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Barrett's Stem Cells as a Unique and Targetable Entity

Although metaplasias have always attracted because of their strangeness, it is now clear they represent precursors for some of the most intractable human cancers. Despite this notoriety, they remain curiously understudied, and even their origins have been the subject of acrimonious debate stretching back to Virchow in the 19th century. Barrett's esophagus, with its high incidence, easy endoscopic access, and strong link to esophageal adenocarcinoma, would seem an ideal opportunity to address the origin problem. However, the field has settled into an uneasy status quo marked by no fewer than 4 parallel hypotheses, each of which is said to suffer fatal flaws. We favor one of these deficient hypotheses, that Barrett's arises from a distinct lineage of junctional cells present in all normal individuals, and discuss efforts to shore it up. It will be important to resolve this dialectic so that preemptive strategies for the eradication of Barrett's can reach patient care. *(Cell Mol Gastroenterol Hepatol 2017;4:161–164; http://dx.doi.org/10.1016/j.jcmgh.2017.04.005)*

Warring Parties: Four Independent Hypotheses for Barrett's

Norman Barrett's first reference to the eponymous metaplasia as "columnar lined esophagus" invited speculation that Barrett's arose from the proximal migration of gastric epithelia. With the observations that Barrett's esophagus displayed mature goblet cells typical of the lower gastrointestinal tract but not stomach, the problem become more complex and intriguing. However, it is well-established that patients present with either intestinal metaplasia with goblet cells or columnar metaplasia lacking goblet cells, although risk of adenocarcinoma seemed to track more with intestinal metaplasia.[1] Although there remain persistent transatlantic discussions about whether Barrett's is one or both of these metaplasias, the origin of an intestinal metaplasia suggested more exotic mechanisms. Some of these ranged from the seeding by bone marrow–derived progenitors to the ectopic expression of colon-determining transcription factors.[2] Although the distillation of the past 30 years of research into the origins of Barrett's is beyond the scope of this statement, no fewer than 4 hypotheses, each with strengths and weaknesses, remain in play. These include (1) the esophageal transcommitment hypothesis, (2) the submucosal gland hypothesis, (3) the gastric transcommitment hypothesis, and (4) the junctional stem cell hypothesis. It would be comforting to conclude that Barrett's indeed originates via multiple pathways and all of these hypotheses are correct, although it is far more likely that they are all wrong, at least in their present renditions. We will summarize, from the standpoint of proponents of the junctional stem cell hypothesis, its basis and strengths, discuss ongoing efforts to address its fatal flaws, and illustrate the particular advantages of its clonogenic approach to drug discovery for Barrett's.

Barrett's Without Esophagus: the p63 Knockout Model

We backed into this exciting if unsettled field via developmental biology, with a mutant mouse that remains in our opinion the strongest argument against the esophageal transcommitment hypothesis and in favor of the junctional stem cell hypothesis.[3] In brief, we generated a mouse that lacks the p63 gene, which encodes a p53-like transcription factor that is highly and specifically expressed in the stem cells of all stratified epithelia including the epidermis, the prostate, and mammary gland, and, importantly for the present discussion, the esophagus. Mice lacking both copies of p63 die within hours of birth because of the frank absence of the epidermis and all other stratified epithelia.[4,5] Our retrospective analyses of these mice through embryogenesis revealed that in the absence of p63, the stratified epithelia undergo a non-regenerative differentiation and are completely absent by mid- to late gestation. Thus from the standpoint of the origin of Barrett's, the lineage that gave rise to the esophageal squamous epithelia no longer exists in these mice by embryonic day 14. What makes this observation particularly damning for the esophageal transcommitment hypothesis is that by embryonic day 18 these mice develop a robust metaplasia with all the morphologic and gene expression hallmarks of human Barrett's. Moreover, the gene expression profile of this Barrett's-like metaplasia is decidedly distinct from stomach,

small intestine, and colon, ruling out a simple migration as the source of this Barrett's-like metaplasia.[3] In a blunt way the observations ruled out as candidates both esophageal epithelium and adjacent gastric epithelia. In particular, 5 independent lineage markers gleaned from the expression profiles demonstrated that the cells that would form this metaplasia were already positioned at embryonic day 14 in these mutant mice but not in wild-type mice. We therefore compared the epithelial dynamics of the mutant and wild-type mice at this critical stage and realized that both mutant and wild-type mice show a single layer of progenitors of this metaplasia at E13; however, this layer was subsequently lost in wild-type mice by the undermining actions of the p63-expressing esophageal stem cells migrating down from the proximal esophagus to join up with the gastric epithelia in the murine forestomach. Importantly, as the normal squamous and gastric epithelia converged, a very discrete number of these metaplasia precursor cells remained at the junction at E14, and these junctionally positioned cells remained there through the life of the animal. Our analyses of human 22-week-old and adult human samples suggest that the cellular dynamics that gives rise to the junctional distribution of metaplastic precursors in mice is conserved in humans and that these cells remain at this position in normal individuals throughout life. Last, our lineage tracing of these junctional cells in murine models in which the esophageal epithelia can be conditionally damaged demonstrated the potential of these junctional cells to rapidly expand to fill the void.

In summary, our analysis of the Barrett's-like metaplasia in the p63-null mouse made several predictions for the evolution of Barrett's that were non-obvious and counter to the prevailing concepts for the initiation of precancerous lesions as defined by Hanahan-Weinberg.[6] First, the rapid appearance of Barrett's on a matter of days of damage to the esophagus was inconsistent with a mutational maturation and suggested an opportunistic spread of preexisting precursors. If true, it would follow that Barrett's can form without driver mutations or any mutations whatsoever. Second, this mouse model, which highlights a lineage of Barrett's precursors distinct from those of the normal esophagus and gastric epithelia, predicted that this lineage would have a unique stem cell as well. Confirmation of these predictions had to await advances in stem cell cloning technologies that would enable the analysis of human Barrett's cases.

Cloning Patient-matched Barrett's, Gastric, and Esophageal Stem Cells

Furthering the Barrett's analysis beyond murine models required us to devise means and conditions to clone columnar stem cells much the way Howard Green was able to do with stratified epithelial stem cells.[7] The technology we developed proved to be robust, enabled clonogenic analyses of human cells of the gastrointestinal tract, as well as supported disease models of epithelia differentiated from them. By using this technology we worked with Christopher Crum (Brigham & Women's Hospital/Harvard Medical School) to clone stem cells along the entire human gastrointestinal tract in studies that demonstrated that each was epigenetically committed to differentiating to the epithelia from which they were derived despite months of continuous cell division in vitro as stem cells. Thus, stem cells from duodenum always gave rise to three-dimensional duodenum epithelia and right colon stem cells to right colon epithelium. In addition to their immense commitment stability, these stem cells appeared to have unlimited self-renewal capacity and a remarkable degree of genomic stability. Perhaps most surprising to us was that a single stem cell can give rise to all of the local cell types such as goblet cells, endocrine cells, and Paneth cells and self-assemble into a three-dimensional epithelium remarkably similar to the in vivo epithelium, all in the absence of stromal cells.

Armed with this technology, we worked closely with Lawrence Ho Khek Yu (National University of Singapore) to clone patient-matched stem cells from endoscopic biopsies of esophagus, Barrett's, and gastric cardia from 12 Barrett's cases without high-grade dysplasia.[8] As with the normal gastrointestinal tract, we were able to clone 100–300 clones from each 1-mm biopsy and from them sample discrete pedigrees for further analysis. Importantly, whole genome expression profiles revealed that the esophageal, gastric, and Barrett's stem cells were quite distinct, a notion confirmed on their three-dimensional differentiation, which yielded stratified squamous epithelia, gastric epithelia, and intestinal metaplasia, respectively (Figure 1). We should add here that the biopsies taken from the most distal portions of the stomach accessible were much more closely related to stem cells derived from all other portions of the stomach including fundus, greater and lesser curve, and antrum (not shown). Taken together, these data support the notion that Barrett's relies on a discrete stem cell for regenerative growth, and that this stem cell is distinct from those of eponymous tissues such as the esophagus and stomach.

These patient-matched series of stem cells from Barrett's cases also allowed a clonal analysis of the genomic changes each had undergone in these patients. Structural variations in the form of copy number variation and exome sequencing for single nucleotide variation revealed a spectrum of changes in the Barrett's stem cells across this patient cohort. Most patients showed stereotyped sets of monoallelic or biallelic deletions at fragile sites impacting genes such as the INK4A locus including p16, FHIT, and WWOX and in general have nearly the full complement of deletions as reported for the typical esophageal adenocarcinoma (EAC).[9] Several of the Barrett's cases also showed more ominous amplifications of proto-oncogenes (c-Myc, Myb) and receptor tyrosine kinases (FGFR), as well as mutations in p53 and other genes mutated in EAC. Finally, one-third of the Barrett's stem cells of these cases showed little in the way of copy number variation or single nucleotide variation, which was like their counterparts in the esophagus stomach, suggesting that clinically defined Barrett's can arise without driver mutations or mutations of any form. The ability of Barrett's epithelium to establish without a protracted phase of mutational maturation is consistent with the rapid appearance of a Barrett's-like epithelium in our p63-null mice and

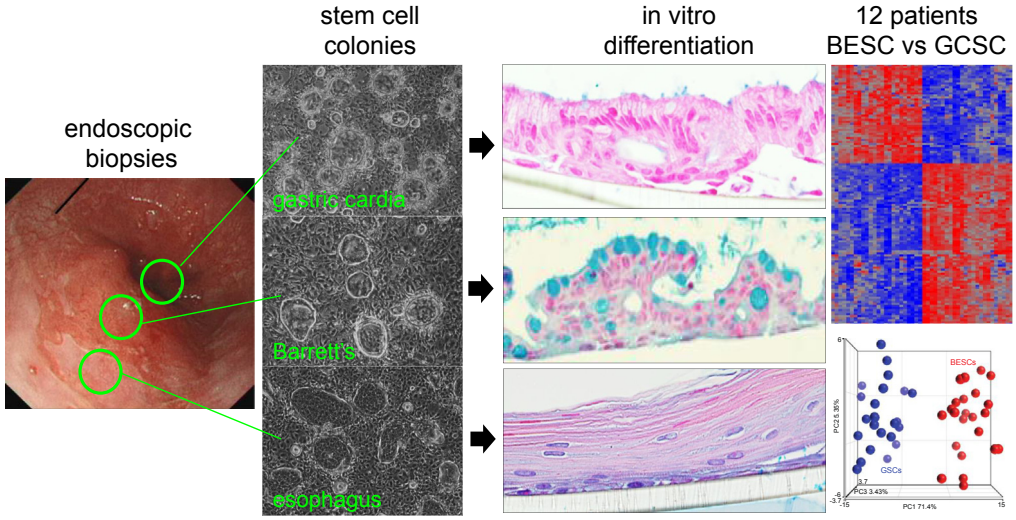

Figure 1. Cloning Barrett's stem cells from endoscopic biopsies. One-millimeter endoscopic biopsies were selected from esophagus, Barrett's, and gastric epithelium and processed for generating stem cell colonies. Single cell-derived "pedigrees" were expanded and differentiated in vitro to yield the indicated three-dimensional epithelia. Patient-matched stem cells from Barrett's (BESC) and gastric cardia (GCSC) yield a consistent differential gene expression pattern that can also be seen in principal component analysis of whole genome expression.

in the overall notion that Barrett's arises from a preexisting population of cells at the gastroesophageal junction.

Fatal Flaws and Alternative Facts

Probably the most often cited objections to the junctional stem cell hypothesis are the esophageal duodenal anastomosis (EDA) models in rats that develop metaplasia, dysplasia, and nominally "esophageal adenocarcinoma" at the neojunction at high rates. If the interpretations of this model are correct, they would preclude both the junctional stem cell hypothesis as well as the gastric transcommitment hypothesis for the origins of Barrett's (and EAC). However, the EDA models are enigmatic for multiple reasons including their high rates of cancer formation, uncertain nature of the associated "Barrett's", and, importantly, the variance between the histology of the adenocarcinoma appearing in these models and that typical of EAC.[10] All of these concerns bring into question whether EDA models are true representations of the Barrett's-dysplasia-EAC sequence and lethal objections to the junction stem cell or gastric transcommitment hypotheses. Another glaring deficiency in the junctional stem cell model is that it predicts that the junctional stem cells exist in normal mice and humans, and yet reports of their cloning have not made it into the literature. This is an active project in the laboratory, and preliminary results indicated that such stem cells do populate the normal junction and are distinct from stem cells of all regions of the gastric epithelia that together form a clade distinct from duodenum and jejunum. Further analyses of these cells should clarify their relationship with all other stem cells of the proximal gastrointestinal tract.

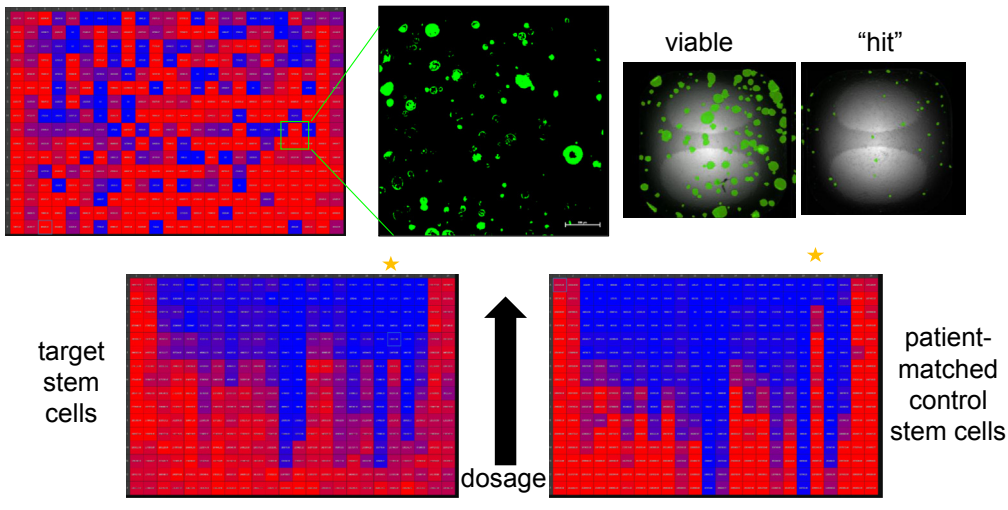

Figure 2. Adaptation of patient-matched Barrett's and gastric stem cells to screening formats. Green fluorescent protein-labeled Barrett's esophagus and gastric cardia stem cells in 384-well format screened with known and experimental drugs. *Bottom*, Dose-response analysis to identify potential therapeutic windows.

Therapeutic Predictions and Implications

If the long-term regenerative growth of Barrett's is indeed dependent on a stem cell distinct from those that support the local esophageal and gastric epithelium, these differences should render Barrett's stem cells selectively targetable. Present standard-of-care for dysplastic Barrett's relies on mucosal resections and physical ablation via radiofrequency ablation and cryogenics, which are expensive, time-consuming, and not without morbidities including strictures and recurrent disease.[11] The adaptation of patient-matched stem cells of Barrett's and local epithelia may enable moderate and even high-throughput testing of small molecule, biologics, or even immunologic approaches to the selective eradication of the Barrett's stem cells in a manner that would spare those of normal epithelia to fill in the gaps. Toward this end, we have adapted these patient-matched stem cells to a 384-well screening configuration and are well along the road to identifying compounds that selectively compromise Barrett's stem cells of any mutational profile as well as others that selectively kill Barrett's stem cells with advanced profiles (Deluba et al, unpublished data, May 2017; Figure 2). We anticipate that if such studies are validated both in vitro and in vivo, they could yield small molecules and biologics that could be used in combination with endoscopic mucosal resections and physical ablation modalities to improve patient care and outcome.

WA XIAN
Institute of Molecular Medicine
University of Texas Health Science Center at Houston
Houston, Texas
and the Department of Biochemistry and Molecular Biology
University of Texas McGovern Medical School
Houston, Texas

FRANK MCKEON
Department of Biology and Biochemistry
University of Houston
Houston, Texas, and
Department of Microbiology and Immunology
Yong Loo Lin School of Medicine
National University of Singapore
Singapore

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Conflicts of interest
The authors disclose no conflicts.

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