Neosquamous Epithelium Does Not Typically Arise from Barrett's Epithelium

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Abstract

Purpose: Neosquamous epithelium (NSE) can arise within Barrett's esophagus as a consequence of medical or surgical acid reduction therapy, as well as after endoscopic ablation. Morphologic studies have suggested that NSE can develop from adjacent squamous epithelium, submucosal gland ducts, or multipotent progenitor cell(s) that can give rise to either squamous or Barrett's epithelium, depending on the luminal environment. The cells responsible for Barrett's epithelium self-renewal are frequently mutated during neoplastic progression. If NSE arises from the same cells that self-renew the Barrett's epithelium, the two tissues should be clonally related and share genetic alterations; if NSE does not originate in the self-renewing Barrett's, NSE and Barrett's esophagus should be genetically independent.

Experimental Design: We isolated islands of NSE and the surrounding Barrett's epithelium from 20 patients by microdissection and evaluated each tissue for genetic alterations in exon 2 of *CDKN2A* or exons 5 to 9 of the *TP53* gene. Nine patients had *p16* mutations and 11 had *TP53* mutations within the Barrett's epithelium.

Results: In 1 of 20 patients, a focus of NSE had a 146 bp deletion in *p16* identical to that found in surrounding Barrett's epithelium. The NSE in the remaining 19 patients was wild-type for *p16* or *TP53*.

Conclusion: Our mutational data support the hypothesis that, in most circumstances, NSE originates in cells different from those responsible for self-renewal of Barrett's epithelium. However, in one case, NSE and Barrett's epithelium seem to have arisen from a progenitor cell that was capable of differentiating into either intestinal metaplasia or NSE.

The stratified squamous epithelium that normally lines the esophagus is established in the 4th month of embryonic development (1) and usually persists until death. In some individuals, the squamous epithelium is replaced by a specialized intestinal metaplasia in response to chronic gastroesophageal reflux disease, a condition termed Barrett's esophagus (2). Patients with Barrett's esophagus are at a 30- to 40-fold increased risk for the development of esophageal adenocarcinoma, a cancer that has increased rapidly in incidence over the past 30 years (3). The mechanisms of conversion from squamous to intestinal epithelium are not known, but it has been hypothesized that the mucus-producing Barrett's specialized intestinal epithelium provides greater protection against the erosive effects of reflux than squamous epithelium (4).

Treatment for Barrett's esophagus consists primarily of surgical and medical interventions to control gastroesophageal reflux. Proton pump inhibitors, used in the United States since the late 1980s, bind irreversibly to the H^+/K^+ ATPase and prevent the production of acid by gastric parietal cells (5, 6). Proton pump inhibitors have become the treatment of choice for reflux and are remarkably effective, capable of reducing acid output by >95% (7). The development of effective acid suppression therapies has led to the observation in some patients of regrowth of apparently normal neosquamous epithelium (NSE) in regions previously occupied by Barrett's epithelium (8 – 12), many of which arise as islands completely surrounded by the Barrett's esophagus. Regrowth of NSE is also seen in patients treated with various endoscopic ablative therapies, such as multipolar electrocoagulation, endoscopic mucosal resection, and photodynamic therapy in combination with antireflux therapy with proton pump inhibitors or surgery (13 – 15). Previous studies examining NSE have found it to be histologically identical to normal squamous epithelium (9, 12, 16). However, with both proton pump inhibitor therapy as well as endoscopic ablation therapies, it is not uncommon to have a mosaic of both Barrett's specialized intestinal metaplasia and NSE in a patient's esophagus, and it is

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unclear whether squamous reepithelialization is associated with a reduction in cancer risk (15).

The origin of NSE is unknown. NSE may simply represent an alternate epithelial phenotype of the Barrett's epithelium. It is unlikely that differentiated Barrett's epithelium directly transdifferentiates into NSE, given that most ablative therapies regenerate NSE although most of the Barrett's has been eliminated. The cells that generate the self-renewing Barrett's epithelium may be able to undergo separate differentiation pathways depending on the environmental conditions in the esophagus (Fig. 1A). According to this model, a self-renewing squamous epithelium is generated under normal conditions and self-renewing Barrett's epithelium under chronic reflux conditions. Alternatively, the two epithelia may arise from independent progenitors, leading to establishment of either squamous or intestinal-type epithelium depending on luminal conditions (Fig. 1B). Development of NSE islands within a region of the esophagus previously covered with genetically altered Barrett's epithelium provides an opportunity to investigate the relationship between these epithelia (Fig. 2). If NSE derives from the same cells that self-renew the Barrett's epithelium, but has a different phenotype, then NSE will share a common genetic background with the Barrett's esophagus. Alternatively, if NSE arises independently of the cells that self-renew the Barrett's epithelium, then NSE will be genetically independent of the Barrett's esophagus.

Barrett's epithelium has similar proliferation and renewal characteristics to those of the small intestine (17, 18), with cells differentiating as they move up the crypt where they are eventually sloughed off into the lumen. We have taken advantage of the fact that development of esophageal adenocarcinoma is characterized by genetic instability, selection of genetic variants, and expansion of clonal populations early in progression (19) to characterize the relationship between Barrett's esophagus and NSE. Alterations in the tumor suppressor genes *CDKN2A* (*p16*; 9p loss of heterozygosity, mutation, and promoter hypermethylation) and *TP53* (17p loss of heterozygosity, mutation) are frequently found in patients with Barrett's esophagus (20). The resulting clonal expansion indicates cells with a given genetic alteration have spread from a single crypt to cover thousands or hundreds of thousands of crypts in the Barrett's epithelium. We examined Barrett's esophagus patients having (*a*) clonally expanded populations containing mutations in either *p16* or *TP53* and (*b*) NSE completely within the clonally expanded region. Detection of the same mutation in the NSE and surrounding Barrett's epithelium would indicate that both derive from the cells that self-renew the Barrett's epithelium. Our results indicate that in the majority of cases, NSE has an origin independent of the surrounding self-renewing Barrett's epithelium.

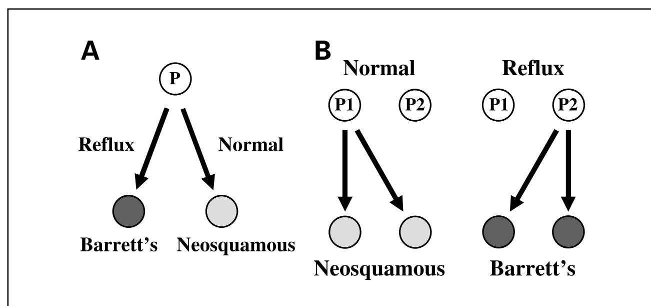

Fig. 1. Possible origins of the NSE. *A,* the cells that generate the self-renewing Barrett's epithelium (*P*) can also generate NSE, depending on the reflux environment of the esophagus. *B,* multiple independent progenitor cells (*P1, P2*) generate distinct epithelia in the esophagus, only one of which grows to predominate depending on the esophageal luminal environment. Additional precursors may exist in the pathway between the ultimate esophageal epithelial stem cell and the differentiated squamous and Barrett's epithelial cells. Determining the existence and identity of these precursors will require additional studies.

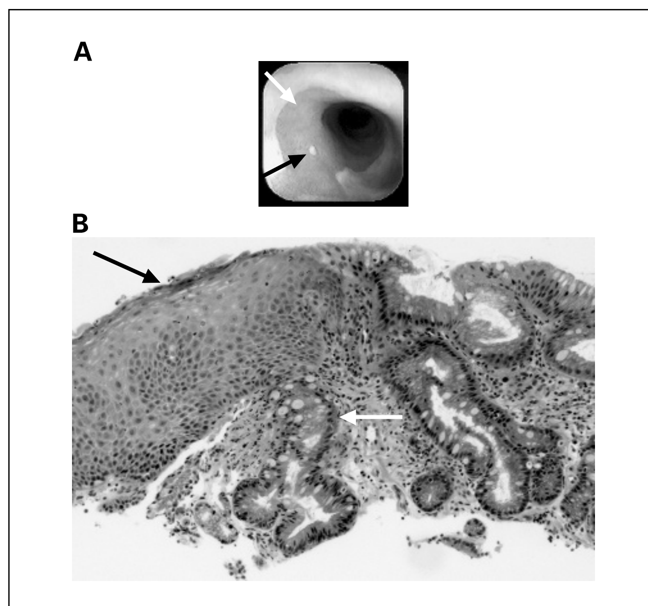

Fig. 2. NSE in Barrett's esophagus. *A,* endoscopic view of Barrett's epithelium (*darker region, white arrow*) with a neosquamous island (*black arrow*). *B,* histologic section (H&E stain) of NSE (*black arrow*) adjacent to Barrett's epithelium. Note the gland of Barrett's epithelium (*white arrow*) that has been overgrown by the NSE.

Materials and Methods

Patients. Participants were selected from the Seattle Barrett's Esophagus Study, which has been approved by the University of Washington Human Subjects Division and/or the Fred Hutchinson Cancer Research Center Institutional Review Board continuously since 1983. Endoscopic and histologic records were reviewed to identify 20 patients who had a previously characterized area of specialized intestinal metaplasia with a clonally expanded population of cells with either a *p16* or *TP53* gene mutation (as detected previously), and also had endoscopically and histologically confirmed islands of neosquamous mucosa arising within the area containing the mutated Barrett's epithelium.

Isolation of NSE. Biopsies containing NSE were fixed in 10% buffered formalin and sectioned. The first and last sections were cut at a thickness of 5 μm and stained with H&E to allow morphologic evaluation of the type of epithelium present in the biopsy. Microdissections of the biopsies were done using a new no. 10 scalpel blade on 10-μm-thick tissue sections at ×20 magnification using an American Optical dissecting microscope. Microdissected epithelia were placed in sterile microcentrifuge tubes for processing. Cutting blades were changed between specimens and between different epithelia.

Microdissected sections were deparaffinized using Histoclear (National Diagnostics, Atlanta, GA) and washed sequentially in 100%, 75%, and 50% ethanol. DNA was isolated by a protocol developed by Frank et al. (21). Briefly, dehydrated sections were resuspended in 50 to 100 μL buffer [50 mmol/L Tris-HCl (pH 8.3), 0.2 mg/mL proteinase K] and digested at 55°C overnight. Digests were spun at 14K in an Eppendorf microcentrifuge for 5 minutes to pellet debris. Five microliters

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of the supernatant were used in subsequent whole genome reactions. The remaining tissue on the microdissected slides was stained with H&E and examined by a gastrointestinal pathologist (R.D. Odze) to verify the nature of the microdissected tissue (i.e., squamous, columnar, etc.).

Flow sorting of NSE. Biopsies of NSE were minced to release nuclei (22), stained with the DNA binding dye 4′,6-diamidino-2-phenylindole, and nuclei from NSE cells were identified on the basis of side scatter and 4′,6-diamidino-2-phenylindole fluorescence as described previously (23). Briefly, this sorting strategy is based on the retention of dense cytoplasm around the nuclei of squamous cells that nonspecifically binds 4′,6-diamidino-2-phenylindole. Thus, these nuclei have both a high degree of side scatter due to the complexity of the cytoplasm around the nucleus, and fluoresce brightly with 4′,6-diamidino-2-phenylindole, in contrast to the Barrett's intestinal epithelial nuclei, which take up less 4′,6-diamidino-2-phenylindole and have significantly lower side scatter. In experiments with lung bronchial sputum samples, such a sorting procedure was able to generate populations with <1% columnar epithelium (23). DNA from the flow-sorted cells was isolated as described previously (22).

Genetic analyses. Given the small size of the microdissected sections, we first did whole genome amplification before sequencing. Primer extension preamplification was done as previously reported (22). *p16* exon 2 (24, 25) or *TP53* exons 5 to 6, 7, and 8 to 9 (26–28) were amplified by PCR from primer extension preamplification products. All PCR products were purified by MultiScreen PCR Filtration System (Millipore, Bedford, MA), and sequenced using BigDye Terminator cycle sequencing (Applied Biosystems, Foster City, CA) on an ABI 3730 DNA sequencer. All mutations were confirmed with at least two independent primer extension preamplification, PCR, and sequencing reactions. Wild-type sequences were confirmed for all patients using constitutive samples. Mutations in *TP53* for the 11 patients in this study have been previously reported (28).

Loss of heterozygosity analyses were done as previously described (22). Briefly, products from the primer extension preamplification reactions were amplified using primers for the single tandem repeat loci D9S925, D9S932, and D9S1118, desalted and concentrated using Microcon-100 sample filters (Millipore), and run on an ABI 3730 Capillary Electrophoresis System (Applied Biosystems). Loss of heterozygosity was determined by comparing the ratio of the peak heights of each allele with normal controls run previously for each patient.

Results

We identified 20 patients having islands of NSE within a region of the Barrett's segment that contained mutations in either *p16* or *TP53*. The characteristics of these patients are described in Table 1. Islands of NSE (two to seven biopsies per patient) and surrounding Barrett's epithelium (one to six biopsies per patient) were obtained from all patients. Endoscopic and histologic examples of NSE arising in a region surrounded by Barrett's epithelium are shown in Fig. 2A and B, and the experimental design illustrated in Fig. 3.

Table 1. Patient characteristics

Male/female	17:3
Mean age	73.1 y
Mean segment length	8.2 cm
Patients with *p16* mutation	9/20
Patients with *p53* mutation	11/20
No. neosquamous samples	77 (two to seven per patient)
No. Barrett's samples	56 (one to six per patient)

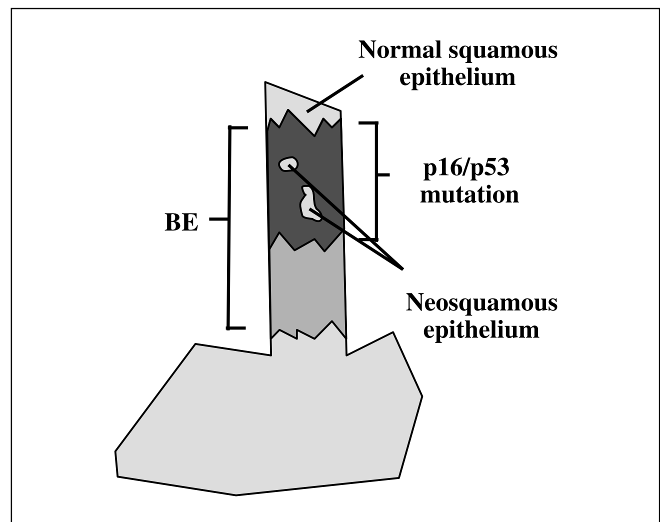

Fig. 3. Experimental design for characterizing NSE. Patients from the Seattle Barrett's Esophagus Study were selected that had developed NSE located completely within regions of the Barrett's segment containing clonally expanded populations with mutations in either *p16* or *TP53*.

Sequencing results were obtained from 74% of the samples and from all patients; samples that failed to provide adequate sequencing results were associated with specific patients (e.g., samples from five patients accounted for 75% of the failures) and likely reflected an artifact of fixation that resulted in poor-quality DNA for PCR. All microdissected tissues were verified to be entirely neosquamous or intestinal epithelium (Fig. 4), but included some stromal cells.

We confirmed the previously identified *p16* or *TP53* mutations in the microdissected Barrett's epithelium in 18 of 20 patients (90%; Table 2). The Barrett's epithelium present in the biopsies from the other two patients was small and had >75% stromal cell contamination, making unambiguous identification of the mutations difficult; however, these mutations had been previously verified in adjacent Barrett's esophagus biopsies from the same patients. All mutations were identical to those identified in tissue obtained from prior endoscopies (28). Islands of NSE were found to be wild type at the loci mutated in the adjacent Barrett's epithelium in 19 of 20 patients examined (95%). However, one patient (patient 13) was found to have an identical *p16* mutation, a 146 bp deletion in the NSE and in the adjacent intestinalized epithelium (Fig. 5). Stromal cells present in the microdissected Barrett's and NSE contribute the wild-type background sequence. In this patient, the alteration was found in one biopsy containing NSE out of seven NSE biopsies examined from three separate levels of a 15 cm Barrett's segment. The patient had a wild-type *p16* DNA sequence in normal constitutive tissue, confirming that the mutation was not inherited. This mutation has not been reported in the literature and it is unlikely to have arisen independently in the two tissues. This result was verified by sequencing independently isolated microdissected tissue from another section from the same biopsy. In addition, the patterns of loss of heterozygosity at informative markers on chromosome 9p were consistent between the NSE and surrounding Barrett's epithelium, further indicating a clonal relationship (data not shown).

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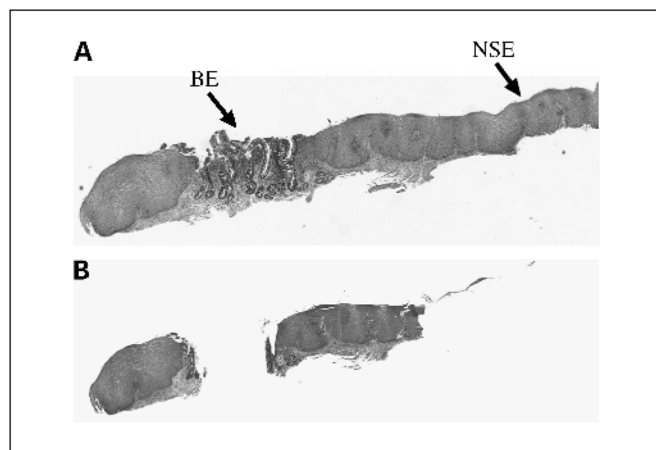

Fig. 4. Examples of microdissection of NSE. Mosaic images of (*A*) a Barrett's esophagus biopsy containing both Barrett's epithelium (*BE*) and NSE and (*B*) a section adjacent to that shown in (*A*), after microdissection of the Barrett's epithelium and NSE.

We further verified that the NSE from patient 13 contained a mutation in *p16* by using a flow cytometric sorting method that discriminates squamous from columnar epithelium on the basis of side scatter and fluorescence. This protocol purifies the NSE cells from the underlying stromal cells that are present after mechanical microdissection. The sequence of the *p16* gene obtained from the flow-purified NSE cells contained only the identical 146 bp deletion (Fig. 5) found in the microdissected samples.

Discussion

Patients with Barrett's esophagus frequently develop islands of NSE during acid suppression therapy and the goal of ablative therapies is to completely replace the Barrett's epithelium with a regenerated squamous epithelium. However, it has been difficult to determine if the development of NSE affects the risk of developing cancer because little is known about the origin or genetics of NSE. We have shown that NSE and the surrounding Barrett's do not contain common genetic alterations and, therefore, the NSE is not derived from the self-renewing Barrett's epithelium in most cases. However, in one case, the presence of the same *p16* mutation and pattern of loss of heterozygosity in both NSE and the surrounding Barrett's epithelium indicate that they can be clonally related and share a common precursor. In this case, we find evidence for a progenitor that is capable of generating both types of epithelia. Because the progenitor cell(s) of the esophageal epithelium are unknown, it is impossible to determine where in the pathway of epithelial progenitors this common precursor lies. Future studies using progenitor cell markers, as opposed to the tumor suppressor gene alterations used here, may clarify this question.

Although many forms of surgical or medical antireflux therapy are able to induce some degree of squamous reepithelialization, there is no evidence that neosquamous regrowth during antireflux therapy reduces the risk of developing esophageal adenocarcinoma (15, 29). Most antireflux treatments are able to induce a moderate level of neosquamous regeneration, but only rarely will this completely eliminate the Barrett's epithelium (8, 12, 15). Ablative therapies, such as photodynamic therapy or multipolar electrocoagulation, can successfully eradicate most of the Barrett's epithelium in a patient, and the results presented here suggest the NSE developing after these treatments may frequently be wild type, although none of the patients we examined had undergone ablative therapies. However, concerns still remain that residual Barrett's hidden under the regenerated squamous poses a risk of cancer that may be difficult to detect (30, 31), or that the residual Barrett's after ablation may contain mutations (32) and may progress rapidly after treatment (33).

Our results that a minority of the NSE we sampled contained mutations present in the surrounding Barrett's esophagus are consistent with a previous study examining p53 overexpression in NSE that developed in patients treated by multipolar electrocoagulation and proton pump inhibitor therapy. Garewal et al. (13) found regenerated NSE was negative for p53 by immunostaining in 11 of 11 patients with total squamous reepithelialization, but p53 protein expression was elevated in 6 of 14 cases who only developed squamous islands. In addition, they reported anecdotally that the Barrett's esophagus adjacent to the NSE seemed to express more p53 than elsewhere. Similarly, another report described *TP53* mutations in two cases of NSE arising after photodynamic therapy, but did not characterize the preexisting Barrett's epithelium (32). The approach used in the present study specifically examined genetic alterations that (*a*) frequently occur in Barrett's esophagus and (*b*) were already known to exist in large fields of clonal populations surrounding the islands of NSE. Thus, the Barrett's epithelium surrounding the NSE, as well as the self-renewing cells that generated the Barrett's, should have contained a *p16* or *TP53* mutation in our cases. It is unlikely that the one case in

Table 2. Sequencing results for Barrett's epithelium and NSE

Patient	Gene	Barrett's epithelium	NSE
1	*p53*	C388T	Wild type
2	*p53*	C388T	Wild type
3	*p53*	C455T	Wild type
4	*p53*	G518A	Wild type
5	*p53*	C535T	Wild type
6	*p53*	T650G	Wild type
7	*p53*	A659G	Wild type
8	*p53*	G743A	Wild type
9	*p53*	G743A	Wild type
10	*p53*	G743A	Wild type
11	*p53*	G818T	Wild type
12	*p16*	174 2bp del	Wild type
13	*p16*	198 146bp del	198 146bp del
14	*p16*	235 11bp del	Wild type
15	*p16*	NR (C238T)	Wild type
16	*p16*	C238T	Wild type
17	*p16*	C247T	Wild type
18	*p16*	C247T	Wild type
19	*p16*	G262T	Wild type
20	*p16*	NR (G330A)	Wild type

NOTE: Sequencing results for the *TP53* and *p16* genes for Barrett's epithelium and NSE. Mutation locations are given as the nucleotide mutated or the beginning base of the indicated deletion.

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Fig. 5. Sequencing chromatograms from patient 13, showing the wild-type sequence for *p16*, the mixture of wild-type and 146 bp deletion in the microdissected Barrett's epithelium and NES samples, and the 146 bp deletion alone in the flow-sorted NES cells.

which we found a *p16* mutation in NSE was the result of undetected contamination by Barrett's cells because the mutation was found in two independent microdissections, and purification of NSE by flow sorting resulted in a pure population of cells containing the mutation. The lack of genetic alterations in the NSE in most cases indicates that the cells that self-renew the mutated Barrett's epithelium do not generate NSE.

The single patient with mutated NSE had only a single focus with mutation of seven biopsies with NSE taken from three different levels in the esophagus. Development of NSE from the same precursor that generates the surrounding Barrett's epithelium does not seem to depend on the specific *p16* mutation examined, because the same mutation was found throughout the 15 cm Barrett's segment. This finding suggests that NSE can arise from multiple sources even within the same patient. Because mutation in the NSE was only found in 1 of 20 patients, one can speculate that mutation of a progenitor cell capable of generating both NSE and Barrett's epithelium is not a common event.

NSE has been hypothesized to originate from three sources: encroachment of adjacent normal squamous epithelium from the squamocolumnar junction, outgrowth from an existing pool of squamous cell progenitors in the esophageal epithelium, or from multipotent progenitor cells that also generate the Barrett's epithelium (8, 9). In the current study, we eliminated the possibility that NSE is derived from adjacent normal squamous epithelium by only including patients who developed islands of NSE completely surrounded by a field of Barrett's epithelium. Submucosal esophageal gland ducts, which are partially lined with squamous epithelium and found throughout the esophageal epithelium, may provide a reservoir for regrowth of an unaltered squamous epithelium (8, 34–37). An earlier study examining the characteristics of Barrett's epithelium found underneath squamous islands in response to proton pump inhibitor therapy reported that its foci were adjacent to esophageal gland ducts in 21% of biopsies (16). This probably represents an underestimate of how often NSE is associated with gland ducts because the study only examined those cases having Barrett's epithelium found underneath squamous islands not those with NSE alone. It is likely the ultimate fate of the esophageal epithelium, whether it be squamous or Barrett's epithelium, is dependent on complex interactions between the luminal environment of the esophagus and its different progenitor cell populations. Further studies to characterize these interactions may allow development of interventions to promote regeneration of genetically normal squamous epithelium in Barrett's esophagus patients or even prevent development of Barrett's epithelium in patients with gastroesophageal reflux disease.

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