in response to reflux exposure

(A) Schematic representation of treatment protocol and cell proliferation assay. (B) Quantitative RT-PCR of esophagus biopsies from GERD and Non-GERD patients showing a significant increase in the expression of the proliferative marker KI67 among GERD patients. This data is also presented in Ferrer-Torres et al., 2019. (C) Plots of cell number four days after plating at 500 thousand cells between control and HCl treated conditions. (D) Plotted difference between the two EA samples and one AA sample tested showing a significant difference in cell count change between groups ($P=0.0012$).

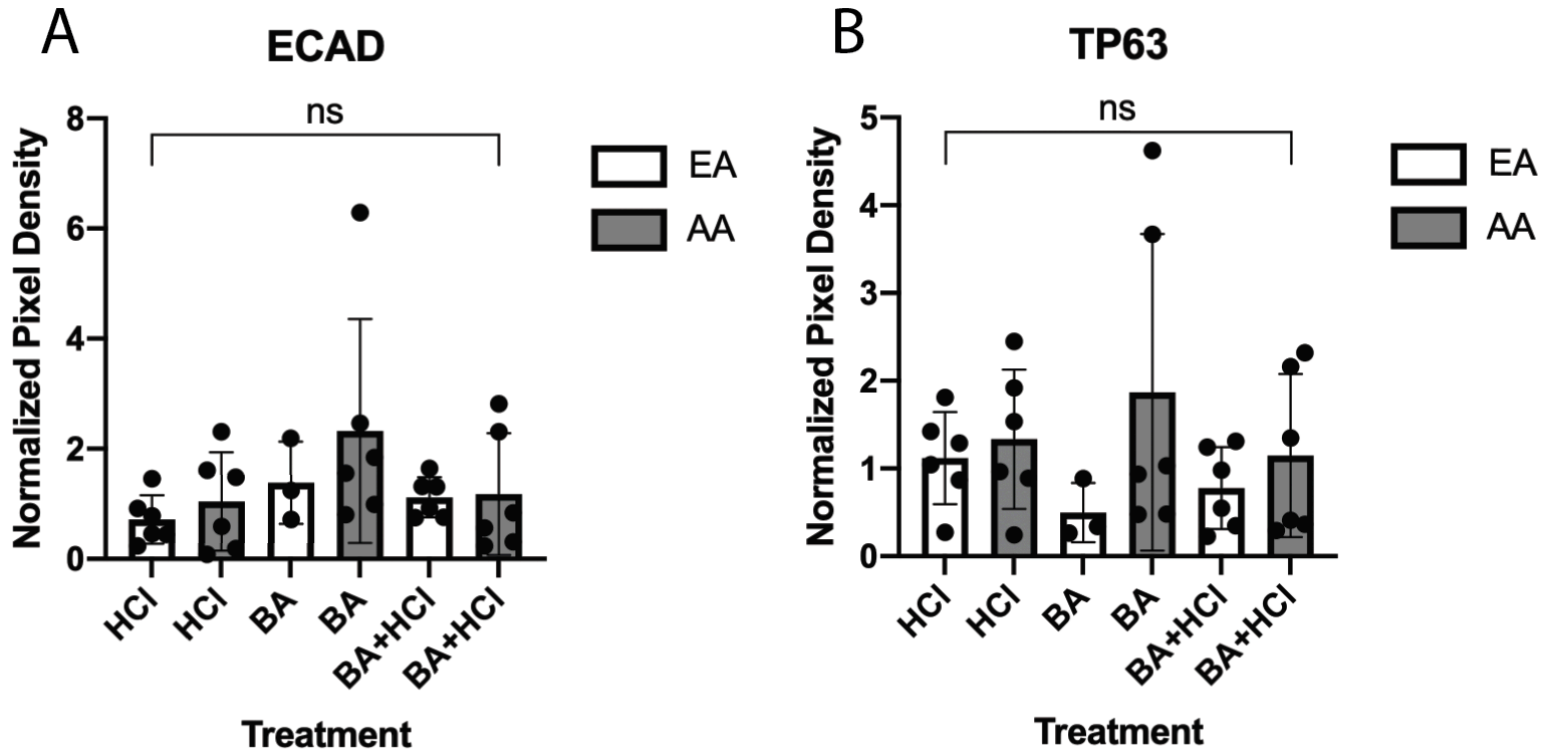


Figure 10. ECAD and TP63 response to reflux exposure

Two EA and two AA cell lines were exposed to HCl at pH of 4, 300 μ M Bile Acid, or a combination of both for one hour. Immunofluorescent staining was used to visualize (A) ECAD and (B) TP63 expression. Images were captured between samples in triplicate at constant exposures between all samples, and pixel saturation was calculated for each image. Plotted data points represent pixel saturation for the respective protein marker normalized to DAPI saturation per image and then compared to control conditions for each patient.

Chapter 3 - GSTT2 As A Mediator Of Differential Reflux Response

Given the observations above it is apparent that the EA and AA cell lines examined present differing cellular responses and degrees of susceptibility to modeled reflux exposure. The presence of BA and low pH have both been linked to the production of reactive oxygen species (ROS) (Goldman et al., 2010; Dvorak et al., 2007). Increased levels of ROS cause DNA damage and have been attributed as a mechanism explaining the progression of GERD to BE and EAC (Goldman et al., 2010; Song et al., 2016; Peng et al., 2021).

The glutathione s-transferase (GST) gene family serves to neutralize ROS through the electron donor glutathione (Pickett and Lu, 1989; Hayes and Strange, 1995; Hayes and McLellan, 1999). Dysregulation of members of the GST family have been associated with increased susceptibility to several cancers including breast, lung, and colon (Landi, 2000; Parl, 2005; McIlwain et al., 2006). In the colon, GSTT2 (Glutathione S-Transferase Theta 2) specifically has been shown to play a protective role against ROS induced DNA damage from gastrointestinal contents (Pteterman et al., 2009).

It has recently been observed that alleles of the GSTT2 locus show a racially distinct expression pattern that is strongly correlated with the disparity in EAC progression (Ferrer-Torres et al., 2019). Two predominant polymorphisms of GSTT2 have been identified: the wildtype allele of GSTT2 as well as an allele containing a 17-bp duplication sequence in the promoter region of GSTT2 (Zhao et al., 2009). The promoter duplication allele has been shown to lead to significantly lower expression of GSTT2 mRNA (Zhao et al., 2009). The frequency of this mutant promoter duplication allele is roughly 94 percent among individuals of European

descent, whereas the mutation is significantly less common in individuals of African descent, with allele frequencies as low as 64% in Western African population (Ferrer-Torres et al. 2019). Because of this, *GSTT2* expression levels at the mRNA level tend to be significantly higher within the African American population when compared to European Americans, and *GSTT2* was the most disparately expressed gene in the esophagus between the two racial groups (Ferrer-Torres et al. 2019). Furthermore, within the heterogenous African American GERD population, the promoter duplication allele is correlated with the progression of EAC, whereas the population of African Americans with GERD who do not progress to EAC contain a larger proportion of the wildtype allele (Ferrer-Torres et al., 2019). Based on the findings that *GSTT2* is one of the most disparately expressed genes between AA and EA patients (Ferrer-Torres et al., 2019) and its role in protecting cells from ROS, *GSTT2* is a promising candidate as a biological determinant of the racial disparity in EAC development.

GSTT2 Protects Esophageal Epithelium from Oxidative Stress

The following data is also presented in Ferrer-Torres et al., 2019.

Prior to Dr. Ferrer-Torres' work, the role of *GSTT2* in the esophagus had not been examined in detail. To evaluate whether *GSTT2* contributes to protection of the esophageal epithelium during reflux I assisted Dr. Ferrer-Torres in using siRNA to knockdown *GSTT2* expression (Ferrer-Torres et al., 2017; 2019). Several siRNA constructs were tested and two constructs, siRNA-05 and siRNA-06, were selected for their ability to reduce *GSTT2* expression by roughly 80% and 60%, respectively (Figure 11A, B). The immortalized esophagus epithelial cell line Het1A was cultured and treated with the *GSTT2* siRNAs, after which the cells were exposed to 100 μ M of the reactive oxygen species cumene hydroperoxide (Cum-OOH).

Oxidative stress from Cum-OOH induced DNA damage in control and siRNA treated samples as measured by γ H2AX foci, but the knockdown of GSTT2 led to a greater than two-fold increase in DNA damage ($P=0.008$ and 0.009)(Figure 11C). This suggests that GSTT2 plays a prominent role in the epithelium of the esophagus to protect cells from ROS, and when GSTT2 is diminished cells become more susceptible to damage from ROS.

Impact of GSTT2 Genotype on Reflux Response Assays

If the GSTT2 genotype is responsible for differential protection from reflux, then it would be expected that the results from the reflux response assays would reflect the genotype of the samples. In order to examine this, we first had to verify that GSTT2 expression patterns were maintained in our *in vitro* model. Immunofluorescent staining of *in vitro* cultures showed that GSTT2 expression patterns resembled *in vivo* tissue, with punctate cytoplasmic foci throughout the entire tissue. Protein levels were also measured via immunoblot and identified that the observed disparity between AA and EA GSTT2 protein levels (Ferrer-Torres et al., 2019) was recapitulated *in vitro*.

Genotyping the GSTT2/2B loci was accomplished using the set of primers previously described (Zhao et al., 2009). GSTT2 has a paralogue called GSTT2B located roughly 10,000 base pairs upstream of GSTT2, both of which are transcribed and produce functionally identical proteins. Because the promoters for GSTT2 and GSTT2B are identical and can both possess the promoter duplication, an individual can have between zero and four functional promoters. There is also a 37 kilobase deletion event that can remove the GSTT2B allele. The primers utilized are capable of distinguishing between the deletion genotype, but then can only determine whether

the promoter duplication is homozygous or some degree of heterozygous. As such, an individual heterozygous for both the deletion and promoter duplication could possess either one or two functional promoters.

For the reflux response assays four EA and three AA cell lines were used. For the EA samples the GSTT2 genotypes are as follows (Figure 13): HT-180 is homozygous for no deletion and heterozygous for the promoter duplication. HT-239 is heterozygous for the deletion and homozygous for the promoter duplication. HT-253 is homozygous for the deletion and homozygous for the promoter duplication. HT-227 is heterozygous for the deletion and homozygous for the promoter duplication. For the AA samples the GSTT2 genotypes are as follows (Figure 13): HT-183 is heterozygous for the deletion and homozygous for the promoter duplication. HT-218 is heterozygous for the deletion and heterozygous for the promoter duplication. HT-261 is heterozygous for the deletion and homozygous for the promoter duplication. Genotyping for several of the samples was repeated for verification (data not shown).

HT-239, 253, 227, 183, and 261 do not possess a wildtype GSTT2 promoter as they are all homozygous for the promoter duplication. HT-180 possesses between 1-3 wildtype promoters, while HT-218 possesses between 1-2 wildtype promoters. Given the prediction that the GSTT2 promoter genotype dictates GSTT2 levels and therefore reflux susceptibility, we hypothesized that HT-239, 253, 183, and 261 would show similarly high susceptibility to reflux, while HT-180 and 218 would show lower susceptibility.

This is not the trend that was observed in the reflux response assays. In the IC₅₀ assay there was still greater than a 10 fold difference when comparing the EA (HT-239 and 253) and AA (HT-183 and 261) samples that were all homozygous for the promoter duplication. Furthermore, HT-180 and 218 had IC₅₀ values that fit within the order of magnitude of their respective racial group rather than having collectively increased IC₅₀ values relative to the other four samples based on their genotype. Grouping samples based on genotype yielded no significant difference between groups ($P=0.81$). This trend continued for the DNA damage assay for which HT-183 and 261 both presented with lower levels of DNA damage than HT-253, even though all three samples are homozygous for the promoter duplication. HT-180 also presented with greater DNA damage than HT-183 and 261 despite being heterozygous for the promoter duplication, which would have predicted reduced DNA damage. Lastly, for the proliferation assay the EA samples HT-180 and 227 both showed significant increases in proliferation, while the AA cell line HT-261 did not show a significant increase in proliferation and actually showed a decrease, even though HT-227 and 261 are both homozygous for the promoter duplication suggesting greater proliferative response, whereas HT-180 would be expected to have lower susceptibility and therefore a reduced response. These observations indicate that trends in differential responses in these assays are based more on the racial category of samples and not on GSTT2 promoter genotype.

It should be noted that the lack of samples that are homozygous for functional promoters leaves the possibility that GSTT2 exhibits haploinsufficiency which would mean that all of the samples tested are effectively mutant genotypes. This is unlikely due to the continuous nature of the phenotypes assayed, which would instead be expected to show that varying levels of

glutathione pathway activity would confer varying levels of resistance to reflux in a non-discrete fashion. Furthermore, even if all of the samples tested share an insufficient GSTT2 genotype then they would be expected to exhibit similar response phenotypes if GSTT2 genotype drives response differences. This was not observed, however, as differences between racial groups persisted. As such, the GSTT2 promoter genotype alone does not appear to be sufficient to explain the disparities among the samples tested, though larger samples are needed to assess the predictive power of the promoter genotype for reflux response at the population level and to evaluate the full range of possible genotypes.

In spite of these results, GSTT2 and the glutathione pathway remain relevant to racial disparities in reflux responses between AA and EA patients. Examined populations of AA patients had significantly greater GSTT2 mRNA and protein levels than EA patients, independently of their genotype (Figure 12B). As such, additional differences in the transcriptional regulation or post-translational stabilization of GSTT2 are likely to explain mRNA/protein abundance independent of differing promoter alleles. Indeed, while the GSTT2 protein is very stable, exposure to acid can lead to GSTT2 degradation and subsequently reduced protein levels. We had previously observed that 10 minutes of exposure to HCl at pH 4 is capable of reducing GSTT2 protein levels in an EA cell line. When treated with 300 μ M BA and HCl at pH 4 for 45 minutes, GSTT2 protein levels for the EA HT-253 were roughly 50 percent depleted, whereas the AA HT-261 GSTT2 levels remained stable (Figure 14A, B). Moreover, treatment for 60 minutes almost completely depleted GSTT2 protein in the EA sample, whereas it induced only a fifty percent reduction in the GSTT2 protein in the AA HT-261. While these preliminary data still require further validation, the work reveals a potential difference in GSTT2 regulation

between the EA and AA cells beyond promoter genotype, as both HT-253 and 261 possess the same genotype. It is also possible, however, that other cellular processes are protecting the HT-261 cells, and that this alternative protection is subsequently protecting GSTT2 from degradation.

Chapter 3 Figures

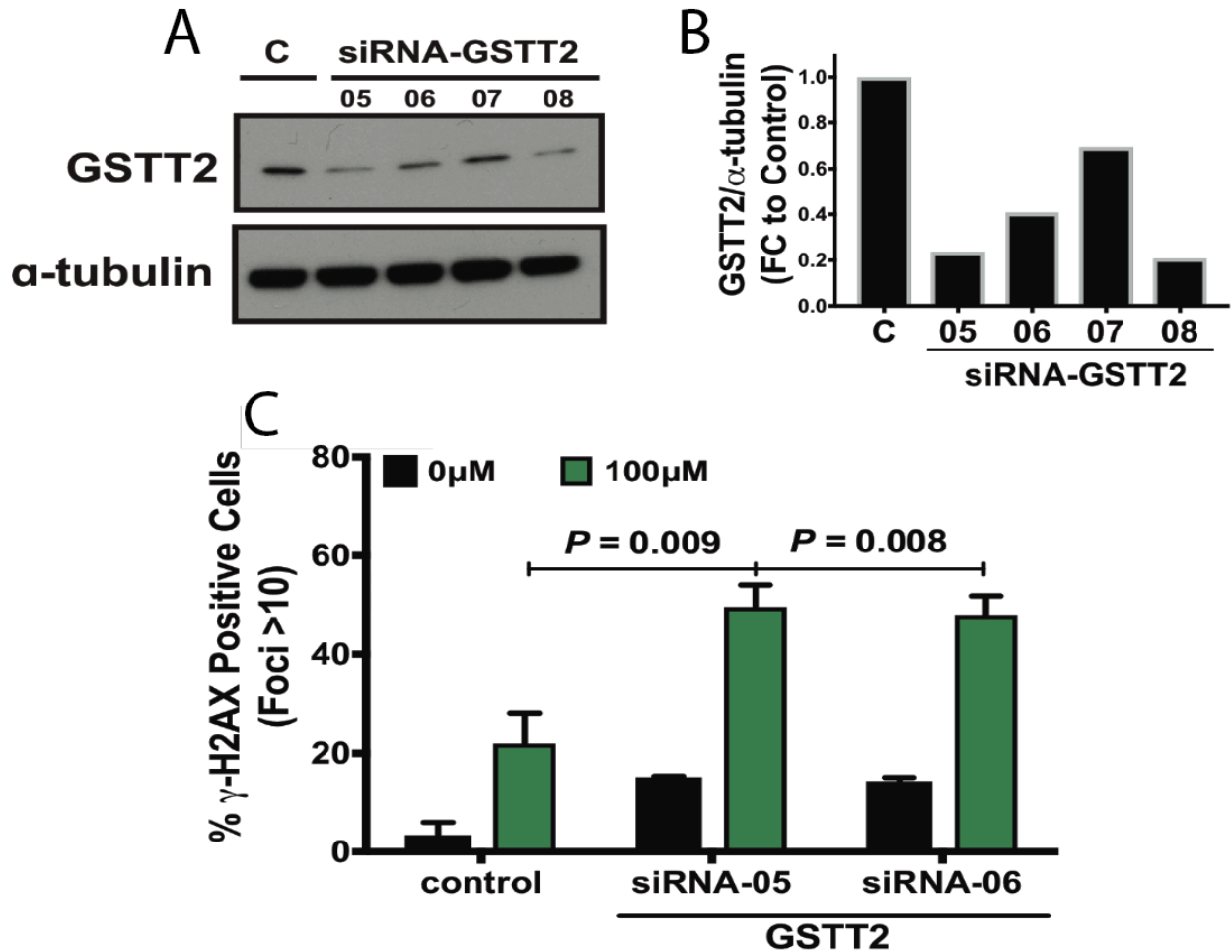


Figure 11. Loss of GSTT2 increases DNA damage from Cum-OOH

Data also presented in Ferrer-Torres et al., 2019

(A) Immunoblot for GSTT2 and α -tubulin protein levels in Het1A cells for control conditions and after treatment with four GSTT2 siRNA constructs. (B) Quantification of immunoblot results, with GSTT2 levels normalized to α -tubulin for each sample. (C) GSTT2 expression was knocked down in Het1A cells using two of the GSTT2 siRNA constructs (siRNA-05 and 06). The cells were then exposed to either 0 or 100 μ M of cum-OOH. Immunofluorescence was used to visualize DNA damage, with a threshold of at least ten γ H2AX foci per cell used to characterize sufficiently damaged cells. DAPI was used to measure total cells per sample, and the proportion of γ H2AX positive cells was plotted, revealing a significant difference in DNA damage for treatment conditions when GSTT2 is knocked down.

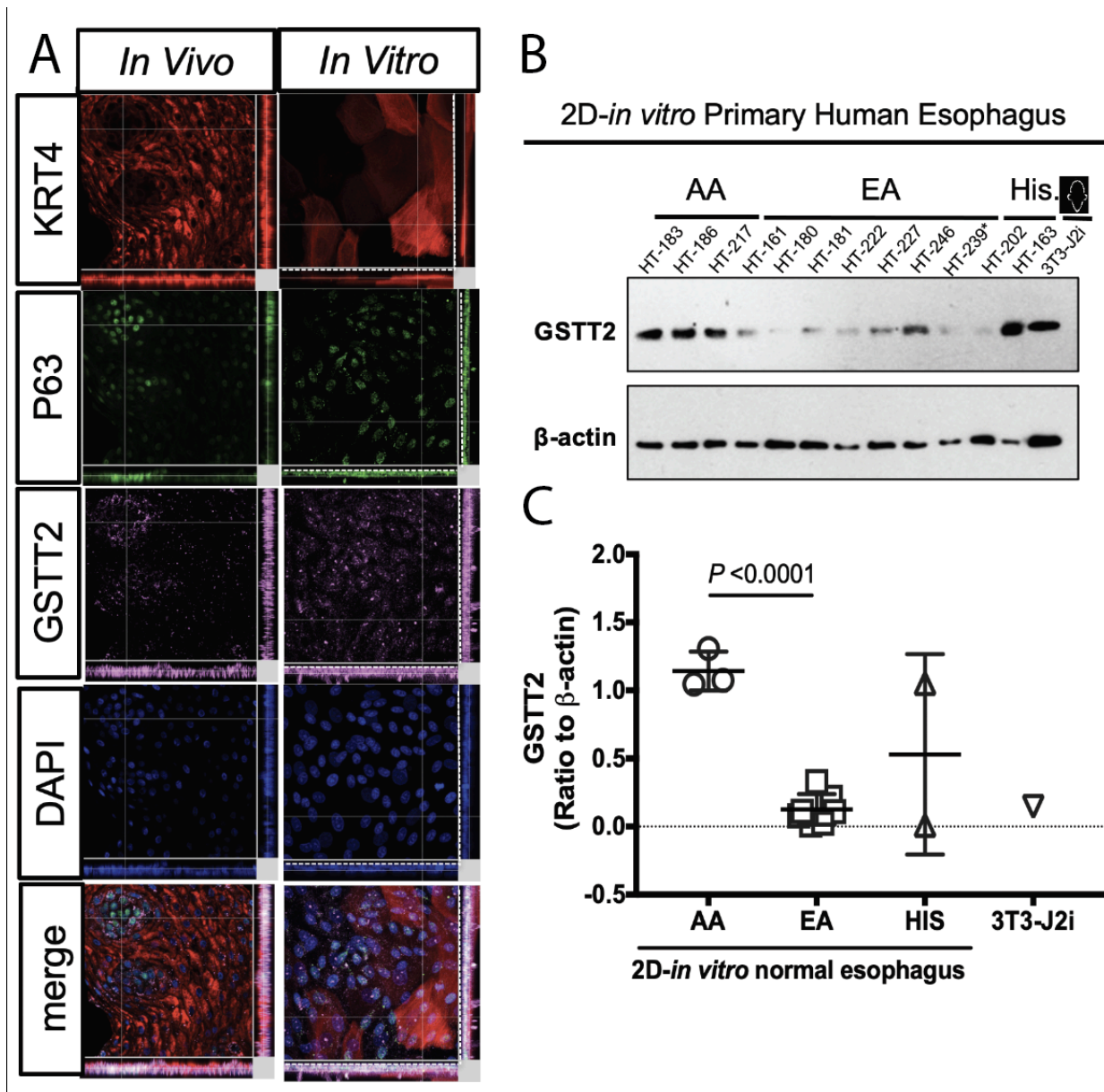


Figure 12. GSTT2 expression maintained during *in vitro* cultures

(A) Immunofluorescent staining of high density *in vitro* cultures. GSTT2 protein expression is retained throughout all layers of the cultured epithelial tissue. (B) Immunoblot of GSTT2 and β-actin protein from a racially diverse cohort of *in vitro* cultures. (C) Quantification of immunoblot results, with GSTT2 levels normalized to β-actin for each sample. The AA group showed significantly higher levels of GSTT2 protein than the EA group.

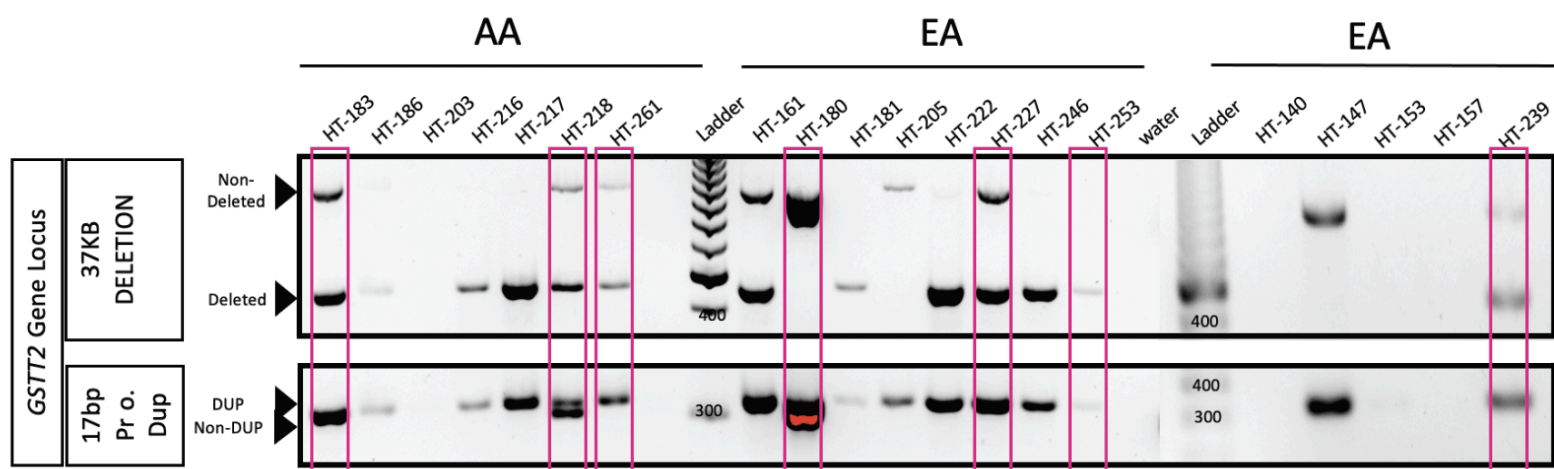


Figure 13. Sample genotyping for GSTT2

Samples were genotyped for the promoter duplication and deletion event at the GSTT2/B loci. Primers for the deletion event produced products that are 505 base pairs in length if the deletion is not present, and 847 base pairs if the deletion is present. Primers for the promoter duplication produce products of roughly 300 base pairs depending on the presence of the duplication event, which adds 17 base pairs to the product. The highlighted samples were used for the reflux response assays.

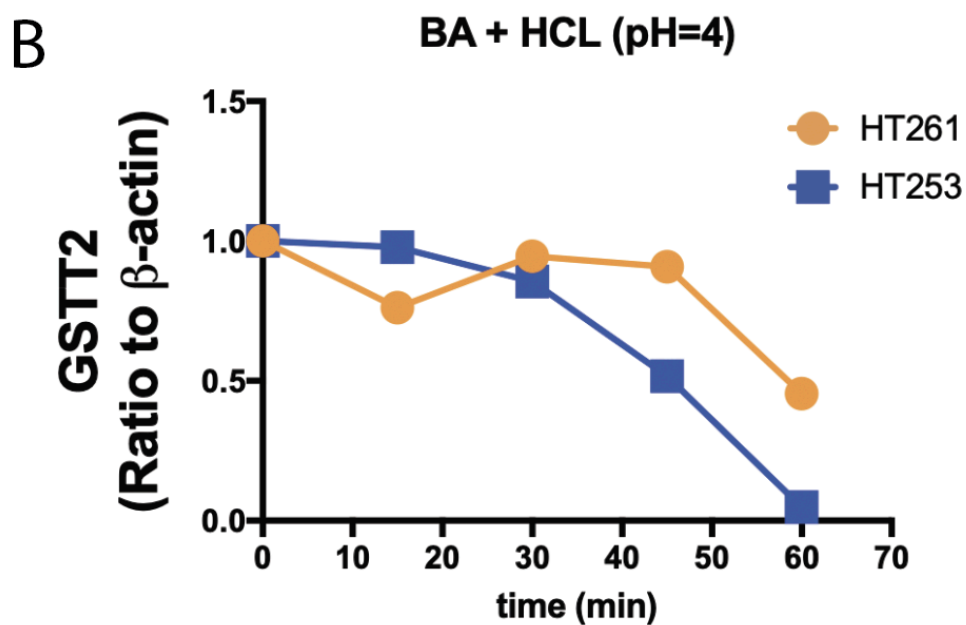
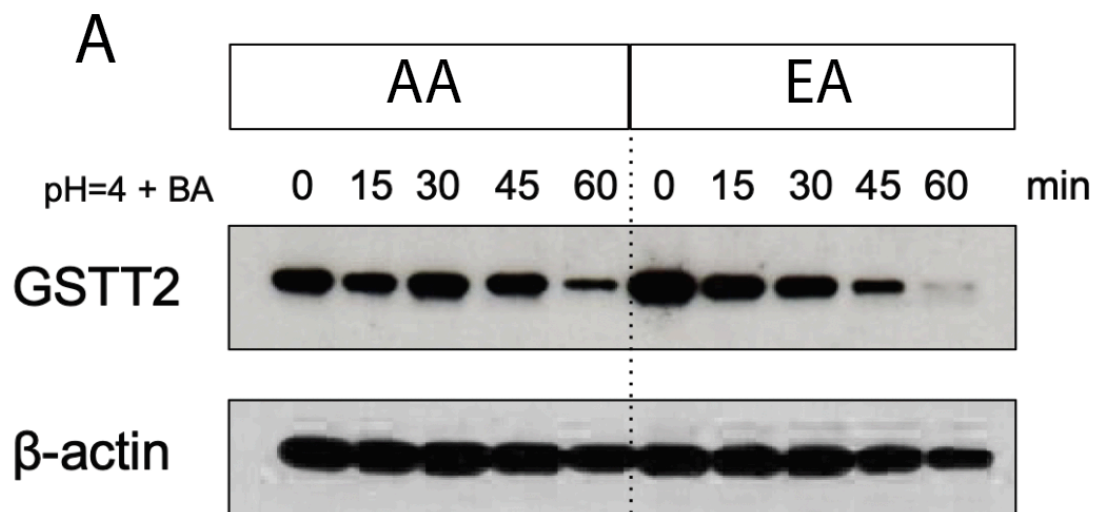


Figure 14. GSTT2 stability after reflux exposure

(A) One AA and one EA sample were treated with reflux analog (300 μ M Bile Acid and HCl at pH of 4) in 15 minute time increments from 0-60 minutes. Immunoblot was conducted to measure the protein levels of GSTT2 and β -actin from these samples. (B) GSTT2 protein levels were normalized to β -actin for each sample, and normalized GSTT2 protein levels were compared to control conditions. These data points were plotted for the two samples.

Chapter 4 -Conclusions and Future Directions

There is overwhelming evidence that disparities exist in healthcare. Marked differences are present in disease incidence, burden of illness, and disease outcome based on gender, culture, race/ethnicity, socioeconomic status, and geographic location. It is a necessity that the medical community and providers are purposeful in mitigating any inequities which harm those that are continually marginalized by systemic discrimination. While there are many social determinants of health and psychosocial features that contribute to these disparities, biomedical research is revealing that biological determinants can also play a role in disproportionately predisposing different populations to disease. Even though the work addressed here focuses on these potential biological determinants, it is important to reiterate that efforts to understand biological determinants of disparities should not undermine the significance of social determinants. Social inequalities still exist at all levels of healthcare, and truly equitable care can not be achieved without addressing every aspect of patient health.

GERD and the associated BE and EAC are extremely prevalent diseases in our western culture that have a profound impact on quality of life and patient survival. As such, it is important to understand the underlying mechanisms that govern these diseases and appreciate any potential differences in susceptibility between patient populations. EAC rates among EA populations are four fold greater than that of AA populations, despite no clear differences in environmental risk factors between these populations. This leaves the possibility that underlying biological determinants contribute to differential risk of EAC among AA and EA populations. In order to investigate this relationship, I worked with Daysha Ferrer-Torres, PhD, to optimize an *in vitro* culture system to model racially diverse patient esophagus tissue. This culture system is

capable of expanding epithelial tissue from patient esophagus biopsies. While enriching for the basal proliferative populations of the epithelium, the culture system still supports the other cellular populations of the epithelium as reflected in the expression of each of the major markers identified in native cell populations. Though low-density cultures are composed predominantly of basal and suprabasal cell types, confluent cells form a polarized epithelium with differentiating squamous cells on the apical surface. These cultures therefore mimic the native esophagus and provide a promising tool for the study of tissue physiology – not confounded by uncontrollable environmental influences.

This model system was used to culture biopsies derived from AA and EA patients. Samples were exposed to Bile acids, HCl, or both as reflux analogous, and assayed for various aspects of cellular response. On measures of cell morphology, DNA damage, and proliferative response, the EA samples showed increased susceptibility to treatment, whereas the AA samples exhibited greater resistance. While these promising findings suggest a potential difference in tissue physiology between EA and AA populations, all of these experiments were conducted with small sample sizes and would need to be repeated in much larger replicates in order to begin making claims about large populations.

Polymorphisms of the glutathione transferase protein GSTT2 have been proposed as an underlying explanation for the observed racial disparities in EAC rates. GSTT2 is known to protect tissues from reactive oxygen species, which are largely responsible for the DNA damage caused by gastric reflux. AA populations express higher levels of GSTT2 mRNA and protein than EA populations, and this was suspected to be in part due to the increased allele frequency of

a promoter duplication allele in EA populations that reduces GSTT2 expression. However, our reflux response assays did not support a model in which GSTT2 promoter genotype alone causes differences in tissue damage susceptibility. Among the samples tested, damage response was more closely tied to racial group than GSTT2 genotype, indicating that either GSTT2 is not the main determining factor in reflux response or that additional variation occurs in the regulation of GSTT2 beyond promoter genotype. Larger samples are required in order to verify this observation and begin to elucidate the role of GSTT2 in reflux.

Beyond expanding the number of patients tested, future experiments should investigate the potential role of GSTT2 expression patterns on reflux response between racial groups. Transfecting AA and EA cell lines with GSTT2 overexpression and knockdown vectors can determine whether modulation of GSTT2 levels is capable of influencing the observed differences in the reflux response assays between AA and EA samples. Given that siRNA knockdown of GSTT2 in Het1A cells increases susceptibility to ROS treatment, I would expect that reduction in GSTT2 expression in AA samples would lead to increased susceptibility to reflux treatment. Similarly, I would expect that the overexpression of GSTT2 in the EA cell lines would confer resistance and therefore mitigate response to reflux. By creating similar levels of GSTT2 expression in the EA and AA samples, transgenic manipulation can ascertain the contribution of GSTT2 to the reflux response disparities between the two racial groups. It is of course also possible that the differential mRNA expression of the GSTT2 locus between racial groups is in part mediated by changes at the chromatin level. This could be evaluated using ATAC-seq to assess chromatin accessibility, ChIP-seq to assess histone modifications, and DNA methylation assays to assess whether repressive DNA modifications are disproportionately

associated with the GSTT2 locus in EA cells or if activating signatures are associated with the locus in AA cells.

An additional question of interest would be investigating the potential mechanisms by which GSTT2 expression could be differentially regulated or GSTT2 protein could be differentially stabilized between populations. This could be examined experimentally by introducing functional GSTT2 alleles into EA and AA cells that are homozygous for the promoter duplication and evaluating whether the transfected alleles yield differing mRNA and protein levels between samples. Given the observation that samples with identical GSTT2 alleles possess different levels of GSTT2 and reflux response, I would expect that the AA samples would yield higher GSTT2 levels than the EA samples when transfected with the same functional GSTT2 allele. An alternative potential source of functional differences in reflux-induced toxicity is the overall activity of the glutathione pathway perhaps mediated in part by differences in the stability of GSTT2 protein between populations. Our preliminary studies revealed that acid exposure leads to degradation of GSTT2, and that the GSTT2 protein from the EA sample was depleted more rapidly. Future studies can include glutathione pathway activity and metabolism assays (Truman and Hook, 2013) to evaluate potential differences between EA and AA cell lines in untreated as well as reflux conditions. I would expect to see increased activity of the glutathione pathway in the AA cell lines due to the observed resistance to ROS and increased abundance of GSTT2 protein. The use of transgenic lines as described above could also help isolate metabolic differences between groups by controlling GSTT2 expression. Lastly, investigating the mechanism of GSTT2 turnover during reflux exposure would be informative. If GSTT2 is removed via proteasomal degradation, treatment of proteasome inhibitors during acid

exposure treatment would be expected to mitigate the increased depletion of GSTT2 in the EA cell lines and potentially reduce cell damage in longer duration reflux response assays.

Whether or not GSTT2 plays a prominent role as a biological determinant in racial disparities for EAC, it remains a critical defense in protecting the esophagus from ROS and its regulation should continue to be studied for its potential therapeutic implications in GERD patients.

Chapter 5 - A Brief Inquiry Into Caveolins

This brief literature review and hypothesis were generated as my original thesis proposal. Given the limitations on timeframe and laboratory access I decided to change the topic of my thesis during this past year, but I am still very excited about the future potential for this caveolin project.

The stratified epithelium of the esophagus consists of multiple cellular layers composed of cells transitioning from the stem cell and proliferative populations of the basal zone to the increasingly differentiated layers of apical surface. Understanding the mechanisms that maintain the basal region of the esophagus has implications for both the initiation of hyperplastic diseases and for the emerging field of regenerative medicine. However, there is a scarcity of information currently available regarding the mechanism of stem cell maintenance and transition to differentiated cell types in the esophageal epithelium.

Our characterization of native and cultured esophageal epithelium described above identified caveolin 1 and 2 (Cav1 and Cav2) as prominent markers restricted to basal cell populations. Our scRNAseq analysis showed that Cav1/2 co-localized with the basal stem cell markers ITGB4 and NT5E (DeWard et al., 2014) for our *in vivo* samples and cultured *in vitro* cells. Caveolins are membrane-bound proteins that oligomerize and then localize to lipid rafts on the cell membrane where they accumulate to form caveolae that provide a catherine-independent mechanism of endocytosis (Sowa, 2012; Busija et al., 2016). Cav1 is capable of forming homooligomers and is both necessary and sufficient for caveolae formation, whereas Cav2 requires Cav-1 expression in order to localize to the plasma membrane and is not capable of

forming caveolae without heterodimerization with Cav1 (Razani et al., 2001a, 2001b; Mora et al., 1999).

The recruitment of membrane proteins to caveolae allows for caveolin-mediated removal of proteins from the cell membrane that can play a role in regulating signaling pathways (Sowa, 2012; Busija et al., 2016). In this vein, Cav1 and 2 have been studied in other tissues for their ability to regulate both TGF- β and BMP signaling through their respective receptors.

Cav1 enriched caveolae have been shown to recruit TGF- β receptor I and endocytose the receptor for degradation (Di Guglielmo et al., 2003) which leads to a subsequent reduction in the phosphorylation of smad2/3 (Razani et al., 2001). Cav2 seems to perform a similar function as it is capable of reducing smad2/3 phosphorylation and promoting TGF- β receptor I turnover, and interestingly Cav2 was shown to carry out this function independently of Cav1 (Xie et al., 2011). As a result, Cav-2 overexpression protects endothelial cells from the proliferation inhibition caused by exogenous TGF- β treatment by removing TGF- β receptor I from the membrane (Xie et al., 2011).

Caveolins have similarly been observed to impact BMP signaling. Cav1 is capable of binding BMP Receptor I and II to recruit them to caveolae for endocytosis, and subsequently inhibits smad1/5/8 (Nohe et al., 2004). The recruitment of smurf2 to caveolae has been suggested as a mechanism by which caveolins inhibit TGF- β and BMP signaling (Di Guglielmo et al., 2003; Hiepen et al., 2020), since smurf2 binding has been shown to induce TGF- β receptor

degradation (Kavask et al, 2000; Di Guglielmo et al., 2003) target smads for degradation (Zhang et al., 2001) and inhibit BMP signaling (Kushioka et al., 2020).

The function of caveolins in the human esophagus has not yet been explored. The specific and prominent expression of Cav1 and Cav2 in basal cells, the importance of TGF- β and BMP inhibition in maintaining proliferative and stem cell fates in esophageal epithelium, and ability of caveolins to regulate these pathways in other tissues suggest that caveolins could be playing a similar role in the esophagus. Additionally, the overexpression of Cav1 and Cav2 (Ando et al., 2007) as well as the inhibition of TGF- β /Smad2 (Fukuchi et al., 2006) have both been independently correlated with esophagus squamous cell carcinoma. This further indicates that Caveolins might be serving to maintain stem-like proliferative states in the esophagus.

Based on these findings I hypothesized that caveolins could be serving to endocytose and inhibit TGF- β and BMP receptors in the basal zone of the esophagus epithelium to maintain a stem cell fate. Upon leaving the stem cell niche of the basal zone caveolin expression ceases, allowing TGF- β and BMP receptors to accumulate on the cell membrane and subsequently induce smad signaling, transitioning cells out of the stem cell fate and towards differentiation as they migrate apically. My original thesis proposal included performing conditional knockdown and overexpression of Cav1 and Cav2 in our *in vitro* model. I predicted that the knockdown of Cav1/2 would reduce basal stem cell populations and increase the proportion of transitional and differentiated cell types, whereas overexpression would cause the inverse. I predicted that the knockdown could similarly reduce cell proliferation rates, but the existence of KI67+ proliferative populations outside of the Cav1/2+ basal zone complicates the connection between

caveolins and proliferation. Additionally, I wanted to perform immunohistochemistry and immunoblotting to evaluate levels of TGF- β receptor I with phosphorylated Smad 2/3 and BMP receptors with phosphorylated Smad1/5/8, expecting to see an inverse relationship between their protein levels and Cav1/2 expression.

It should be noted that caveolin deficient mice did not show any esophagus defects (Razani et al., 2001a, 2001b). However, caveolins are expressed apically in the mouse esophagus rather than localizing to the basal zone, ruling out the potential for them to function in stem cell maintenance in the mouse esophagus. This difference in expression pattern alongside the general differences in morphology of the mouse esophagus mean that caveolins could be playing a different role in the mouse and human esophagus and therefore produce differing phenotypes upon loss of caveolin function. It is therefore still valuable to explore caveolins in the human esophagus.

Methods

Patient Sample collection

Histologically healthy esophageal squamous epithelium biopsies were obtained alongside non-dysplastic Barrett's esophagus (BE) from consenting Men and Women during upper endoscopy procedures for BE screening at the University of Michigan Health System. All samples were collected between 2017 and 2020 using procedures approved by the University of Michigan institutional review board (IRB). Samples used for scRNA-seq were dissociated immediately. Samples that were used for immunofluorescence were fixed in paraformaldehyde as described below. Remaining tissue was either immediately processed for cell culturing or cryopreserved and stored at -80°C until being thawed and processed for culturing. For cryopreservation, tissue was minced and froze in HYENAC media with 20% CBS serum and 10% DMSO. Vials were placed in Mr. Frosty's containers at -80°C overnight before being moved to liquid nitrogen for storage.

During this process fresh biopsies that were processed immediately could be grown at a nearly 100% success rate. When kept in HYENAC media with 20% CBS biopsies could be stored 4°C for 24 hours before successful culturing and could even produce viable cultured cell lines after being shipped. To find more detailed protocols visit: <https://www.umichtml.org/protocols>.

Patient Samples Identification

All patients samples are denoted by their Human Tissue (HT) number which was assigned based on when the tissue was received. The race of all patients was determined by

self-identified reporting. The designation of European American (EA) was used for patients who self-identified as White-Non Hispanics (W-NH), while African American (AA) was used for patients who self-identified as Black. All experiments conducted utilized the normal squamous biopsies from these patients as characterized by the physicians who performed the biopsy procedures.

Whole Mount Immunofluorescence Staining

Plates were processed and stored in PBS at 4°C prior to staining. PBS was removed and the cells were cross-linked for 20 minutes at RT using 500µL of 10% phosphate buffered formalin. The wells were washed with 500µL TBS and the cells were then permeabilized using -20°C 100% methanol. They were then incubated for one hour in 500µL of blocking buffer (5% goat serum, 1% BSA, and 0.2% Triton x100 in TBS). Primary antibodies [CAV1 (rabbit monoclonal isotype - Sigma, Cat. No. HPA049326); CAV2 (rabbit monoclonal isotype - Sigma, Cat. No. HPA044810); COL17A1 (rabbit monoclonal isotype - Sigma, Cat. No. HPA043673); ECAD (mouse monoclonal isotype - BD Biosciences Cat. No. 610181)(rat monoclonal isotype - Thermo Fisher Cat. No. 13-1900); KI67 (mouse monoclonal isotype - BioLegend Cat. No. 652402); KRT4 (rabbit monoclonal isotype - Atlas Cat. No. HPA034881); LY6D (rabbit monoclonal isotype - Atlas Cat. No. HPA024755); TP63 (biotinylated goat monoclonal isotype - R&D Biosystems Cat. No. BAF1916)(rabbit monoclonal isotype - Cell Signaling Technologies Cat. No. 13109); VIM (goat monoclonal isotype - Santa Cruz BioTechnologies Cat No. sc-7558); GSTT2 (mouse monoclonal isotype - Santa Cruz, Cat. No. 514667); γ -H2AX (Anti-phospho-Histone H2A.X (Ser139) mouse monoclonal isotype - Millipore, Cat. No. 05-636)] were diluted (γ -H2AX 1:1500, GSTT2-1:500, all else 1:1000) in TBS with 1% BSA

and applied to the cells (150 μ L per well) for overnight incubation in a humidified chamber at 4°C. The wells were then washed with TBS-T three times for 5 minutes and incubated with secondary antibodies at room temperature for one hour. Secondary antibodies and DAPI (1:1000) were diluted in 1% BSA. After incubation wells were washed in TBS-T three times for 5 minutes. Coverslips were removed from the wells and mounted onto slides with ProLong Gold. Slides were stored at -20°C.

Paraffin sectioning and staining

Fresh biopsies were fixed overnight in 4% paraformaldehyde (Sigma). Biopsies were then washed with 1X PBS and dehydrated in alcohol. This included 30 minutes (each) in 25%, 50%, and then 75% methanol, PBS/0.05% Tween-20, 100% methanol, 100% ethanol and 70% ethanol in the order listed. An automated tissue processor (Leica ASP300) was used to paraffinize the tissue. Resulting paraffin blocks were sectioned into 7 μ m sections and placed on microscope slides.

For immunohistochemical staining slides were rehydrated in histoclear (two 5 minute changes) followed by two 3 minute changes in 100% ethanol, 95% ethanol, 70% ethanol, 30% ethanol, and two five minute changes in deionized H₂O. Slides were then incubated in a vegetable steamer for 40 minutes in 1X sodium citrate buffer for antigen retrieval. Slides were then washed in PBS and permeabilized with 0.1% TritonX-100 in 1X PBS for 10 minutes. Blocking solution was composed of 0.1% Tween-20, 5% normal donkey serum in 1X PBS and applied to slides for one hour. Primary antibodies were diluted in the blocking solution and applied to slides overnight in a humidified chamber at 4°C. The next day slides were washed

with 1X PBS three times for 5 minutes. Secondary antibodies and DAPI (1:1000) were diluted in blocking buffer and applied to slides for one hour at room temperature in a humidified chamber. The slides were washed with 1X PBS three times for 5 minutes before coverslips were mounted using ProLong Gold. Slides were stored at -20°C.

H&E (Hematoxylin and Eosin) staining of esophagus biopsies was carried out in accordance with manufacturer protocols using Harris Modified Hematoxylin (Fisher Scientific) and Shandon Eosin Y (Thermo Scientific).

Imaging and image processing

A Nikon A-1 confocal microscope was used to image all immunofluorescent samples. Adjustments of brightness and contrast, as well as cell counting were performed using ImageJ (National Institute of Health, USA) with any adjustments applied uniformly for all images in an experiment.

qRT-PCR validation

RNA was isolated from cultured tissue using the QIAzol RNA Lysis Reagent (Qiagen, Valencia, CA), RNeasy spin columns (Qiagen) with on-column DNase I incubation following manufacturer's instructions. Reverse-transcriptase was used to convert isolated RNA into cDNA according to manufacturer instructions (Life Technologies, Grand Island, NY). RT-PCR reactions were run with a total volume of 20 µl, containing 2 µl 10x RT buffer, 2 µl 10x RT primers, 0.8 µl 25x dNTPs, 1 µl reverse transcriptase, 1 µl RNase inhibitor, and 2 µg containing 200 ng of RNA, depending on RNA concentration after extraction. The products were then diluted to 3 ng/µl. The

ABI PRISM® 7900HT Sequence Detection System was used to run samples in accordance with manufacturer's instructions with thermal cycles containing initial denaturation (10 minutes at 95°C) followed by 40 cycles of denaturation (12 seconds at 95°C), annealing (1 minute at 60°C) and extension (1 minute at 60°C). To calculate the fold-change of relative expression for each gene the $\Delta\Delta$ -Ct method was utilized (Livak and Schmittgen, 2001).

Immunoblotting

Cells were cultured as described above and were washed with PBS before processing. The wells were treated with Cell Signaling protein lysis buffer (40ul, 1X) containing proteinase inhibitor cocktail (20ul per mL) before cells were scraped and transferred to 1.5 ml tubes. The samples were centrifuged at 2000 rpm for 20 minutes at 4°C, and supernatant was extracted and protein quantification was performed using protein assay. The lysates were then prepared with 20% 2-mercaptoethanol and 4X sample buffer at a concentration of 20µg. The protein was run for 2 hours at 125-126V in SDS Novex gels (4-12% gradient)(Invitrogen). Nitrocellulose membranes were activated for 1 minute with 100% methanol. The transfer was run overnight at 12V using Novex Transfer Buffer, after which Ponceau red was used to stain the membranes for assessment and transfer efficiency. For blocking the membranes were incubated for 1 hour at room temperature in either 5% milk/TBST or 5% BSA/TBST. Primary antibodies (GSTT2 mouse monoclonal isotype IgG2a κ - Santa Cruz, Cat. No. 514667) were diluted in 5% milk/TBST or 5% BSA/TBST and incubated with the membranes overnight at 4°C. The membranes were then washed with TBS-T for 5 minutes three times before being treated for 1 hour at room temperature with secondary antibodies diluted in 5% milk/TBST. The membranes

were washed with TBS-T for 5 minutes three times and then incubated with ECL for 5 minutes. X-ray film was then used to reveal the westernblotts.

GSTT2 siRNA and Cum-OOH treatment

The immortalized esophageal squamous mucosa cell line HET1A was used for siRNA experiments. The cells were cultured on top of four 6mm coverslips within 35mm x 10mm polystyrene plates (Cat. No 430165)(Corning Incorporated, NY) with 10% fetal bovine serum and 1% antibiotic- antiseptic in Dulbecco's Modified Eagle's Medium (DMEM)(Cat. No. 11965092). Samples were plated with 120,000 cells per plate. Four siRNA constructs were tested for their ability to knockdown GSTT2 expression: siRNA-05 GCUAAGGAUGGUGAUUUC; siRNA-06 GCACCGUGGAUUUGGUCAA; siRNA-07 AGGCUAUGCUGCUUCGAAU, siRNA-08 GACACUGGCUGAUCUCAUG, Dharmacon. Cells were treated with a 10nm concentration of either GSTT2 targeting siRNA, control non-target (NT) siRNA, or the OPTI-MEM plus RNAimax (mock control)(Invitrogen). On day one Lipofectamine® RNAiMAX Transfection Reagent (as directed by RNAiMAX protocol, Cat. No. 13778030 (Thermo Fisher)) was used to transfect the samples in the following groups: mock, NT, si05, si06, si07, and si08. The next day the media was removed and the transfection protocol was repeated. On the third day the transfection media was removed and replaced with the original culturing media, after which the cells were incubated for 48 hours. The coverslips were then removed and individually placed into wells of a 24-well plate where they were treated for 1 hour with either 0 μ M or 100 μ M of cumene-OOH (247502 Sigma-ALDRICH). The treatment was removed and cells were fixed using -20 °C 100% methanol for 20 minutes. They were then

washed three times for 5 minutes each with PBS and stored at 4°C in PBS until immunofluorescence was performed.

Bile Acid Exposure High-Content Imaging

(Collaboration with Charles Zhang from Jonathan Sexton's Lab)

Cells were cultured at an initial seeding density of 2,000 cells/well on PerkinElmer CellCarrier-384 Ultra Microplates and grown for 48 hrs to 80% confluence. Cells were then treated with a 10-point, 1:3 serial dilution, dose range of a bile acid mixture from 0 to 3000 μ M with 4 well-level replicated. After 48 hrs of treatment, cells were fixed and stained with Hoechst 33342, Cell Mask Deep Red (nuclei and cell boundary segmentation), and NFkB (cell stress).

CellProfiler was used for automated nuclei and cell segmentation followed by measurements of morphological features (size and shape), intensity and distribution of stains amassing 1,400 unique measured features per-cell. These measured features were normalized and filtered for low-variance and high-correlation with Konstanz Information Miner (KNIME). Random forest models were designed based on a regression model of untreated cells vs 3000 μ M treated cells, and individual cells were scored along this model. A 80/20 split was done where 20% of the data was used in model creation and 80% for model validation. Per-well averages were obtained and plotted vs actual treatment value in GraphPad Prism 8. IC₅₀ values were calculated using GraphPad's nonlinear regression curve fitting.

DNA Isolation

The Qiagen DNeasy Blood & Tissue Kit was used to extract genomic DNA from patient esophagus biopsies and tissue culture following manufacturer's instructions. Samples were treated in 1.5 ml tubes with 800 µl of 100% ethanol and QIAzol followed by centrifugation at 4°C for 30min at 1200 rpm. 10 µl of proteinase K (10 mg/µl) were added, followed by 25 µl of 10% SDS after which samples were incubated at 56°C overnight. 500 µl of tris-extracted phenol were added and samples were inverted and incubated for 3 hours at room temperature. Samples were then centrifuged at 3500 rpm for 20 minutes at room temperature and the aqueous layer was transferred to new tubes. 2 µl of sterile 5 M NaCl and 800 µl of ice-cold ethanol were added to each tube causing DNA to precipitate. Samples were then centrifuged at 12000 rpm for 15 minutes at 4°C. The tubes were air-dried and resuspended in 50 µl of TE buffer then incubated at 4°C overnight. DNA concentration and purity was determined using a NanoDrop spectrophotometer.

***GSTT2B* deletion genotype analysis.**

The genotype of the *GSTT2* deletion was analyzed via PCR utilizing the three-primer set as described previously (Zhao *et al.*, 2009): *GSTT2B*-6858 5'-CACTCAACACAGTAGCCTCATCGTG-3', *GSTT2B*-6857 5'-TGCCTCCCCTGCCTTATTTC-3', and *GSTT2B-2B* 5'-CCTTCTGAAATGGAGCCTTTG-3'. The 25 µl PCR reaction contained 12.5 µl of Promega GoTaq Green Master Mix, 10 pmol of the *GSTT2B*-6858 and *GSTT2B*-6857 primers, 12 pmol of *GSTT2B-2B*, and 25 ng of DNA for a total volume of 25 µl. The reaction was performed with an initial 2 minute denaturation at 95°C, then 35 cycles containing a 30 second denaturation at

95°C, a one minute annealing at 60°C, and a 45 second extension at 72°C. Electrophoresis was then performed on the products in a 2% agarose gel in 1X TBE buffer. Invitrogen's 100 bp DNA ladder was used as a standard and ethidium bromide was used for DNA visualization.

***GSTT2/2B* promoter duplication analysis.**

The genotype of the *GSTT2* 17 bp promoter duplication was analyzed via PCR utilizing the standard primers from Marotta *et al* (2012). The 25 µl PCR reaction contained 12.5 µl of Promega GoTaq Green Master Mix, 10 pmol of the *GSTT2B*-6858 and *GSTT2B*-6857 primers, 12 pmol of *GSTT2B-2B*, and 25 ng of DNA for a total volume of 25 µl. The reaction was performed with an initial 2 minute denaturation at 95°C, then 35 cycles containing a 30 second denaturation at 95°C, a one minute annealing at 60°C, and a 45 second extension at 72°C. Electrophoresis was then performed on the products in a 2% agarose gel in 1X TBE buffer. Invitrogen's 100 bp DNA ladder was used as a standard and ethidium bromide was used for DNA visualization.

Single Cell Sequencing

Tissue Dissociation (Yu-Hwai Tsai and Angeline (Fei-Fei) Wu)

Patient biopsies were handled in ice-cold 1X HBSS (with Mg²⁺, Ca²⁺) and all tubes and pipettes were pre-washed with 1% BSA in 1X HBSS to prevent adhesion. Biopsies were placed in a petri dish with 1X HBSS and minced manually using a scalpel and spring-squeeze scissors. The minced tissue was then put in a 15 mL conical tube containing 1% BSA in HBSS, centrifuged at 10°C for 5 minutes at 500G, and excess HBSS was removed. The Neural Tissue Dissociation Kit (Miltenyi, 130-092-628) was used for dissociation reagents. Mix 1 was added

and incubated for 15 minutes at 10°C. Mix 2 was then added and the samples were fluxed through P1000 pipette tips. Fluxing was repeated with 10 minute intervals of incubation at 10°C in between until clumps of cells were no longer visible under a stereo microscope. A 70µm filter was coated in 1% BSA and used to filter samples in 1X HBSS, which were then centrifuged at 10°C for five minutes at 500G. 500µl of 1X HBSS was added to the resulting product for resuspension followed by 1mL of RBC Lysis Buffer (Roche, 11814389001). The tubes were incubated for 15 minutes at 4°C on a rocker. Samples were centrifuged at 10°C for five minutes at 500G and then washed in 2mL of 1% BSA. Centrifugation and washing was then repeated twice. Cells were counted with a hemocytometer, and subsequent rounds of centrifugation and resuspension were applied as needed to reach a concentration of 1000 cells/µL. Cells were kept on ice.

Single-cell library preparation

The 10x Chromium at the University of Michigan Advanced Genomics Core facility was used to create single cell droplets with a target of capturing 5,000-10,000 cells. The Chromium Next GEM Single Cell 3' Library Construction Kit v3.1 prepared single cell libraries according to manufacturer instructions.

Sequencing Data Processing and Cluster Identification

(Performed by Joshua Wu)

Overview

All single cell RNA sequencing was conducted on the Illumina Novaseq 6000 through the University of Michigan Advanced Genomic Core. The 10x Genomic Ranger was used to

construct gene matrices from raw data based on the human reference genome (hg19). Previously described protocols for single cell analysis were conducted on python (Wolf et al., 2018), for which filtering parameters were implemented based on mitochondrial transcript fraction, unique molecular identifier (UMI) counts, and gene count range. This filtering removed genes with high variability, the remaining genes were log normalized, and mitochondrial fractions and UMI variability were linearly regressed. Gene expression datapoints were then transformed via Z-transformation, and tissue was screened by organ-specific filtering. Clustering of principal components (10-11) in a graph-based manner was performed by the previously described UMAP algorithm (McInnes et al., 2018) along with the Louvain algorithm within Scanpy at a 0.6 resolution (Blondel et al., 2008).

Sequencing data and processing FASTQ reads into gene expression matrices

All single cell RNA sequencing was conducted on the Illumina Novaseq 6000 through the University of Michigan Advanced Genomic Core. The 10x Genomic Ranger was used to construct gene matrices from Illumina base calls based on the human reference genome (hg19). To remove unique molecular identifiers and adaptor sequences before alignment, base call files were demultiplexed. This yielded a matrix of filtered feature barcodes for each sample that composed only of detectable genes.

Quality control

Filtering was performed to remove individual cells expressing too many (>7500) or too few (<500) genes or possessing greater than 20% mitochondrial genes.

Normalization and Scaling

After the extraction of highly variable genes, log normalization was conducted on the per cell read count matrices. Effects from individual cell's fraction of mitochondrial transcript and read counts were regressed out based on Scanpy's simple linear regression, after which z-transformation was used to scale the data.

Variable Gene Selection

The selection of highly variable genes was performed by splitting mean gene expression values (log normalized) into 20 bins of equal-width, for which normalized variance-to-mean values were determined. The thresholds of between 0.125 and 3 for normalized mean expression levels and above 0.5 for normalized dispersion values were used to designate genes as highly variable for removal.

Batch Correction

Differences in batches based on technical features such as differences in sample collection and timing were mitigated by the previously described batch balanced k nearest neighbors (BBKNN) python package (Polański et al., 2020). Alternatively, to the k nearest neighbors' algorithm, BBNKK's procedure that initially splits the data into batches based on technical artifacts. Nearest neighbors are calculated for each individual cell per independent batch, which facilitates the identification of similarities between cells from different batches while keeping the PCA space unaffected.

Dimension Reduction and Clustering

The top principal components were used to calculate a neighborhood graph based on the nearest neighbors from the filtered expression matrix. When needed, BBKNN was used for three neighbors per matched based on the top 50 principal components before application of the UMAP algorithm for two-dimensional visualization. The Louvain algorithm then identified clusters (0.2-0.4 resolution).

Cluster Annotation

Well established gene markers were used to identify the general identity of the clusters. Epithelial identify was marked by *CDH1*, mesenchymal identity by *VIM*, immune identity by *CD53*, *VAMP8*, *CD48*, *ITGB2*, endothelial identity by *ESAM*, *CDH5*, *CD34*, *KDR*, and neuronal identity by *POSTN*, *S100B*, *STMN2*, *ELAV4*.

Sub-clustering

Once initial clusters were formed using UMAP embedding, some clusters were analyzed for additional sub-clustering. The data from cells within these clusters were extracted from the original data set that had been filtered but not yet normalized. This extracted matrix of the sub-population of cells then underwent the subsequent steps of analysis as detailed above to yield a two-dimensional UMPA embedding.

Contributions

The work for this thesis was highly collaborated and relied upon contributions from several members of the Spence Lab and Sexton Lab at the University of Michigan. The following list describes individual contributions for each figure to ensure that work is properly credited for everyone who helped generate the data presented.

Figure 1 (unpublished data)

Sample collection and processing by Dayha Ferrer-Torres and **Max Hammer**
Tissue dissociation by Yu-Hwai Tsai and Angeline (Fei-Fei) Wu
scRNAseq data analysis by Joshua Wu

Figure 2 (unpublished data)

Sample collection and processing by Dayha Ferrer-Torres and **Max Hammer**
Tissue dissociation by Yu-Hwai Tsai and Angeline (Fei-Fei) Wu
scRNAseq data analysis by Joshua Wu
Immunofluorescent staining and imaging by **Max Hammer**

Figure 3 (unpublished data)

Immunofluorescent staining and imaging by **Max Hammer**

Figure 4 (unpublished data)

H&E staining and imaging by **Max Hammer**
Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**
Tissue dissociation by Yu-Hwai Tsai and Angeline (Fei-Fei) Wu
scRNAseq data analysis by Joshua Wu
Ecad expression quantification by Daysha Ferrer-Torres

Figure 5 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**
Tissue dissociation by Yu-Hwai Tsai and Angeline (Fei-Fei) Wu
scRNAseq data analysis by Joshua Wu
Immunofluorescent staining and imaging by **Max Hammer**
Quantification of P63/KI67 expression by **Max Hammer**

Figure 6 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**
Immunofluorescent staining and imaging by **Max Hammer**
Quantification of P63/KI67 expression by **Max Hammer**
Quantitative RT-PCR by **Max Hammer**
Quantification of KRT4 expression by **Max Hammer**

Figure 7 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**
High content morphological screening protocol by Charles Zhang

High content screening data analysis by Charles Zhang

Figure 8 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**

Tissue treatment by Daysha Ferrer-Torres

Immunofluorescent staining and imaging by **Max Hammer**

Quantification of γ H2AX foci by Dayha Ferrer-Torres and **Max Hammer**

Figure 9 (A and C unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**

Quantitative RT-PCR by Daysha Ferrer-Torres (Ferrer-Torres et al., 2019)

Tissue treatment and density measurements by Dayha Ferrer-Torres

Figure 10 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**

Tissue treatment by Daysha Ferrer-Torres

Immunofluorescent staining and imaging by **Max Hammer**

Quantification of Ecad and P63 expression by **Max Hammer**

Figure 11 (data published in Ferrer-Torres et al., 2019 and Daysha Ferrer-Torres' PhD Thesis)

Tissue processing and culturing by Dayha Ferrer-Torres

Tissue treatment by Daysha Ferrer-Torres

Immunofluorescent staining and imaging by Daysha Ferrer-Torres and **Max Hammer**

Quantification of γ H2AX foci by Dayha Ferrer-Torres and **Max Hammer**

Figure 12 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**

Immunofluorescent staining and imaging by **Max Hammer**

Immunoblot by Daysha Ferrer-Torres

Figure 13 (unpublished data)

Genotyping by Daysha Ferrer-Torres and **Max Hammer**

Figure 14 (unpublished data)

Tissue processing and culturing by Dayha Ferrer-Torres and **Max Hammer**

Tissue treatment by Daysha Ferrer-Torres

Immunoblot by Daysha Ferrer-Torres

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