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The Functional Characterization of Normal and Neoplastic Human Enterochromaffin Cells

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Context: Neuroendocrine regulation of small intestinal (SI) function is poorly understood because pure neuroendocrine cells are unavailable, whereas the biological basis of SI carcinoid tumors is unknown because neoplastic human enterochromaffin (EC) cells are unavailable.

Objective: The objective of this study was to define the secretory regulation and transcriptome of naive and neoplastic SI neuroendocrine cells.

Design: EC cells from human ilea were isolated and purified, and a malignant EC cell carcinoid cell line (KRJ-I) was characterized.

Methods: Human ilea from right hemicolectomies were pronase/collagenase digested and Nycodenz gradient centrifuged, and EC cells were fluorescence-activated cell sorting (FACS) sorted after acridine orange labeling. Enrichment was defined by immunostaining, gene expression, serotonin (5-HT) content, and real-time RT-PCR. Naive FACS-sorted EC and KRJ-I cells were cultured, and 5-HT secretion was measured after stimulation with forskolin, isoproterenol, acetylcholine, γ-aminobutyric acid A ($GABA_A$), pituitary adenylate cyclase-activating polypeptide (PACAP)-38, and gastrin. Normal and neoplastic EC cell transcriptomes were acquired by Affymetrix profiling (U133A).

Results: FACS produced 100 ± 0.3% (chromogranin A staining) and 99 ± 0.7% pure EC cells by immunostaining for tryptophan hydroxylase with greater than 67-fold enrichment and a 5-HT content of 180 ± 18 ng/mg protein (mucosa, 3.5 ± 0.9). Forskolin- and isoproterenol-stimulated 5-HT secretion was 10–100 times more potent for naive cells (EC_{50}, 1.8×10^{-9} M; 5.1×10^{-9} M) than neoplastic cells (EC_{50}, 2.1×10^{-7} M; 8.1×10^{-8} M), but the effect of PACAP-38 was similar (EC_{50}, 1×10^{-7} M). Isoproterenol stimulated cAMP levels 1.6 ± 0.1-fold *vs.* basal (EC_{50}, 2.7×10^{-9} M). Acetylcholine inhibited naive EC cell 5-HT secretion more potently than neoplastic (IC_{50}, 3.2×10^{-9} *vs.* 1.6×10^{-7} M), whereas $GABA_A$ was more potent in neoplastic cells (IC_{50}, 3.9×10^{-10} *vs.* 4.4×10^{-9} M). Octreotide inhibited naive, but not neoplastic, basal 5-HT secretion. Gastrin had no effect on 5-HT secretion. Comparison of naive and neoplastic transcriptomes revealed shared neuroendocrine and EC cell-specific marker genes. Real-time PCR confirmed that expression of adrenergic (β1), somatostatinergic (SST_R2), and neural ($VPAC_1$ and $GABA_A$) receptors occurred on both cell types, but PACAP type 1 (PAC_1) and cholecystokinin type 2 (CCK_2) were undetectable. The putative carcinoid malignancy genes (*MTA1* and *MAGE-D2*) were unique to the neoplastic EC cell transcriptome.

Conclusion: These data support novel methodology to purify live human EC cells for functional characterization and transcriptome assessment, which will allow identification of new targets to control the secretion and proliferation of SI carcinoids. **(*J Clin Endocrinol Metab* 91: 2340–2348, 2006)**

THE REGULATION OF small intestinal function is a problem of considerable scientific and clinical interest. It has been proposed that neural or neuroendocrine regulation may be implicated in a number of disease processes, including irritable bowel syndrome and ileus and carcinoid disease (1, 2). In particular, the enterochromaffin (EC) cell has been identified as an important component of both physiological and pathological issues, including intestinal secretion, diarrhea, and bloating (2).

This cell is difficult to isolate and identify in a pure form because it comprises one of numerous neuroendocrine cells scattered throughout the gut and is located in small numbers deeply embedded in mucosal crypts (3). Its chief characteristic is the secretion of serotonin (5-HT), although substance P (motility regulator) and, more recently, guanylin (secretory regulator) have been identified as additional secretory products (1, 4, 5). To date, information about EC cell function and proliferation has been derived from either intact mucosal preparations (Ussing chambers) or studies of impure homogenates of the mucosa (1, 6, 7). Given the complex nature of such preparations, information derived from these studies has yielded results that are often inconsistent. Overall, however, it appears that the regulation of 5-HT secretion is neurally mediated via acetylcholine and adrenergic agents (8).

Nevertheless, the precise regulatory mechanisms of EC cell secretion have not been defined. In addition, other agents secreted by EC cells, namely substance P and guanylin, have been rarely studied, although both have been identified in pharmacological studies as regulators of intestinal motility and secretion, respectively (2, 4). The development of a pure human EC cell preparation, therefore, represents an important scientific opportunity to investigate the physiology of

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Abbreviations: AO, Acridine orange; CgA, chromogranin A; EC, enterochromaffin; ER, endoplasmic reticulum; F_0, crude cell fraction; F_{FACS}, FACS fraction; F_N, Nycodenz fraction; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GABA, γ-aminobutyric acid; 5-HT, serotonin; LDH, lactate dehydrogenase; NE, neuroendocrine; PACAP, pituitary adenylate cyclase-activating polypeptide; POMC, proopiomelanocortin; PYY, peptide YY; SERT, 5-HT transporter; TEM, transmission electron microscopy; TPH, tryptophan hydroxylase; VMAT, vesicular monoamine transporter.

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the neuroendocrine regulation of small bowel function, which will facilitate delineation of the mechanisms responsible for intestinal secretion and motility.

An important disease arising from the EC cell is the carcinoid tumor of the small bowel (9). The pathophysiology of EC cell neoplastic transformation has not been defined, reflecting both the lack of knowledge of normal EC cell function and the unavailability of a pure EC cell carcinoid preparation or primary tumor cell line. In particular, the similarities or differences between normal and neoplastic EC cells are unknown. Because the dominant carcinoid tumor of the body is the EC cell carcinoid of the small bowel (43%) (10), such information would be of considerable scientific and clinical relevance.

In this study we generated a 99–100% pure cell preparation of human EC cells and compared them to a novel human EC cell malignant carcinoid cell line that we have developed (11, 12). The purposes of the study were to define the characteristics of normal human EC cells and identify differences between normal and neoplastic EC cells.

Materials and Methods

Naive EC cell

Cell isolation. We have previously developed the methodology to isolate EC cells from ileal tissue of the multimammate mouse (*Mastomys natalensis*) (8). For human preparations, normal ileum (5–8 cm; right hemicolectomy resections) were preincubated in dithiothreitol/Hanks' balanced salt solution (30 min) to remove mucus (13, 14). Total mucosa was obtained by blunt dissection and hand-minced with razors (13), the slurry digested by stirring in pronase (0.7 mg/ml)/collagenase (0.25 mg/ml) in 2 mM EDTA medium (1 h) and then filtered [crude cell fraction (F_0)]. Ileal mucosal scrapings provided a baseline to assess enrichment.

Nycodenz gradient centrifugation. Density gradient centrifugation (1100 rpm) was performed with F_0; cells were collected at 1.070 g/liter (fraction F_N) (14).

FACS sorting. Cells (F_0 and F_N) were fixed (ice-cold methanol), immunostained with mouse monoclonal anti-TPH (antitryptophan hydroxylase; 0.1 μg/ml; Calbiochem, La Jolla, CA), washed, and stained with fluorescein isothiocyanate (FITC)-labeled secondary antibody (1:100 dilution, 20 min, room temperature). For FACS sorting of live cells, F_N preparations were incubated with the weak base acridine orange (AO; 2 μM, 20 min, room temperature). All fractions were filtered (50 μm pore size) before sorting (high-speed FACS Aria, Yale Cancer Center, New Haven, CT; excitation, 488 nm; activated FITC- or AO-labeled cells) and sorted by gating on side scatter (dense, small cells ~8–14 μm; emission, 532 ± 15 nm). Positive cells were collected over 30 min (fraction F_{FACS}).

Characterization. TPH and chromogranin A (CgA)-positive cells were quantified to determine the purity of EC cell preparations (8). 5-HT content (ELISA) and transcript levels of neuroendocrine (NE) and EC cell-specific marker genes (PCR) were determined.

Immunostaining. For methanol-fixed, FITC-TPH-positive, FACS-sorted preparations, cells (2–5 × 10^4) pipetted onto frosted microscope slides were counted (percentage of TPH-positive cells after FACS sorting). In separate experiments, aliquots of FACS-sorted, fixed cells were incubated with CgA antibodies (1:200; 4 C; goat, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), stained with secondary antibody (horseradish peroxidase-antigoat; 1 h at room temperature; 1:100; DakoCytomation, Carpinteria, CA), and visualized with Cy5-tyramide (NEN Life Science Products, Boston, MA) to provide the percentage of CgA-positive cells (NE cells in each preparation). As a control for nonspecific staining, CgA was excluded. For live AO-FACS-sorted preparations, cells (2–5 × 10^4) fixed in methanol were stained with anti-CgA (mouse; 1:1000; DakoCytomation) or mouse anti-TPH, stained with secondary antibody (FITC-antimouse/rabbit; 1:100; Promega Corp., Madison, WI), and cells were counted to assess the percentage of NE or EC cells in each preparation. As a control for nonspecific staining, primary antibodies were excluded. For confocal microscopy, micrographs were recorded using a confocal microscopy system equipped with three Ar488, Kr568, and HeNe633 lasers (TCS-SP, Leica, Mannheim, Germany).

Transmission electron microscopy (TEM) and Immunogold staining. Cells (1 × 10^6) were fixed (1 h) in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4), postfixed in 1% osmium tetroxide, processed (Epon embedded), and thin sectioned (15). Cells for Immunogold labeling were fixed (4% paraformaldehyde), gelatin embedded, and infused (2.3 M sucrose), and ultrathin cryosections were prepared. Grids were examined by TEM (Tecnai 12 Biotwin electron microscope) at 80 kV.

Real-time PCR. Two NE markers [CgA and vesicular monoamine transporter ($VMAT_1$)] and four markers of EC cells [TPH, 5-HT transporter (SERT), substance P, and guanylin] were assessed using real-time PCR (16). To detect contaminating NE cells, somatostatin, gastrin, glucagon, peptide YY (PYY), proopiomelanocortin (POMC), and neurotensin transcripts were amplified. All primer probe sets were designed to encompass exon:exon boundaries and preferentially amplify cDNA (Assay-on-Demand, Applied Biosystems, Foster City, CA).

RNA extraction and clean-up. RNA (intact mucosa, F_0, F_N, and F_{FACS}) was extracted (TRIzol, Invitrogen Life Technologies, Inc., Gaithersburg, MD) and cleaned (RNeasy Kit and DNeasy Tissue Kit, QIAGEN, Valencia, CA) to minimize contaminating genomic DNA. RNA (2 μg) was converted to cDNA (High Capacity cDNA Archive Kit, Applied Biosystems) (17, 18).

Real-time PCR protocol. Real time RT-PCR analysis was performed using Assays-on-Demand products and the ABI 7900 Sequence Detection System according to the manufacturer's suggestions (17). Cycling was performed under standard conditions (TaqMan Universal PCR Master Mix Protocol), and the standard curve method (ABI User Bulletin 2) was used to determine relative transcript levels. GAPDH was used to normalize data (17, 18).

5-HT content. 5-HT content was measured and quantified as a percentage of the total protein content. Cells (5 × 10^4) were pelleted (300 × *g*, 5 min), total protein was extracted and assayed (milligrams per milliliter; Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA), and 5-HT content (nanograms per milliliter) was measured (5-HT ELISA, Diagnostika GmBH, Hamburg, Germany) according to the manufacturer's instructions for serum samples. The 5-HT content was defined as nanograms of 5-HT per milligrams of protein.

Culture and 5-HT secretion. In rodent studies, 5-HT secretion was regulated by adrenergic, muscarinic, and γ-aminobutyric acid (GABA) receptors (8), whereas small intestinal NE cell secretion is a cAMP-mediated event (8, 13, 19). In the current studies, the time course for 5-HT secretion was determined in response to forskolin (cAMP activator). The effects of isoproterenol (selective β-adrenergic receptor agonist), acetylcholine chloride (muscarinic ligand), $GABA_A$, pituitary adenylate cyclase-activating polypeptide (PACAP)-38, and gastrin (all 10^{-12} to 10^{-6} M) were then measured, and the effect of the somatostatin analog, octreotide, on isoproterenol-stimulated 5-HT secretion ($EC_{50} = 10^{-8}$ M) was evaluated.

Live AO-FACS sorted cells (~2 × 10^4 cells/100 μl) in serum-free Ham's F-12 medium (Invitrogen Life Technologies, Inc.) with antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml; Sigma-Aldrich Corp., St. Louis, MO; 2 × 10^4 cells/well 96-well collagen I-coated plates; BD Biosciences, Fullerton, CA) were maintained in a humidified atmosphere at 37 C in 5% CO_2 for 2 h. A time course (0, 3, 5, 15, 30, 45, and 60 min) for 5-HT secretion in response to forskolin (10^{-6} M) was then evaluated. To confirm that release represented a secretory process rather than cell damage or lysis, lactate dehydrogenase (LDH) release into media was measured (commercially available LDH assay, CytoTox-ONE Homogenous Membrane Integrity Assay, Promega Corp.). Dose-response curves for ligands (15-min incubation) were then determined. Data for 5-HT secretion were expressed relative to baseline secretion; values shown are the mean ± SEM. The EC_{50}/IC_{50} values were calculated from nonlinear regression analysis (PRISM 4, GraphPad, Inc., San Diego,

TABLE 1. Human EC cell purification methodology

	Human EC cell preparations (n = 4)			
	Ileal mucosa (F_0)	Nycodenz (F_N)	FACS-TPH (F_{FACS})	FACS-AO
5-HT (fold increase compared to IM)	2	28	72	67
TPH^+ cells (%)	4.2 ± 0.6	75 ± 8.2	99 ± 0.6	99 ± 0.9
CgA^+ cells (%)	6.3 ± 1.1	84 ± 3	100 ± 0.3	100 ± 1.3
Cell no.[a]	2.8×10^7	2.7×10^6	6.8×10^5	7.2×10^5
Viability (%)	99.6	97.9	NA	99.3

Nycodenz gradient centrifugation, followed by FACS sorting with either tryptophan hydroxylase- or acridine orange-stained cells, results in sequential enrichment of intestinal EC cells. F_{FACS} viability cannot be evaluated. Cells were ethanol fixed before anti-TPH immunostaining. Trypan blue staining was used. NA, Not applicable; IM, ileal mucosa.

[a] Average of five separate experiments (one human sample per experiment).

CA). LDH release was measured in parallel experiments to confirm that no cell damage occurred.

Neoplastic EC cell-KRJ-I cell line

Cell culture. KRJ-I cells were cultured in Ham's F-12 medium supplemented with antibiotics and 10% fetal calf serum (Sigma-Aldrich Corp.).

Confocal microscopy and Immunogold labeling. Confocal microscopy and Immunogold labeling were undertaken as described for naive cells, except that 2×10^6 cells were used per preparation.

5-HT secretion. The secretory profile of KRJ-I cells (5×10^4 cells/well in serum-free medium) was assessed after stimulation (60 min) with forskolin, isoproterenol, acetylcholine chloride, $GABA_A$, PACAP-38, and gastrin.

GeneChip examination: naive vs. neoplastic EC cells

Affymetrix transcriptional profiling (Affymetrix, Santa Clara, CA) was used to identify genes altered in small intestinal carcinoids compared with normal mucosa (18). In this study we used the same approach to define the transcriptome of the naive human EC cell and compared it with the neoplastic KRJ-I cell.

Hybridization. For each experiment (n = 3), 2–3.5 μg total RNA ($A_{260/280}$, >1.9) was cRNA labeled, hybridized (Affymetrix Human Expression 230 2.0 chip; 39,000 transcripts) and analyzed using a standard protocol (Keck Affymetrix Core Facility, Yale University, New Haven, CT) (18).

GeneChip data analysis. Hybridized arrays were scanned (Affymetrix GeneChip 3000 scanner) and scaled (average intensity, 500), and hybridization intensity data were converted into the presence/absence of calls for each gene (Affymetrix GCOS software) (18). Sample comparison between the neoplastic and naive EC cells was undertaken using GCOS-generated .CEL files, normalizing and modeling to generate model-based estimates of expression for each probe set [DNA Chip Analyzer (dCHIP), version 1.3] (18, 20).

Results

Naive human EC cells

Cell isolation. The intact mucosa contained 4.01 ± 1.4, and F_0 contained 7.6 ± 1.2 ng 5-HT/mg protein (Table 1). This increased to 116 ± 9 ng/mg in F_N, 15-fold more than that in F_0 and 28-fold more than that in intact mucosa. F_0 and F_N cells contained 2.7 ± 1.2% and 75 ± 6.1% TPH-positive cells, respectively. Nycodenz gradient centrifugation resulted in a 32-fold enrichment and significantly enriched populations of EC cells (~75%) *vs.* F_0. FACS sorting of F_N using TPH immunostaining or AO (2 μM) produced a 99% homogenous preparation of human ileal EC cells (F_{FACS}).

Characterization

Immunostaining. Confocal microscopy of F_{FACS} cells (dual-stained with AO and anti-TPH) demonstrated colocalization of TPH and AO within the cytoplasm (Fig. 1A). Some 100 ± 0.3% cells were CgA positive, and 99 ± 0.6% were TPH positive, indicating a NE origin and EC cell phenotype.

Electron microscopy. Ultrastructure of F_{FACS} cells evaluated using TEM identified EC cell characteristics (21, 22): 1) well-developed Golgi apparatus, numerous mitochondria, and

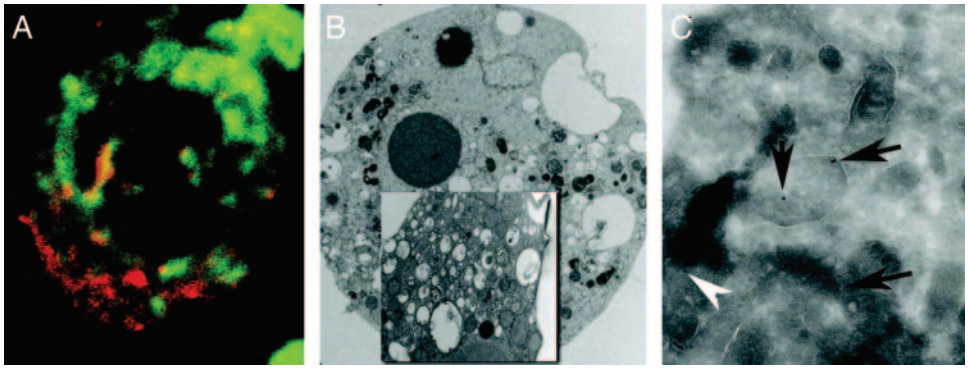

FIG. 1. Naive human EC cell morphology. A, Confocal immunofluorescence microscopic picture of dual-stained naive human EC cells demonstrating localization of acridine orange in vesicles (*green* fluorescence) and identification of TPH (*red* fluorescence) within the cytoplasm (×400 magnification). B, EM (×7200 magnification) of EC cells demonstrating the typical admixture of large granules and electroluscent empty vesicles. Vesicles are characterized by dense content and pear or ovoid shapes consistent with their biconcave shape (*inset*). Cells are approximately 8 μm in size. C, Immunoelectron micrograph of a human EC cell labeled with anti-TPH. Gold particles associated with this enzyme are clearly visible in the ER (*white arrow*) and in vesicular structures (*black arrows*). The absence of staining (control, exclusion of primary antibody) demonstrated specificity of the TPH Immunogold staining (data not shown).

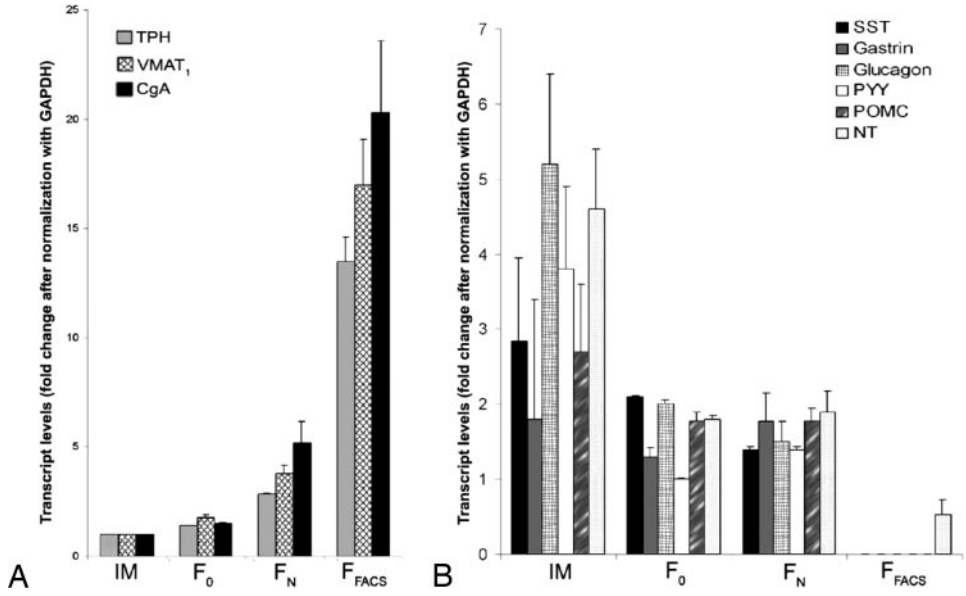

FIG. 2. Enrichment of human ileal 5-HT-containing cells during respective Nycodenz gradient centrifugation and FACS sorting steps. A, Increase in transcript levels of TPH, $VMAT_1$ and CgA over intact mucosa (IM; fold increase) during progressive purification of EC cell fractions. Values are the mean ± SEM (n = 3). B, Decrease in and absence of transcript levels of somatostatin (SST), gastrin, glucagon, PYY, POMC, and neurotensin (NT) during progressive purification of EC cell fractions. FACS-sorted preparations only expressed neurotensin transcripts. Values are the mean ± SEM (n = 3).

rough endoplasmic reticula; 2) vacuolated granules; and 3) large numbers of variously sized electron dense vesicles, both consistent with NE secretory function (Fig. 1B). Gold particle labeling (anti-TPH) demonstrated this enzyme in endoplasmic reticulum (ER) and Golgi apparatus (Fig. 1C). Gold labeling of substance P and 5-HT antibodies demonstrated these secretory substances within vesicles (data not shown). These results confirm both the EC nature and active synthesis of specific secretory products.

PCR. Sequential increases in NE (CgA and $VMAT_1$) and EC (TPH) cell-specific markers were confirmed during purification, with the highest expression (~14- to 21-fold greater than intact mucosa) in F_{FACS} (99% EC cell; Fig. 2A). EC-enriched cell preparations were positive for SERT (5.1- to 8.2-fold greater than intact mucosa), substance P (9.1- to 12-fold greater), and guanylin transcripts (4.3- to 6-fold greater).

Contaminating cells. AO-FACS-sorted fractions (containing 1% contaminating CgA-positive cells) did not express somatostatin, gastrin, glucagon, PYY, or POMC transcripts, but expressed neurotensin transcript, indicating that the contaminating NE cell type was a neurotensin-producing cell. Somatostatin-producing D cells were absent, abrogating a potential inhibitory effect on cell secretion (23).

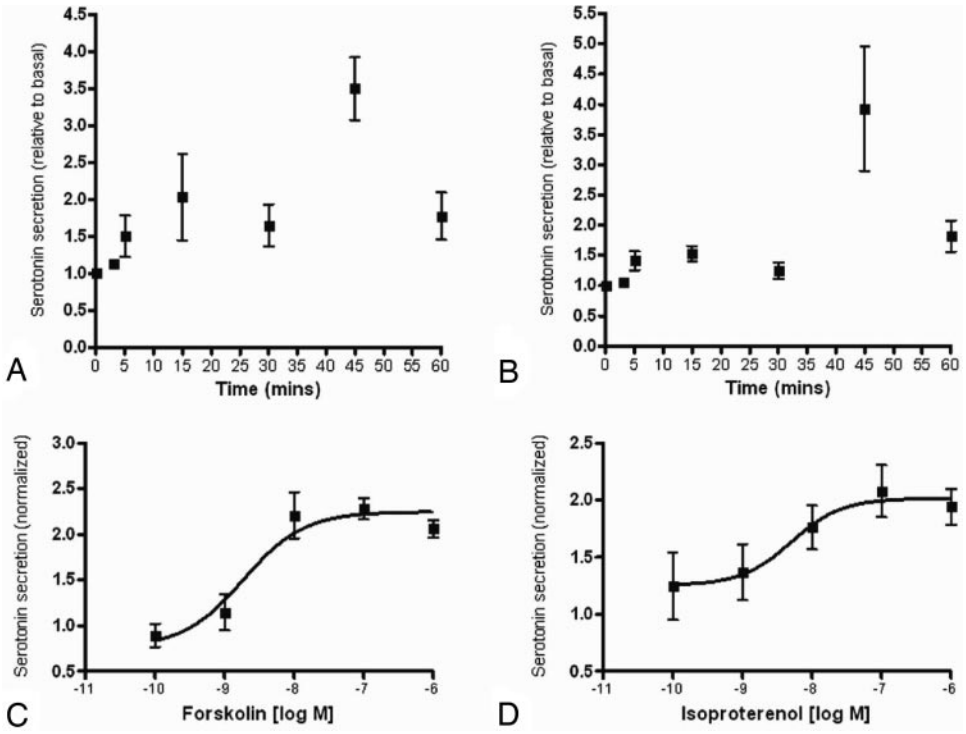

FIG. 3. Time course and dose responses for forskolin- and isoproterenol-stimulated 5-HT secretion from 1-h cultured naive EC cells. Significant stimulation was evident at 5 min and was maximal at 45 min for both forskolin- (10^{-6} M; A) and isoproterenol (10^{-6} M; B)-stimulated secretion. The EC_{50} values for forskolin (C) and isoproterenol (D) were calculated, using nonlinear regression analysis, to be 1.8×10^{-9} and 5.1×10^{-9} M, respectively. Data are normalized to unstimulated (basal) secretion and are presented as the mean ± SD (n = 3; each experiment performed in triplicate).

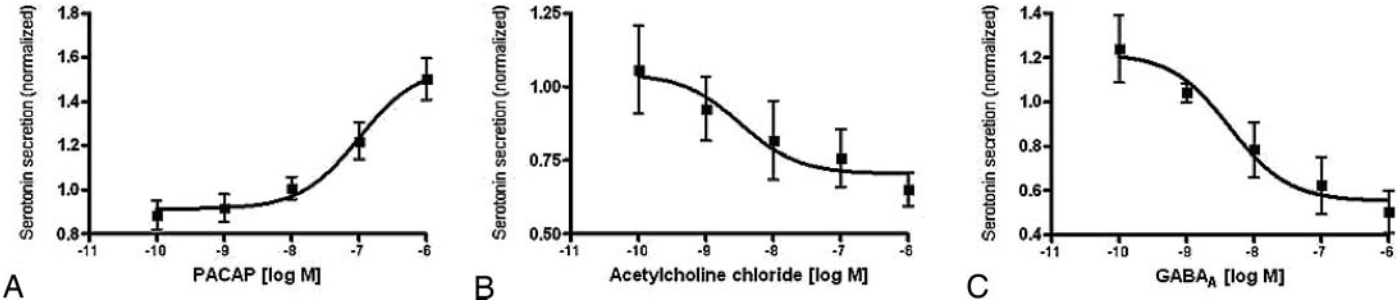

FIG. 4. PACAP, acetylcholine chloride, and $GABA_A$ dose-response curves for 5-HT secretion from 1-h cultured naive EC cells. The half-maximal response (EC_{50} or IC_{50}) was calculated using nonlinear regression analysis. The EC_{50} value for PACAP was 1.4×10^{-7} M (A). Dose-response curves for inhibition of isoproterenol ($EC_{50} = 1 \times 10^{-8}$ M)-stimulated 5-HT secretion by acetylcholine chloride (B) and $GABA_A$ (C) demonstrated IC_{50} values of 3.2×10^{-9} and 4.4×10^{-9} M, respectively. Data are presented as the mean ± SD (n = 3; experiments performed in triplicate).

Receptors. Using real-time RT-PCR, receptors known to modulate NE cell function in EC-like cells (PACAP, gastrin, and somatostatin) were examined in human EC cell preparations (24–27). Neural ($VPAC_1$) and somatostatinergic (SST_R2) receptors were present, but CCK_2 was not, suggesting that human EC cells might respond to PACAP and somatostatin, but not gastrin.

Culture and 5-HT secretion

5-HT secretion in response to forskolin (10^{-6} M) and isoproterenol (10^{-6} M) was assessed in F_{FACS} cells to establish the secretory time course. 5-HT secretion was identifiable as early as 5 min and peaked at 45 min (3.5 ± 0.2- and 4.0 ± 1.2-fold, respectively; Fig. 3, A and B). No significant alteration in culture medium LDH levels at each time point was noted, indicating no cytotoxic effect of either agent.

Isoproterenol stimulated secretion ($EC_{50} = 5.1 \times 10^{-9}$ M; Fig. 3D) and forskolin stimulated secretion ($EC_{50} = 1.8 \times 10^{-9}$ M), suggesting cAMP signaling pathway involvement in 5-HT secretion (Fig. 3C). Isoproterenol stimulated intracellular cAMP levels 1.6 ± 0.12-fold *vs.* unstimulated cells ($EC_{50} = 2.7 \times 10^{-9}$ M), confirming a cAMP signaling component.

Dose responses (45 min) for secretagogues, the neural agents, PACAP-38, acetylcholine chloride, and $GABA_A$; the hormonal agent, gastrin; and the SST agonist, octreotide, were determined. PACAP-38 stimulated secretion ($EC_{50} = 1.0 \times 10^{-7}$ M; Fig. 4A), whereas acetylcholine chloride and $GABA_A$ inhibited isoproterenol ($EC_{50} = 10^{-8}$ M)-stimulated secretion with IC_{50} values of 3.2×10^{-9} and 4.4×10^{-9} M (Fig. 4, B and C). Both agents inhibited basal 5-HT secretion ($IC_{50} = 3 \times 10^{-9}$ and 2×10^{-9} M). Gastrin did not elevate secretion above basal (Fig. 5A), but octreotide inhibited both basal and isoproterenol-stimulated secretion with IC_{50} values of 3×10^{-10} and 1.3×10^{-8} M (Fig. 5B). LDH levels were not altered by any of the evaluated ligands.

Neoplastic cells (KRJ-I cell line)

Characterization. KRJ-I is a long-term metastatic ileal carcinoid cell line established from a multifocal metastatic ileal carcinoid tumor with insular histology (11). We have previously characterized this cell line in detail (12), and in this study, we confirm KRJ-I to be of NE and EC cell origin. All cells were CgA, TPH, substance P, and guanylin positive by immunohistochemistry and real-time PCR (Table 2). Markers for two other small intestine NE cells (SST and neurotensin) were absent. Real-time RT-PCR demonstrated $VPAC_1$, $VPAC_2$, β_1-adrenergic, M_4, and SST_R2 receptors, but not CCK_2, suggesting that, like the naive human EC cell, KRJ-I cells may be responsive to PACAP and somatostatin, but not to gastrin. Electron microscopy demonstrated oval or irregular and lobulated nuclei with single nucleoli and pleomorphic cytoplasmic NE granules (Fig. 6A). TPH Immunogold staining demonstrated uptake in ER and Golgi (Fig. 6B). Substance P and 5-HT gold labeling demonstrated positivity within vesicular structures (Fig. 6C), and confocal microscopy demonstrated dual positivity for TPH and substance P (Fig. 6D).

5-HT secretion

Previously, we noted that KRJ-I 5-HT secretion was stimulated with isoproterenol, involved cAMP activation, and could be inhibited by an SST agonist (12), indicating functional SST_R and β-adrenergic receptors. In this study, we compared 5-HT secretion with that in naive cells (Tables 3 and 4). Dose responses (60 min) for forskolin demonstrated

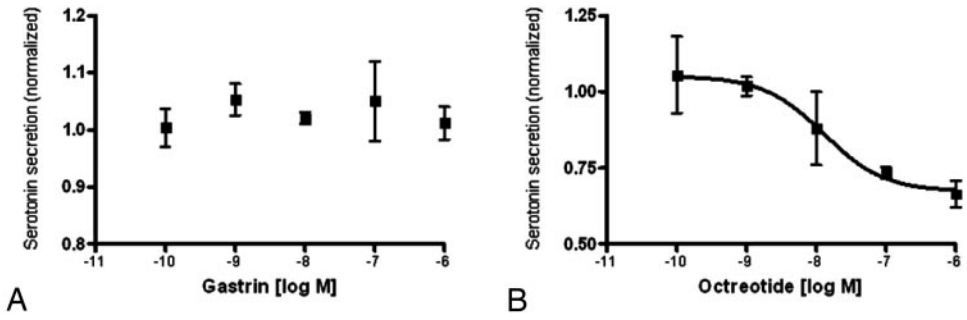

FIG. 5. Gastrin and octreotide dose responses for 5-HT secretion from 1-h cultured naive EC cells. Gastrin had no effect (A), whereas octreotide inhibited isoproterenol ($EC_{50} = 1 \times 10^{-8}$ M)-stimulated 5-HT secretion with an IC_{50} of 1.3×10^{-8} M (B). Data are presented as the mean ± SD (n = 3; experiments performed in triplicate).

TABLE 2. KRJ-I cell line characterization (real-time RT-PCR and immunostaining) demonstrates it to be an EC cell with classic neuroendocrine features

	Transcript	Protein
NE cell markers		
CgA	+	+
$VMAT_1$	+	+
EC cell markers		
TPH	+	+
5-HT	NA	+
SERT	+	ND
Substance P	+	+
Guanylin	+	+
Non-EC cell markers		
Somatostatin	−	−
Neurotensin	−	ND

NA, Not applicable; ND, not determined.

stimulation of KRJ-I serotonin secretion ($EC_{50} = \sim 2.1 \times 10^{-7}$ M; naive cells, $EC_{50} = 1.8 \times 10^{-9}$ M). Isoproterenol stimulated KRJ-I ($EC_{50} = 8.1 \times 10^{-8}$ M; naive cells, $EC_{50} = \sim 8.1 \times 10^{-8}$ M), whereas PACAP-38 stimulated 5-HT secretion with a similar efficacy ($EC_{50} = \sim 1 \times 10^{-7}$ M; Tables 3 and 4).

Acetylcholine chloride and $GABA_A$ inhibited basal 5-HT secretion ($IC_{50} = 2 \times 10^{-9}$ M; naive cells, $\sim 3 \times 10^{-9}$ M). The effect of $GABA_A$ on isoproterenol ($EC_{50} = 10^{-8}$ M)-stimulated secretion was more potent in KRJ-I cells ($IC_{50} = \sim 4 \times 10^{-10}$ M; naive cells, $\sim 4 \times 10^{-9}$ M); acetylcholine chloride was 100-fold less potent ($IC_{50} = \sim 2 \times 10^{-7}$ M; naive cells, $\sim 3 \times 10^{-9}$ M). Octreotide did not inhibit basal, but inhibited isoproterenol-stimulated, secretion ($IC_{50} = 5.3 \times 10^{-9}$ M). Gastrin had no effect on EC cell secretion. LDH levels were not altered by any of the ligands.

EC cell transcriptome

FACS-sorted naive human EC cells and the KRJ-I cell line expressed 38–43.0% of the more than 23,000 transcripts on the U133 chip and had low 3′/5′ ratios of GAPDH less than 3.5 (consistent amplification not 3′-skewed). NE transcripts (CgA and $VMAT_1$), EC cell marker genes (SERT, TPH, substance P, and guanylin), and SST_R2 and $VPAC_1$ receptors, consistent with the physiological evaluation of EC cell function, were present in both samples (Table 5). Serotoninergic ($5\text{-}HT_{2C}$ or $5\text{-}HT_{2A}$) and tachykininergic ($TAC_{1/2}$), but not histaminergic, receptors were also present.

Naive cells expressed $SSTR_5$, M_4/nicotinic B_2/$GABA_A\alpha_6/\beta_2$ receptors, whereas neoplastic cells expressed SST_R3, cholinergic M_4/GABA-Aγ3 receptors. Naive cell cycle gene expression suggests that they may not be terminally differentiated (23).

Discussion

Using FACS sorting technology developed for the isolation of pure rodent EC cells (8), we isolated 99% pure human EC cells and noted that this cell produces 5-HT, substance P, and guanylin. These results confirm previous studies in other species (1, 4, 5). In addition, we established and characterized a neoplastic EC cell line, KRJ-I, and demonstrated that it expresses similar enzyme (TPH) and secretory transcripts (substance P and guanylin) and proteins (5-HT and sub-

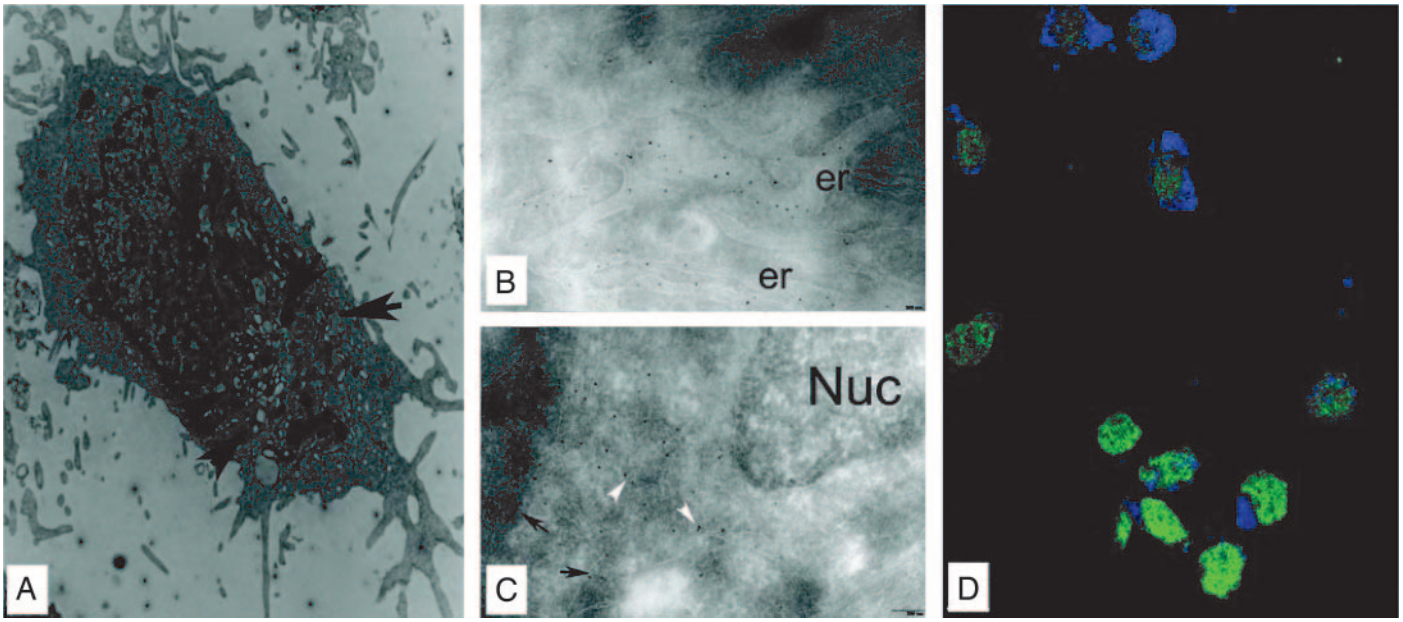

FIG. 6. Neoplastic human EC cell (KRJ-I) morphology. A, EM of KRJ-I cells demonstrating a large, lobulated nucleus, multiple mitochondria, and the typical admixture of numerous small vesicles (*black arrows*) and irregular dense secretory granules. Cells are approximately 12 ± 2.8 μm in size (×4,800 magnification). B, Immunoelectron micrograph of cells dual-labeled with anti-TPH (5 nm gold) and substance P (10 nm gold). Gold particles associated with TPH are evident in the ER (er), as is substance P in this micrograph (×64,000 magnification). C, Immunoelectron micrograph of cells dual-labeled with anti-5-HT (5 nm gold) and substance P (10 nm gold). Gold particles associated with both these EC cell products are evident in the same vesicular structures (*white arrowheads*), or with substance P alone (*black arrows*; ×64,000 magnification). Nuc, Nucleus. D, Confocal immunofluorescence microscopic picture of dual-stained KRJ-I cells demonstrating localization of substance P (*blue* fluorescence) and 5-HT (*green* fluorescence) within the cytoplasm. Substance P staining in KRJ-I cells, as in naive EC cells, was predominantly localized at the periphery of the cells (×400 magnification).

TABLE 3. Stimulatory concentrations of agents on naive and neoplastic EC cell 5-HT secretion *in vitro*

	Naive EC cell (M)[a]	Neoplastic EC cell (M; KRJ-I)[a]
Forskolin	1.8×10^{-9}	2×10^{-7}
Isoproterenol	5.1×10^{-9}	8.1×10^{-8}
PACAP-38	1×10^{-7}	1.4×10^{-7}
Gastrin	0	0

[a] Values are EC_{50} for effects of agents on 5-HT secretion.

stance P) as well as receptors (β-adrenergic, $VPAC_1$, M_4, GABA, and SST) as the naive cell.

In the naive human EC cell preparation, similar to rodent preparations (8), the contamination of 1% or less was due to neurotensin-producing endocrine cells. The precise function of neurotensin is unknown, but it is considered to be related to the maintenance of capillary membrane permeability (28). The fact that neither the normal EC cell transcriptome nor the neoplastic phenotype expresses a neurotensin receptor suggests that the presence of neurotensin will not alter the physiological evaluation of human EC cells. Of particular importance, however, was the demonstration that no somatostatin cells were present in the EC preparation.

The naive human EC cell is approximately 8 μm in diameter and contains pleomorphic secretory granules that represent the source of 5-HT, substance P, and guanylin. A functional assessment of the EC cell demonstrates that secretory stimulation is mediated by downstream signaling from β-adrenergic and PACAP receptors, whereas inhibition is effected via the neural transmitters, $GABA_A$ and acetylcholine. Octreotide (somatostatin analog) is an inhibitor, as might be predicted from the presence of the SST_R2 receptor subtype. Of note was the absence of CCK_2 receptor transcripts in both normal and neoplastic EC cells and the fact that gastrin was ineffectual in stimulating cell secretion. This is of clinical interest because gastrin (pentagastrin test) has previously been used as a provocative stimulant for such tumors (29). This suggests that the provocative effect of gastrin in activating neoplastic EC cell secretion must occur via an upstream, indirect, gastrin-initiated mechanism, rather than by gastrin itself.

Of particular interest was the identification of the different somatostatin receptor subtypes in the naive human EC cell compared with the EC cell carcinoid cell line. The ability to demonstrate different receptor profiles is of clinical and scientific relevance, because 30–40% of carcinoid patients treated with somatostatin analogs have either ineffective symptom control or breakthrough of symptomatology during therapy (9, 30). This has been variously ascribed to alterations in receptor sensitivity or profile, drug formulation, or lack of compliance (31). The current therapeutic somatostatin analogs (octreotide and Autogel) exhibit a dominant somatostatin subtype 2 and 5 receptor profile (32). Tumors that do not express these two receptor subtypes would be less amenable to currently available therapy. Our investigations suggest that somatostatin receptor profiling may allow prediction of the efficacy of a particular form of therapy. In addition, receptor profiling of an individual patient's tumor may allow for the targeted use of a specific agent best capable of managing a particular tumor.

The KRJ-I cell line proliferates rapidly and expresses a receptor profile similar to the normal human EC cell, and its functional responses to secretagogues and inhibitors are comparable, with some interesting differences. The lower cell levels of 5-HT in this cell may reflect the altered ability of a neoplastic cell to produce 5-HT or may be consistent with the proposed inability of the tumor cell to regulate 5-HT secretion, leading to indiscriminate loss into the circulation, consistent with the symptoms of the carcinoid syndrome (33).

There were interesting differences in naive and neoplastic secretory responses to neural agents. Acetylcholine inhibited naive EC cell 5-HT secretion more potently than neoplastic secretion, whereas $GABA_A$ was more potent in tumor cells. Octreotide failed to inhibit basal neoplastic EC 5-HT secretion, but had an inhibitory effect on naive EC cells. These differences may represent differences in receptor profiling, because neoplastic cells expressed cholinergic M_4/GABA-Aγ3 compared with M_4/nicotinic B2/GABA-Aα6/β_2 on naive cells.

Analysis of the FACS-sorted human naive EC cell transcriptome revealed shared NE and EC cell-specific marker genes and a receptor profile (SST, PACAP, acetylcholine, and GABA) consistent with the physiological evaluation of EC cell function. Serotoninergic ($5\text{-}HT_{2C}$ or $5\text{-}HT_{2A}$) and tachykininergic ($TAC_{1/2}$), but not histaminergic, receptors were present on both naive and neoplastic EC cells, suggesting that these cells may be subject to autoregulation by 5-HT and substance P. The presence of olfactory receptors suggests that the EC cell may also have a defined sensing function, particularly because some EC cells have apical cytoplasmic processes that access the bowel lumen (22). Differences in receptor expression (SST_R3 in neoplastic *vs.* SST_R5 in naive cells); alterations in serotoninergic, adrenergic, glutaminergic receptors; the identification of a number of markers potentially associated with carcinoid malignancy (particularly MAGE-D2 and MTA1) (18); and the cell cycle highlighted differences associated with neoplasia.

In summary, we have generated a greater than 99% pure preparation of normal human EC cells, characterized their secretory regulation, and characterized the secretory regulation of a neoplastic EC cell line derived from a human

TABLE 4. Inhibitory concentrations of agents on naive and neoplastic EC cell 5-HT secretion *in vitro*

	Naive EC cell[a]		Neoplastic EC cell (KRJ-I)[a]	
	Basal	Isoproterenol-stimulated secretion	Basal	Isoproterenol-stimulated secretion
Octreotide	3×10^{-10}	1.3×10^{-8}	0	5.3×10^{-9}
Acetylcholine	3×10^{-9}	3.2×10^{-9}	2.9×10^{-9}	1.6×10^{-7}
$GABA_A$	2×10^{-9}	4.4×10^{-9}	2×10^{-9}	3.9×10^{-10}

[a] Values are IC_{50} values for effects of agents on basal (unstimulated) or isoproterenol (1×10^{-8} M)-stimulated secretion.

TABLE 5. Commonly expressed genes in AO-FACS-sorted naive EC cells and the KRJ-I cell line as identified by Affymetrix GeneChip profiling

	Naive human EC cells	KRJ-I cells
NE markers		
CgA, -B	+	+
$VMAT_1$	+	+
TPH	+	+
SERT	+	+
Substance P	+	+
Receptors		
PACAP	$VPAC_1$	$VPAC_1$
Somatostatin	$SSTR_{2,5}$	$SSTR_{2,3}$
Cholinergic	Cholinergic M_4/nicotinic B_2	Cholinergic M_4/nicotinic A_5, A_6, -B_1
Adrenergic	β1	α1C, -β1
Dopamine	D_1, D_2	D_2
GABA	A α6 & &β2	A γ3
Histamine H_{1-4}	−	−
5-HT	5-HT_{2C}	5-HT_{2A}
Tachykinin	TAC_2	TAC_1
Growth factors and receptors		
EGF	EGF, TGF-α/EGF_R	EGF_R (erbB)
FGF	FGF-1, -7, -14, FGF_R2	βFGF2, FGF18, FGF_R1, -2
HGF	−	HGF
IGF	IGFBP4	IGFI/II, IGFBP5, IGF_RI/II,
PDGF	$PDGF_{A,B,C}$	$PDGF_A$
TGF-β	TGF-β2, TGF-β_R1	TGF-β2, TGF-β_R2, -3
VEGF		$VEGF_B$
Olfactory receptors		
Olfactory receptor	7E12, 3A2, 1D3, 4L1	7E12, 3A2, 1D2, 5L2
Cell cycle markers		
Ki67/PCNA	+/−	+/+
Cyclins	A2, C, D2, E2, H, I	A2, B2, D2, E1/2, F, G2, H, I, L, M
Cdks	2, 4, 8	3, 4, 5, 8, 9, 10
Cyclin inhibitors	p16, p27	p16, 18, 21, 27
Markers of EC growth or malignancy		
NAP1L1	+	+
MAGE-D2	−	+
MTA1	−	+

A selection of six major categories of genes identified in each cell type is represented. Cdk, Cyclin-dependent kinase; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatic growth factor; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

ileal carcinoid. The comparison between the two cell systems allows identification of the regulatory agents that modulate secretion and, potentially, proliferation. The identification of specific receptors and pathways will enable the development of agents capable of targeting such events. In addition, the identification of the transcriptome for both the normal and the neoplastic EC cell line allows the elucidation of signatures responsible for proliferative regulation and defines the malignant and metastatic potential of this cell system.

We believe that because the delineation of the EC-like cell system led to the understanding of gastrin-regulated acid secretion and the ability to identify different types of gastric carcinoid (34), a similar assessment of the human EC cell will be important. Delineation of different small bowel carcinoids into specific lesions of defined benignity and malignancy is a likely goal. It is possible that the further assessment of the function of normal human EC cells may well be of relevance in the evaluation of alterations of the NE regulatory mechanisms of irritable bowel syndrome.

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All of the authors have nothing to declare.

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