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Development and Characterization of an Organotypic Model of Barrett's Esophagus

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Abstract

Understanding the molecular and cellular processes underlying the development, maintenance, and progression of Barrett's esophagus (BE) presents an empirical challenge because there are no simple animal models and standard 2D cell culture can distort cellular processes. Here we describe a three-dimensional (3D) cell culture system to study Barrett's esophagus. BE cell lines (CP-A, CP-B, CP-C, and CP-D) and esophageal squamous keratinocytes (EPC2) were cultured on a matrix consisting of esophageal fibroblasts and collagen. Comparison of growth and cytokeratin expression in the presence of all-trans retinoic acid or hydrochloric acid was made by immunohistochemistry and Alcian Blue staining to determine which treatments produced a BE phenotype of columnar cytokeratin expression in 3D culture. All-trans retinoic acid differentially affected the growth of BE cell lines in 3D culture. Notably, the non-dysplastic metaplasia-derived cell line (CP-A) expressed reduced squamous cytokeratins and enhanced columnar cytokeratins upon ATRA treatment. ATRA altered the EPC2 squamous cytokeratin profile towards a more columnar expression pattern. Cell lines derived from patients with high grade dysplasia already expressed columnar cytokeratins and therefore did not show a systematic shift toward a more columnar phenotype with ATRA treatment. ATRA treatment however did reduce the squamoid-like multilayer stratification observed in all cell lines. As the first study to demonstrate long term 3D growth of BE cell lines, we have determined that BE cells can be cultured for at least three weeks on a fibroblast/collagen matrix and that the use of ATRA causes a general reduction in squamous-like multilayered growth and an increase in columnar phenotype with the specific effects cell-line dependent.

Keywords

ATRA; 3D culture; Acid pulse; Cytokeratins; Organotypic reconstruct

Introduction

Mechanistic studies of cancer initiation, progression, and therapeutic discovery require cell culture and/or animal model systems. Cell culture systems have typically grown cells on plastic, and often these results conflict with *in vivo* experiments. 2-dimensional culture models do not capture many effects of the tissue microenvironment, including cell morphology, polarity and junctions, interactions with the extracellular matrix (ECM) and adjacent cells, and mechanotransduction, which affect intracellular signaling and cell fate (Nelson and Bissell, 2006). 3-dimensional (3D) co-culture, consisting of a substratum prepared with collagen and fibroblast upon which epithelial cells are seeded, better imitates the *in vivo* tissue microenvironment, and may help to bridge the gap between traditional cell culture systems and animal studies.

Interest in the premalignant condition Barrett's Esophagus (BE) stems from its association with esophageal adenocarcinoma (EA). BE is the only known precursor of EA, increasing the relative risk of progression 30-120-fold (Haggitt, 1994). The incidence of EA among individuals with BE is 6–7 per 1000 person-years (Thomas et al., 2007, Yousef et al., 2008). BE is a condition of the esophagus associated with acid and bile reflux where normal multilayer, stratified esophageal squamous tissue is replaced by a single layer of columnar cells with a crypt architecture (specialized intestinal metaplasia) (Reid et al., 2010). The presence of mucous-producing goblet cells in BE is thought to protect the esophagus from insult of acid and bile (Reid et al. 2010). However, this metaplasia increases a patient's risk of cancer, likely because constant reflux increases cellular proliferation, telomere shortening, and DNA damage, resulting in chromosomal instability and genomic alterations (Reid et al. 2010).

Building on 3D studies of skin and normal esophageal epithelium (Kalabis et al., 2003, Okawa et al., 2007), we provide the first 3D cell culture model of BE, allowing us to overcome some shortcomings associated with other models. Currently, the major BE animal model is a surgical anastomosis model in the rat, which creates reflux by connecting the jejunum of the small intestine with the distal esophagus (Su et al., 2004), allowing the development of intestinal metaplasia. This model has various limitations, including atypical bile reflux, species differences, duration, expense and the requirement of surgical expertise. Other studies have used explant tissue biopsies to study BE in more physiological conditions compared with 2D cell culture (Ouatu-Lascar et al., 1999), (Chang et al., 2007), however, biopsies are viable for only a short period of time *ex vivo*, preventing observation of cellular transformation, such as goblet cell formation, under different culture conditions.

To develop this organotypic reconstruct model, we grew hTERT-immortalized BE cell lines, derived from BE patients (Palanca-Wessels et al., 1998); (Palanca-Wessels et al., 2003) and a control normal squamous esophageal cell line, EPC2-hTERT (Harada et al., 2003), on a fibroblast-collagen matrix. The reconstructs were examined for the presence of goblet cells and various squamous and columnar cytokeratin markers, a reliable tool to describe cell type (Nurgalieva et al., 2007). Proper expression of cytokeratins in cell culture depends strongly on the method for culturing, including medium type, serum content, calcium concentration, and the presence of a feeder layer (Biddle and Spandau, 1996; Paladino et al., 2004). Here, we studied the effects of acid pulses to simulate gastric reflux and continuous exposure to all-trans retinoic acid (ATRA) on expression of cytokeratins and morphology of BE cells in 3D culture.

Materials and Methods

Cell lines

Four hTERT-transformed Barrett's esophagus cell lines, CP-A (p53 wildtype, 9p loss of heterozygosity (LOH), 5q LOH), CP-B (17q LOH, 9p LOH), CP-C (17q LOH, 9p LOH), and CP-D (17p LOH, 9p homozygous deletion) (Palanca-Wessels et al., 1998; Palanca-Wessels et al., 2003) were obtained from University of Washington, Seattle, WA (gift from P. Rabinovich). Cells were adapted to serum-free conditions in keratinocyte serum-free medium (KSFM, Invitrogen). BE cell line identities were verified with the STR-based Identifiler PCR Amplification kit (Applied Biosystems) by comparison to original samples. EPC2-hTERT and primary fetal esophageal fibroblasts have been described previously (Harada et al., 2003; Okawa et al., 2007).

Cell culture

Organotypic culture was performed as previously published for esophageal squamous cell reconstructions (Okawa et al., 2007) with some modifications. The fibroblast feeder layer was incubated in bovine collagen for 4 days, after which 5×10^5 epithelial cells were seeded on top. Media used for epithelial cell growth on the feeder layer was the same as previously published (Okawa et al., 2007) with 2% FBS (Atlantic Biologics). Fresh medium was added every 2–3 days. After seeding of the epithelial cells, medium was maintained on both the top and bottom of the 0.4 μ m polycarbonate tissue-culture treated transwell membrane (Corning) for an additional 5 days, after which the media on top of the transwell was removed, while maintaining media on the bottom. At this point, epithelial cells were exposed to a liquid-air interface for the remainder of the experiment to enhance differentiation. Organotypic cultures were sampled weekly, fixed in 10% neutral buffered formaldehyde and embedded in paraffin for H&E and Alcian Blue staining.

All-trans retinoic acid organotypic culture

Culture conditions were as described above with some alterations (Supplemental Methods). ATRA was supplemented in culture medium at a concentration of 0.33 μ M and replenished every 2 days.

Acid pulsing on organotypic culture

Five days after epithelial seeding media was removed and replaced with pH 3.5 PBS for 1 hour every other day (3 days per week) in the top and bottom chambers. The acid was washed out of the chambers with PBS and fresh media was replaced. Samples were taken for fixation 24 hours after the last pulse. Week 1 samples received 3 pulses, week 2 received 6 pulses, and week 3 received 10 pulses total.

Immunohistochemistry

Immunohistochemical staining was performed on 5 μ m sections of paraffin embedded reconstructions. Overnight incubation with primary antibodies was done at 4°C with the following: cytokeratin 13 (NovaCastra/Leica Microsystems, Bannockburn, IL), cytokeratin 19 (TROMA-III, University of Iowa Developmental Studies Hybridoma Bank), cytokeratin 8 (RDI-PRO61038, Research Diagnostics, Acton, MA), and cytokeratin 14 (PRB155P, Covance, Princeton, NJ). Slide development was completed using DAB substrate kit for peroxidases (Vector Laboratories, Burlingame, CA), with development times monitored with a dissecting microscope. Haematoxylin counterstain was then applied. In order to determine the specificity of an antibody towards Barrett's esophagus in 3D culture, each antibody was tested on samples of Barrett's metaplasia, gastric and squamous tissue from 4 patients provided by the laboratory of Dr. Brian Reid (Fred Hutchinson Cancer Research

Center, Seattle, WA). Antibodies were chosen based on specificity towards BE or squamous tissue. Slides were mounted with cytomount, and imaged using a Nikon E600 Upright microscope with ACT-1 software. Multiple images from each slide were taken and independently evaluated. REK, KLG, and LMFM independently scored each image based on proportion of positive cells and intensity of staining. Any discrepancies were resolved by examination of the original slides and, when necessary, repeating the staining procedure. Similarities of 3D BE cell culture with metaplastic BE tissue were evaluated by comparing patient biopsies of Barrett's esophagus with 3D culture of BE using both IHC for specific columnar and squamous cytokeratin expression, hematoxylin and eosin for tissue architecture and Alcian blue for the presence of goblet cells.

Results

The patient-derived BE cell lines CP-A, CP-B, CP-C, and CP-D grown on a fibroblast/collagen matrix vary widely in their phenotype and growth pattern. Co-culture of epithelial cells with fibroblast-embedded collagen allows for *in vitro* growth with more similarities to *in vivo* tissue than with 2D growth. CP-B and CP-D cells, both derived from a region of high-grade dysplasia, grow as an even multilayered stratified epithelium, similar to squamous keratinocytes, but without keratin production (Figure 1A, C, E). Additionally, CP-D cells have a mildly invasive phenotype when grown on a fibroblasts/collagen matrix. CP-C cells are also derived from a region of high-grade dysplasia but typically do not form a multilayered stratified epithelium like CP-B and CP-D over the 3-week course of the experiment. However occasionally there are small patches where CP-C cells grow in more than one layer, possibly due to patchiness of seeding (Figure 1D). The remaining cell line, CP-A, derived from a region of non-dysplastic metaplasia, is distinct from the other cell lines (Figure 1B). CP-A cells grown in 3D culture form goblet cells, invade into the matrix, and express both squamous and columnar cytokeratins.

BE cell culture on a fibroblast/collagen matrix was adapted to culture conditions originally developed for keratinocyte growth (Okawa et al., 2007). Therefore in this study we identify growth conditions necessary for the appropriate culture of BE cell lines to simulate *in vivo* growth. BE cells *in vivo* grow as a single layer of columnar epithelium with crypt architecture, with some goblet cells depending on the state of disease. BE cells cultured under conditions developed for keratinocyte growth resulted in a stratified epithelium. This study attempts to modify the stratified epithelium and squamous cytokeratin expression profile seen in control conditions with the differentiation factor, all-trans retinoic acid (ATRA), and physiologically by recreating gastric reflux with acid pulsing to promote more typical BE growth.

Effect of all-trans retinoic acid (ATRA)

We evaluated the effect of all-trans retinoic acid, the irreversibly oxidized form of vitamin A, on BE cells in 3D culture over 3 weeks for expression of squamous and columnar cytokeratins, goblet cells and altered cell morphology. Cells exposed to ATRA did not grow in the typical stratified pattern as seen in the absence of ATRA (Figure 1; Supplemental Figure 1). Without ATRA exposure, most cell lines formed a thick epithelium, as much as 10 cells thick (Figure 1 –ATRA). With the addition of ATRA, the squamoid stratified 3D structure of these cells was inhibited, and most cell lines (with the exception of CP-D) grew only 1–2 cells thick on the fibroblast/collagen matrix (Figure 1 +ATRA). Even in the CP-D cell line, stratification was greatly reduced by ATRA treatment (Figure 1E +ATRA). EPC2 treated with ATRA resulted in reduced superficial keratin (Figure 1A). Additionally, keratin production was evident by week 3 in CP-A cultures without ATRA and absent with ATRA (Figure 1B, Supplemental Figure 1C). Goblet cells were detected in very abundant numbers in CP-A cells in control conditions, however with ATRA treatment, their numbers

diminished greatly (Figure 2). Reconstructs of cells derived from high-grade dysplasia lack goblet cells, consistent with the *in vivo* observation that BE goblet cells are typically present in the non-dysplastic metaplasia phase of BE and not in high-grade dysplasia (HGD) (Glickman et al., 2006).

Squamous Specific Cytokeratins and ATRA

CK13 and CK14 are typically expressed in the suprabasal layer and epidermis of squamous tissue, and therefore were used in this study to identify changes in the squamous phenotype with ATRA. The results demonstrate that at 3 weeks of growth on a fibroblast/collagen matrix with ATRA, EPC2 cells (Figures 3A) and CP-A cells (Figure 3B; Supplemental Figure 2A,B) show an appreciable decrease in squamous cytokeratin CK13 expression. It should be noted that there was no CK13 expression in CP-B, CP-C, and CP-D cells in control conditions, and no change in expression with ATRA was detected (Suppl. Figure 2C–E).

CK14 expression in CP-A cells is detected in control conditions, and completely abolished at three weeks with ATRA (Figure 3C). Contrary to our expectations, CP-B and CP-D both showed slightly increased expression of CK14 with ATRA at week 3 (Figure 3D, E). EPC2 expression of CK14 is not altered with ATRA treatment, and the CP-C cell line does not express CK14 (Supplemental Figure 2F, I). Taken together, these results indicate that ATRA can reduce the squamous phenotype observed in the CP-A cells in 3D culture. CP-A cells were the only BE cell line studied here which expressed squamous cytokeratins (CK13 and CK14) under control conditions.

Columnar Specific Cytokeratins and ATRA

Columnar cytokeratins 8 and 19 (CK8 and CK19) are expressed in glandular, non-squamous epithelium including Barrett's Esophagus (Boch et al., 1997), and therefore were used to analyze whether ATRA caused a shift towards a columnar phenotype in squamous and BE cells. Here, all 5 cell lines expressed CK19 under control conditions. ATRA treatment with CP-D cells resulted in decreased CK19 expression along with increased patchiness of expression throughout the reconstruct (Figure 4A). EPC2 keratinocytes express CK19 in the basal layers, when cultured with ATRA there is a reduction in stratified epithelium towards a single layer of CK19 positive cells (Figure 4B). Expression of CK19 was not affected by ATRA exposure in the CP-A, CP-B, and CP-C cell lines (suppl. Figure 3A–E).

Evaluation of cytokeratin 8 expression in BE cells demonstrated that cells from high-grade dysplasia (CP-B, CP-C, CP-D) express moderate to high levels of CK8, independent of ATRA treatment (Supplemental Figure 3F–H). CP-A cells express a constant low level of CK8 with ATRA during weeks 1 and 2, however by 3 weeks CP-A cells grown in the absence of ATRA also express CK8 (Figure 4D, E). Therefore, there are temporal changes in expression of columnar cytokeratins in 3D culture, supporting the hypothesis that long-term culturing of these cells may be critical to create a system more similar to *in vivo* conditions. Additionally, ATRA may play a role in transition from squamous to columnar phenotype as evidenced in the treatment on EPC2 cells. Typically, EPC2 cells express no CK8, however with ATRA treatment slight CK8 expression was detected (Figure 4C).

In summary, the effects of ATRA on cytokeratin expression and non-stratified growth are greatest on the growth pattern of CP-A cells derived from non-dysplastic metaplasia, rather than cells from high grade dysplasia. Changes observed in the metaplasia-derived CP-A cell line with ATRA indicate a transition from a squamous-like phenotype to a more columnar, BE-like phenotype. This effect is not generally applicable to cell lines derived from high grade dysplasia, though multilayer stratification was inhibited by ATRA in all cell lines.

Effect of pH 3.5 hydrochloric acid pulses

To study the effects of long-term acid reflux and BE *in vitro*, we cultured cells for 3 weeks on a fibroblast/collagen matrix to study morphological and cytokeratin changes to the CP-A cell line, derived from non-dysplastic metaplasia cells. Cultures were pulsed with pH 3.5 PBS for 1 hour every other day. Acidic PBS was added to the top and bottom of the transwell chamber. Reconstructs were measured for CK8, 13, 14, and 19 expression by IHC and morphology measured with H&E over the course of 3 weeks. Some morphological differences were observed with acid. Frequent acid insult to CP-A cells resulted in a temporary reduction in cell number and epithelial thickness, however, by week 3, there was no observable difference, indicating perhaps an adaptation to acid flux (Figure 5A, Supplemental Figure 4A–D, F–K).

Squamous cytokeratins and acid

As demonstrated in figures 3B and 3C, CP-A cells express squamous cytokeratins CK13 and 14 in the 3D model used here. Acid exposure does not alter the squamous cytokeratin profile of CP-A cells. CK13 expression in acid pulsed CP-A cells shows no difference over 3 weeks compared with controls (Supplement Figure 4B–D). CK14 expression was initially evident in only some cells of the acid pulsed reconstruct at week 1 and not in the controls, however by week 2 and continuing to week 3, control and acid pulsed CP-A cells expressed similar levels of CK14 (Figure 5B,C, Supplemental Figure 4E). In summary, CP-A cells retain their squamous cytokeratin profile independent of acid exposure.

Columnar cytokeratins and acid

CP-A cells express a combination of both squamous and columnar cytokeratins in basal 3D culture conditions. Acid pulsed CP-A cells stained for columnar cytokeratins CK8 and CK19 demonstrate no significant change in expression compared with their controls over 3 weeks with acid exposure (Supplemental Figure 4F–K). CK8 expression initially was particularly low, however by week 2 expression increased substantially, independent of acid exposure. By week 3, control and acid treated cells demonstrated equally reduced CK 8 expression compared to week 2. CK19 expression was elevated throughout 3 weeks of culture, independent of acid exposure. In general, acid pulses of one hour duration do not induce significant changes in squamous and columnar cytokeratin expression in CP-A cells.

Conclusion

In standard conditions designed to grow keratinocytes in 3D culture (Okawa et al., 2007), Barrett's Esophagus cells express both squamous and columnar cytokeratins and exhibit a more squamous-like, multilayered growth morphology. Previous studies demonstrated that ATRA was observed to be important for columnar differentiation of esophageal tissue *in vivo* (Chang et al., 2007). Chang et al. demonstrated that squamous biopsies grown as explant cultures differentiate to a columnar phenotype when treated with ATRA and that this change was caused by altered differentiation, and not enhanced proliferation of a particular cell type within the explant tissue. Vitamin A was previously demonstrated to be critical to the development of chick esophageal epithelium where it strongly inhibited the development of a thick, stratified esophageal epithelium in favor of a psuedostratified esophageal epithelium consisting of columnar, ciliated and mucosal cells (Aydelotte, 1963). Based on these studies, we used ATRA as a differentiation factor for BE cells.

Our 3D model of Barrett's Esophagus established all-trans retinoic acid as a differentiation factor for the BE cell line CP-A, leading to a reduction in squamous cytokeratin expression and an increase in columnar cytokeratin expression after three weeks. Among the other BE cell lines, ATRA did not alter cytokeratin expression, but it did alter morphology leading to

more single-layer BE-like growth. Further analysis of biological differences between these four BE cell lines will help elucidate the differences observed with ATRA treatment.

The development of BE *in vivo* is a response to the constant reflux of acid and bile salts from the stomach into the esophagus in patients with gastro-esophageal reflux disease (GERD). It is hypothesized that BE may actually be a protective mechanism against esophageal tissue damage during reflux (Reid et al., 2010). As the esophagus is constantly wounded with this milieu of irritants, the lining responds by producing a cell similar to the intestines, which can withstand low pH, and which produces mucous for protection (Reid et al. 2010). Over time, the squamous lining is replaced with a more protective columnar lining in some GERD patients.

Acid exposure on *ex vivo* biopsies from BE patients resulted in enhanced survival of BE cells over squamous cells (Dvorakova et al., 2005). Feagins et al. however demonstrated that acid exposure on BE cells in 2D culture had anti-proliferative effects (Feagins et al., 2007). Other studies however demonstrated enhanced proliferation and decreased apoptosis *in vitro* and *ex vivo* with acid (Fitzgerald et al., 1996), (Shirvani et al., 2000). Acid pulsing of a non-neoplastic BE cell line (BAR-T) demonstrated a change towards a colonic phenotype, indicated by an increase in CK8/18 expression (Bajpai et al., 2008; Das et al. 2011), and increased colony formation and tumorigenicity (Das et al. 2011). Based on the potential effects of acid on various BE tissue and cell culture models, we demonstrated here that application of acid on non-dysplastic metaplasia CP-A cells for 3 weeks did not affect cytokeratin profiles nor growth patterns. The lack of altered expression in squamous and columnar cytokeratins with acid suggests that these cells may have an upregulated protective pathway to retain normal levels of cytokeratin expression under stress conditions and protect against changes induced by the toxic effects of acid. Alternatively, the use of bile salts along with acid, and more frequent pulses could reveal more effects on BE growth in 3D culture.

In basic growth conditions without ATRA or acid, CP-A cells, derived from non-dysplastic metaplasia, demonstrated a highly invasive phenotype, unlike *in vivo* growth of BE metaplasia. This suggests that researchers should be cautious in interpreting CP-A cells as a model of metaplasia and note that unlike the other cell lines tested here, they have LOH at chromosome 5q, which contains the APC tumor suppressor gene (Palanca-Wessels et al., 1998). APC blocks cell proliferation and induces differentiation (Jankowski and Odze 2009). Tests for the relationship between mutation of APC and disruption to cellular growth in 3D culture are beyond the scope of this study but warrant further investigation.

The model developed in this study opens up many potential applications to study Barrett's esophagus *in vitro*. Most importantly, a 3D model of BE, with modifications, can be used to test potential therapeutics in a more physiologically realistic (though still artificial) culture environment. In addition, the model can be used to study factors necessary *in vivo* for malignant transformation of esophageal adenocarcinoma derived from benign BE. By understanding this transition, potential agents can be developed that interfere with this conversion. These cell lines may provide a platform to test the generality of potential therapies or treatments in a 3D culture environment as a stepping stone between standard 2D cell culture and other models. The heterogeneity in cell line responses ensures that the diversity present between patients and within a single patient can be captured experimentally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BE	Barrett's esophagus
EA	esophageal adenocarcinoma
CK	cytokeratin
ATRA	all-trans retinoic acid
IHC	immunohistochemistry
NSE	neosquamous epithelium

References

- Aydelotte MB. The Effects of Vitamin A and Citral on Epithelial Differentiation in vitro 2. The Chick Oesophageal and Corneal Epithelia and Epidermis. *Journal of Embryology and Experimental Morphology*. 1963; 11(3):621–635. [PubMed: 14061963]
- Bajpai M, Liu J, Geng X, Souza RF, Amenta PS, Das KM. Repeated exposure to acid and bile selectively induces colonic phenotype expression in a heterogeneous Barrett's epithelial cell line. *Lab Invest*. 2008; 88(6):643–651. [PubMed: 18427553]
- Biddle D, Spandau D. Expression of vimentin in cultured human keratinocytes is associated with cell —extracellular matrix junctions. *Archives of Dermatological Research*. 1996; 288(10):621–624. [PubMed: 8919046]
- Boch JA, Shields HM, Antonioli DA, Zwas F, Sawhney RA, Trier JS. Distribution of cytokeratin markers in Barrett's specialized columnar epithelium. *Gastroenterology*. 1997; 112(3):760–765. [PubMed: 9041237]
- Chang C-L, Lao-Sirieix P, Save V, De La Cueva Mendez G, Laskey R, Fitzgerald RC. Retinoic acid-induced glandular differentiation of the oesophagus. *Gut*. 2007; 56(7):906–917. [PubMed: 17185354]
- Das KM, Kong Y, Bajpai M, Kulkarni D, Geng X, Mishra P, Banerjee D, Hirshfield K. Transformation of benign Barrett's epithelium by repeated acid and bile exposure over 65 weeks: A novel in vitro model. *International Journal of Cancer*. 2011; 128(2):274–282.
- Dvorakova K, Payne CM, Ramsey L, Bernstein H, Holubec H, Chavarria M, Bernstein C, Sampliner RE, Riley C, Prasad A, Garewal H. Apoptosis Resistance in Barrett's Esophagus: Ex vivo Bioassay of Live Stressed Tissues. *Am J Gastroenterol*. 2005; 100(2):424–431. [PubMed: 15667503]
- Feagins LA, Zhang H-Y, Hormi-Carver K, Quinones MH, Thomas D, Zhang X, Terada LS, Spechler SJ, Ramirez RD, Souza RF. Acid Has Antiproliferative Effects in Nonneoplastic Barrett's Epithelial Cells. *Am J Gastroenterol*. 2007; 102(1):10–20. [PubMed: 17266684]
- Fitzgerald RC, Omary MB, Triadafilopoulos G. Dynamic effects of acid on Barrett's esophagus. An ex vivo proliferation and differentiation model. *The Journal of Clinical Investigation*. 1996; 98(9):2120–2128. [PubMed: 8903332]

- Glickman JN, Blount PL, Sanchez CA, Cowan DS, Wongsurawat VJ, Reid BJ, Odze RD. Mucin core polypeptide expression in the progression of neoplasia in Barrett's esophagus. *Human Pathology*. 2006; 37(10):1304–1315. [PubMed: 16949933]
- Haggitt RC. Barrett's esophagus, dysplasia, and adenocarcinoma. *Human Pathology*. 1994; 25(10):982–993. [PubMed: 7927321]
- Harada H, Nakagawa H, Oyama K, Takaoka M, Andl CD, Jacobmeier B, von Werder A, Enders GH, Opitz OG, Rustgi AK. Telomerase Induces Immortalization of Human Esophageal Keratinocytes Without p16INK4a Inactivation. *Molecular Cancer Research*. 2003; 1(10):729–738. [PubMed: 12939398]
- Jankowski JA, Odze RD. Biomarkers in Gastroenterology: Between Hope and Hype Comes Histopathology. *Am J Gastroenterol*. 2009; 104(5):1093–1096. [PubMed: 19417749]
- Kalabis J, Patterson MJ, Enders GH, Marian B, Iozzo RV, Rogler G, Gimotty PA, Herlyn M. Stimulation of human colonic epithelial cells by leukemia inhibitory factor is dependent on collagen-embedded fibroblasts in organotypic culture. *FASEB J*. 2003;02–0852fje.
- Nelson CM, Bissell MJ. Of Extracellular Matrix, Scaffolds, and Signaling: Tissue Architecture Regulates Development, Homeostasis, and Cancer. *Annual Review of Cell and Developmental Biology*. 2006; 22(1):287–309.
- Nurgalieva Z, Lowrey A, El-Serag H. The Use of Cytokeratin Stain to Distinguish Barrett's Esophagus from Contiguous Tissues: A Systematic Review. *Digestive Diseases and Sciences*. 2007; 52(5):1345–1354. [PubMed: 17373588]
- Okawa T, Michaylira CZ, Kalabis J, Stairs DB, Nakagawa H, Andl C, Johnstone CN, Klein-Szanto AJ, El-Deiry WS, Cukierman E, Herlyn M, Rustgi AK. The functional interplay between EGFR overexpression, hTERT activation, and p53 mutation in esophageal epithelial cells with activation of stromal fibroblasts induces tumor development, invasion, and differentiation. *Genes & Development*. 2007; 21(21):2788–2803. [PubMed: 17974918]
- Ouatu-Lascar R, Fitzgerald RC, Triadafilopoulos G. Differentiation and proliferation in Barrett's esophagus and the effects of acid suppression. *Gastroenterology*. 1999; 117(2):327–335. [PubMed: 10419913]
- Paladino G, Marino C, La Terra Mulè S, Civile C, Rusciano D, Enea V. Cytokeratin expression in primary epithelial cell culture from bovine conjunctiva. *Tissue and Cell*. 2004; 36(5):323–332. [PubMed: 15385149]
- Palanca-Wessels MC, Barrett MT, Galipeau PC, Rohrer KL, Reid BJ, Rabinovitch PS. Genetic analysis of long-term Barrett's esophagus epithelial cultures exhibiting cytogenetic and ploidy abnormalities. *Gastroenterology*. 1998; 114(2):295–304. [PubMed: 9453489]
- Palanca-Wessels MCA, Klingelhutz A, Reid BJ, Norwood TH, Opheim KE, Paulson TG, Feng Z, Rabinovitch PS. Extended lifespan of Barrett's esophagus epithelium transduced with the human telomerase catalytic subunit: a useful in vitro model. *Carcinogenesis*. 2003; 24(7):1183–1190. [PubMed: 12807723]
- Reid BJ, Li X, Galipeau PC, Vaughan TL. Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis. *Nat Rev Cancer*. 2010; 10(2):87–101. [PubMed: 20094044]
- Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure. *Gastroenterology*. 2000; 118(3):487–496. [PubMed: 10702199]
- Su Y, Chen X, Klein M, Fang M, Wang S, Yang CS, Goyal RK. Phenotype of columnar-lined esophagus in rats with esophagogastrroduodenal anastomosis: similarity to human Barrett's esophagus. *Lab Invest*. 2004; 84(6):753–765. [PubMed: 15094711]
- Thomas T, Abrams KR, Caestecker JSd, Robinson RJ. Meta analysis: cancer risk in Barrett's oesophagus. *Alimentary Pharmacology & Therapeutics*. 2007; 26(11–12):1465–1477. [PubMed: 17900269]
- Yousef F, Cardwell C, Cantwell MM, Galway K, Johnston BT, Murray L. The Incidence of Esophageal Cancer and High-Grade Dysplasia in Barrett's Esophagus: A Systematic Review and Meta-Analysis. *Am J Epidemiol*. 2008; 168(3):237–249. [PubMed: 18550563]

Fig 1. H&E staining of organotypic reconstructs demonstrate ATRA generally reduces epithelial thickness in BE and squamous cells at 3 weeks (see brackets). A. EPC2 cell line, B. CP-A cell line, showing invasion (arrows) and goblet cells, C. CP-B cell line, D. CP-C cell line, E. CP-D cell line. Scale bar = 75 μ m.

Fig 2. Alcian Blue staining of CP-A organotypic reconstructs at 3 weeks with or without ATRA, demonstrates presence of goblet cells preferentially in control conditions. Scale bar = 100 μ m. arrows=goblet cells

Fig 3. Squamous cytokeratin expression is reduced with ATRA treatment in squamous and CP-A cells, but not in high-grade dysplasia. A. EPC2 CK13 B. CP-A CK13 C. CP-A CK14 D. CP-B CK14 E. CP-D CK14. Scale bar = 100 μ m. arrows=cytokeratin positive cells.

Fig 4. Columnar CK19 expression in ATRA-treated CP-D cells and CK8 in CP-A cells were diminished, without measurable differences in other BE cells. A. CP-D week 3 CK19, B. EPC2 week 3 CK19, C. EPC2 week 3 CK8, D. CP-A Week 1 CK8, E. CP-A Week 3 CK8. Scale bar = 100 μ m. Arrows=cytokeratin positive cells

Fig 5. CP-A organotypic reconstructs demonstrated no effect of acid pulsing over 3 weeks on squamous and columnar cytokeratin expression. A. CP-A Week 3 H&E, B. CP-A Week 1 CK14, C. CP-A Week 3 CK14 Scale bar = 100 μ m. arrows=cytokeratin positive cells