Neuro
-endocrinology

Neuroendocrinology 2009;89:458–470
DOI: 10.1159/000209330

Received: August 14, 2008
Accepted after revision: October 9, 2008
Published online: March 18, 2009

KRJ-I and BON Cell Lines: Defining an Appropriate Enterochromaffin Cell Neuroendocrine Tumor Model

Zakiya-Luna Siddique[a] Ignat Drozdov[a] Jared Floch[a] Bjorn I. Gustafsson[a–c]
Kamilla Stunes[b] Roswitha Pfragner[d] Mark Kidd[a] Irvin M. Modlin[a]

[a]Gastrointestinal Pathobiology Research Group, Yale University School of Medicine New Haven, New Haven, Conn., USA; [b]St Olav's Hospital HF, Trondheim University Hospital, and [c]Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [d]Institute of Pathophysiology and Immunology, Centre for Molecular Medicine, Medical University of Graz, Graz, Austria

Key Words
Neuroendocrine tumors · Enterochromaffin cell · BON cell line · KRJ-I cell line

Abstract
Background: Neuroendocrine tumors (NETs) of the gastrointestinal (GI) system are increasing in incidence with minimal improvement in prognosis. Although the cell of origin has been identified as the enterochromaffin (EC) cell, its secretory and proliferative regulation has not been defined at a mechanistic level. To date, the BON cell line has been the most widely used in vitro EC cell model despite its pancreatic origin. Using whole-genome mathematical analysis as well as secretory and proliferative studies, we compared the BON cell line to the small intestine (SI) EC cell-derived NET cell line, KRJ-I, to assess individual cell line validity and applicability for the investigation of GI-NET disease. ***Methods and Results:*** Principal component analysis and ANOVA of KRJ-I and BON transcriptomes (U133 Plus 2) identified substantially different (<10%) overlap in transcripts with minimal (R^2 = 0.24) correlation in gene expression profiles. RT-PCR detected large variability (>12%) in neuroendocrine (NE) marker transcripts in the BON cell line and the absence of *Tph-2, DDC, TGFβR2,* and *M3* transcripts in KRJ-I. The KRJ-I cell line secreted serotonin (5-HT) in response to isoproterenol (EC_{50} = 100 nM), noradrenaline (EC_{50} = 1.7 nM), and pituitary adenylate cyclase (PACAP, EC_{50} = 0.03 nM). Cholecystokinin (IC_{50} = 430 nM), somatostatin (IC_{50} = 400 nM), acetylcholine (IC_{50} = 3.7 nM), and γ-aminobutyric acid A ($GABA_A$, IC_{50} = 2 nM) all inhibited 5-HT release, while gastrin and bombesin had no effect. 5-HT secretion in the BON cell line was stimulated by isoproterenol (EC_{50} = 900 nM), noradrenaline (EC_{50} = 20 nM), cholecystokinin (EC_{50} = 130 nM), PACAP (EC_{50} = 0.12 nM), bombesin (EC_{50} = 15 nM), and acetylcholine (EC_{50} = 0.2 nM). It was inhibited by somatostatin (IC_{50} = 300 nM) but not $GABA_A$. KRJ-I responded with proliferation to connective tissue growth factor (CTGF, EC_{50} = 0.002 ng/ml), transforming growth factor-α (TGFα, EC_{50} = 0.63 ng/ml) and transforming growth factor-β (TGFβ, EC_{50} = 0.63 ng/ml). Epidermal growth factor (EGF) and somatostatin had no significant effect. BON cell proliferation was stimulated only by EGF and TGFα (EC_{50} = 15.8 and 10 ng/ml). TGFβ (IC_{50} = 0.16 ng/ml), MZ-4-147 (IC_{50} = 0.5 nM), and BIM23A761 (IC_{50} = 0.06 nM) all inhibited proliferation. CTGF and somatostatin had no effect. ***Conclusion:*** KRJ-I and BON cell lines demonstrate

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0028–3835/09/0894–0458$26.00/0

Accessible online at:
www.karger.com/nen

Irvin M. Modlin
Yale University School of Medicine
333 Cedar Street, PO Box 208062
New Haven, CT 06520-8062 (USA)
Tel. +1 203 785 5429, Fax +1 203 737 406, E-Mail imodlin@optonline.net

substantial differences in gene level transcripts, inconsistent receptor profile expression, wide variability in NE marker transcript levels, and significantly differential proliferative and secretory responses. Given the EC cell origin of KRJ-I, these results provide evidence that the BON cell line does not represent an EC cell system and is not a valid study model of (carcinoid) EC cell-derived NET.

Background

The understanding of gastrointestinal (GI) neuroendocrine tumor (NET) pathobiology has been hampered by the paucity of information regarding their mechanisms of secretion, proliferation, and metastasis. To a large extent this reflects the lack of animal models or cell lines for study [1]. The small intestine (SI) and the ileum in particular are the most common GI-NET sites, comprising 21% of all NETs [2], while pancreatic NETs comprise about 1% and represent about 5% of NET incidence [1, 3]. Due to their initial nonspecific presentation, small size and distant location, SI-NETs evade detection, and are often misconstrued as the menopause, irritable bowel syndrome, simple food allergies or anxiety syndrome [4]. Consequently, diagnosis is delayed and the overall 5-year survival for SI-NETs is 64%; a rate which has remained virtually unchanged for the past 30 years [5].

Although the origin of SI-NETs has been identified as the enterochromaffin (EC) cell, the molecular basis of its neoplasia remains unknown. EC cells are ubiquitously distributed within the mucosal crypts of the GI epithelium, interspersed among other NE cells (enteroglucagon, neurotensin, somatostatin). The chief secretory product of the EC cell is serotonin (5-HT); however, substance P and guanylin have also been identified [6, 7]. The characterization of the receptor profile, transcriptome, and mechanistic basis of neoplastic EC cell function is critical to defining the molecular basis of SI-NET disease. The availability of such information is necessary to identify appropriate secretory and proliferative regulatory targets and facilitate the clinical management of this disease.

Establishing an in vitro GI-NET model has proven difficult due to the limited availability of neoplastic tissue, contamination with normal bowel flora, slow proliferation period, and poor long-term survival rates of primary cell cultures. To date, four human cell lines of variable applicability, namely COLO320DM [8], GOT1 [9], CND2 [10] and BON [11], have been utilized as in vitro models considered to be representative of human GI carcinoids. COLO320DM was established from a moderately undifferentiated adenocarcinoma of the sigmoid colon, GOT1 and CND2 cell lines were harvested from a liver metastasis associated with an ileal 'carcinoid' rather than a primary tumor. GOT1 is characterized by a slow doubling time (6–21 days) and is maintained as a mouse xenograft, whilst the CND2 cell line fails to express the principal NE marker chromogranin A and is most probably of NE carcinoma derivation.

To date, the most widely used in vitro model of GI-NETs has been BON. Previous studies have suggested the BON cell line to have characteristics of neoplastic EC cells [12–17] and it has been used as a model of physiological regulation of 5-HT release and to study proliferative regulation [12, 13, 18]. However, BON is an uncloned cell line with a heterogeneous cell population, derived from a peripancreatic lymph node metastasis of a pancreatic 'carcinoid'. It is likely to have acquired further genetic mutations in culture as a consequence of multiple passages and probably more accurately represents a pancreatic adenocarcinoid tumor rather than an EC cell NET per se [19]. Furthermore, the BON cell has distinct limitations as an appropriate GI-NET model since pancreatic NETs and GI-NETs are regarded as separate neoplastic entities [20]. This is based upon a number of analyses including: differentiation at a transcriptome level between the two tumor types [21], histological cell of origin, cytogenetic, mutational and SNP differences reflecting different etiologies and pathways of neoplastic development [19, 22–24] as well as distinctly different responses to chemotherapy [1, 25]. Based on this diverse array of evidence, the WHO categorized pancreatic and GI-NETs as two separate tumor entities [26]. As there are no EC cells in the pancreas, it is likely that BON cells are derived from a pancreatic adenocarcinoma exhibiting NE cell differentiation. The BON 'NE' phenotype can be reversed by Notch or alterations in transforming growth factor-β (TGFβ) and somatostatin [27, 28] signaling with a resultant transition to a mesenchymal phenotype [27], a feature more typical of carcinomas [29].

Recently, the human SI neoplastic EC cell line KRJ-I has been characterized [30, 31] and established as a model for the study of EC cell-derived NETs. KRJ-I is a continuous cell line, established from a primary multifocal ileal NET, with a doubling time of about 2 days, and displays classical morphological, immunohistochemical and biochemical features of an EC cell NET [32].

The establishment of a reliable in vitro model is necessary to define the molecular basis of SI-NET disease and for the future delineation of rational diagnostic and ther-

apeutic strategies. Thus, the identification of an appropriate neoplastic EC cell model is essential. The primary goal of this study was to evaluate the KRJ-I and BON cell lines. Our aims were to: (1) define KRJ-I and BON on a whole-genome level; (2) delineate their NE marker and receptor transcript expression; (3) characterize 5-HT secretion, and (4) define KRJ-I and BON proliferative effects in response to growth factors [epidermal growth factor (EGF), transforming growth factor-α (TGFα), connective tissue growth factor (CTGF), TGFβ], and pharmacotherapeutic agents [somatostatin, the growth hormone releasing hormone (GHRH) receptor antagonist (MZ-4-147), and the selective dopamine receptor 2 agonist (BIM23A761)].

Materials and Methods

Culture Conditions

KRJ-I cells were cultured as floating aggregates at 37°C with 5% CO_2. KRJ-I cells were kept in Ham's F12 medium (Gibco) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA), penicillin 100 U/ml and streptomycin (100 μg/ml) [31, 32]. The adhesive growing BON cells were cultured in DMEM:Ham's F12 medium in a 1:1 ratio (Gibco, USA) supplemented with 10% FBS (Sigma-Aldrich) and antibiotics (100 U penicillin/ml + 100 μg streptomycin/ml, Sigma-Aldrich) [32, 33].

GeneChip

RNA Extraction. Total RNA was extracted from the KRJ-I (n = 2), BON (n = 2) cell lines and normal jejunum tissue (n = 2, GSE2109) using Trizol (Invitrogen, USA) followed by Qiagen RNeasy kit (Qiagen Inc., USA), and the RNA quality was assessed using Agilent Bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA) to visually verify the absence of genomic DNA contamination, integrity, and ratio of 28S and 18S bands. Only samples with an absorbance ratio at 260 and 280 nm (A_{260}/A_{280}) 1.9 were used. 10 μg of total RNA were provided to the Keck Affymetrix facility where cRNA labeling, hybridization (U133A Plus 2.0 GeneChip), and data analysis were performed as described previously [34].

Hybridization. The Affymetrix U133A Plus 2.0 array comprises about 54,000 probe sets and 1,300,000 distinct oligonucleotide features and can analyze the expression level of about 47,000 transcripts and variants, including 38,500 well-characterized human genes (http://www.affymetrix.com/products/arrays/specific/hgu133plus.affx). The hybridized arrays were scanned using a confocal laser fluorescence scanner (Agilent Microarray Scanner, Agilent Technologies). Arrays were scaled to an average intensity of 500 and analyzed independently using Microarray Suite (MAS) 5.0 software (Affymetrix, Santa Clara, Calif., USA).

Real-Time PCR

RNA was extracted from KRJ-I and BON cells (each 1×10^6) after 1, 5 and 7 days of continuous culture using Trizol, and then cleaned using a Qiagen RNeasy kit in conjunction with the DNeasy Tissue kit ensuring absence of any contaminating genomic DNA. The clean RNA was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, USA). Transcript levels of neoplastic EC cell housekeeping genes *(ALG-9, TFCP2, ZNF410)* [35], NE markers [chromogranin A, tryptophan hydroxylase 1 and 2 (Tph-1, -2), dopa decarboxylase (DDC), substance P, guanylin, neuron-specific enolase (NSE), Ki67], and receptors [β-1 adrenergic (ADBR1), muscarinic types 1–4 (M1–4), somatostatin type 2 (sst2), transforming growth factor type 2 (TGFβR2), LRP1] were measured in KRJ-1 and BON. Analysis was performed as described previously [4] using Assays-on-Demand products and the ABI 7900 Sequence Detection System according to the manufacturer's suggestions. All samples were adjusted to 20 ng/μl cDNA before the experiments; 1 μl of template cDNA was used per reaction. Cycling was performed under standard conditions (TaqMan Universal PCR Master Mix protocol). The raw cycle threshold (C_T) values were exported, and data were normalized to *ALG-9, TFCP2, ZNF410* using GeNorm [35, 36].

5-HT ELISA

5-HT secretion was measured using a commercially available 5-HT ELISA (Rocky Mountain Diagnostics, USA) according to manufacturer's instructions for serum samples. Prior to stimulation experiments (all at concentrations of 10^{-12} to 10^{-6} M), cells were transferred to serum-free medium, seeded in 96-well plates at a density of 5×10^4 cells/well (n = 4), and maintained at 37°C in 5% CO_2. Basal 5-HT secretion from KRJ-I and BON cells was measured at 60 min and 24 h. Data for 5-HT secretion were normalized to protein levels.

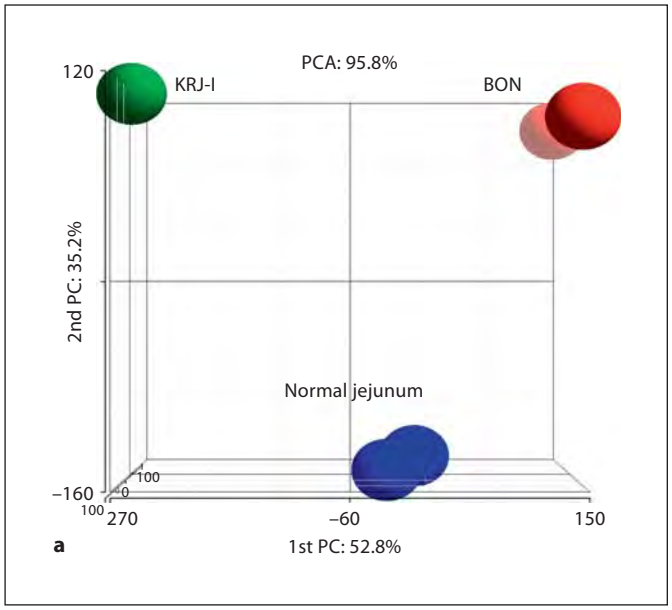
The effects of noradrenaline, isoproterenol (selective β-adrenergic receptor agonist), cholecystokinin, bombesin, pituitary adenylate cyclase (PACAP)-38, and acetylcholine chloride (muscarinic ligand), γ-aminobutyric acid (GABA) and somatostatin on 5-HT secretion were measured. The efficacy of acetylcholine chloride on 5-HT secretion was measured via preincubation (15 min) with atropine to each cell line alone or in combination with acetylcholine chloride (EC_{50} or IC_{50}).

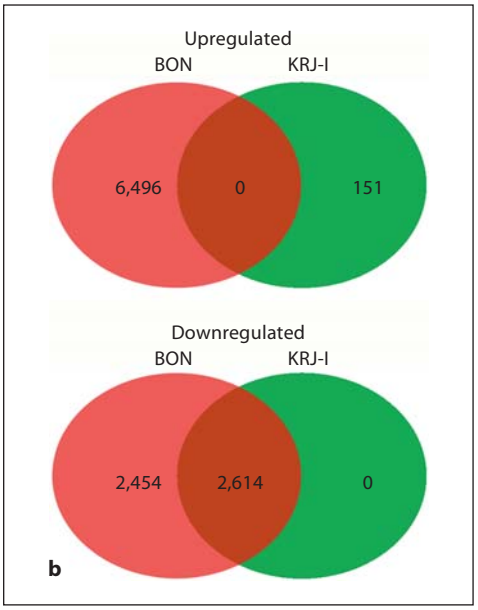
Proliferation Measurement

Effects of TGFα, TGFβ, EGF, CTGF, somatostatin, BIM23A761 (a chimeric somatostatin/dopamine agonist) [37], and MZ-4-147 (GHRH antagonist) [38] were evaluated. Additionally, the effects of cholecystokinin and gastrin on proliferation were evaluated. The methylthiazolyldiphenyl tetrazolium (MTT) assay for mitochondrial enzymatic activity was used to quantify proliferative responses [39]. Cells were seeded in 96-well plates at a density of 5×10^4 cells/well. Growth medium (alone) was used as a control. Selected compounds (all 10^{-12}–10^{-6} M) were added (n = 8 wells for each compound per concentration) and cells incubated for 72 h at 37°C in 5% CO_2. MTT was added (final concentration 0.5 mg/ml per well), and cells were incubated for a further 3 h at 37°C. The reaction was stopped by adding 0.01 N acid-isopropanol and the formazan dye solubilized. The optical density was read at 595 nm using a microplate reader (Bio-Rad 3500, USA).

Statistical Analyses

Raw GeneChip expression data were natural log (ln)-transformed using Microsoft Excel (Redmond, Wash., USA). Analysis of variance (ANOVA) and principal component analysis (PCA) were performed using Partek® Genomic Suit [40]. For ANOVA,





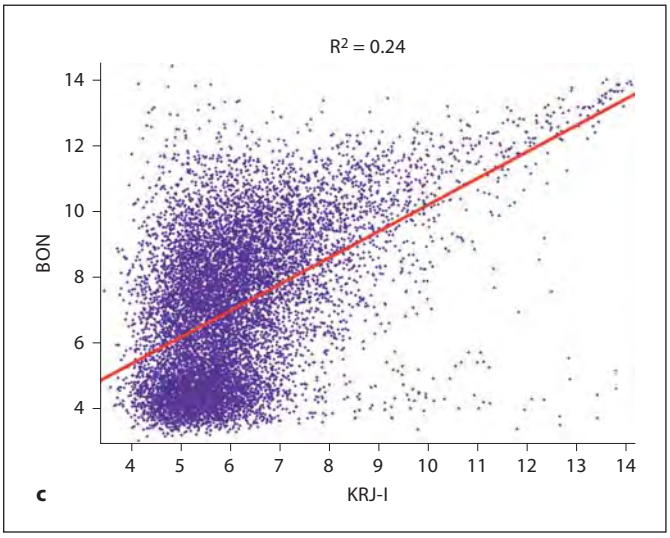


Fig. 1. Transcriptome analysis of KRJ-I, BON and normal jejunum. PCA of KRJ-I (green), BON (red), and normal jejunum (blue) transcriptomes captured 52.8% of the variance by the 1st PC, 35.2% by the 2nd PC, and 7.8% by the 3rd PC. 95.8% of the variance was captured by all 3 PCs (**a**). A large distance of separation between samples is indicative of a greater measure of dissimilarity on a whole-genome level between KRJ-I and BON cell lines. BON and KRJ-I transcriptomes were compared to normal jejunum using ANOVA (**b**). 6,496 and 151 significantly upregulated genes ($p \leq 0.05$, FC ≥ 2) were identified in BON and KRJ-I, respectively. No genes were commonly upregulated in the two cell lines. 2,454 downregulated genes ($p \leq 0.05$, FC ≤ -2) were unique to the BON cell line and 2,614 downregulated genes were shared by BON and KRJ-I. KRJ-I did not contain any unique downregulated genes. Additionally, expressions of all differentially expressed genes were found to be significantly different in two cell lines when compared in a pair-wise fashion (coefficient of linearity, $R^2 = 0.24$) (**c**).

a two-class unpaired algorithm was implemented for normal jejunum, BON, and KRJ-I cell lines. Geometric fold change (FC) was calculated as the ratio of geometric means. A p value ≤ 0.05 and an absolute value of FC ≥ 2.0 were considered significant. PCA was used to describe the structure of high-dimensional data by reducing its dimensionality into uncorrelated principal components (PCs) that explain most variation in the data [41]. PCA mapping was visualized in a 3-dimentional space where the x-, y-, and z-axis represent 1st, 2nd, and 3rd PCs, respectively. Dispersion matrix was computed using the covariance method. The variability in ΔC_T was expressed as coefficient of variation (CV) and was defined as the ratio of standard deviation to the mean. EC_{50}/IC_{50} values were calculated from nonlinear regression analysis (PRISM 4, GraphPad Inc., USA).

Results

Transcriptome Analysis

To delineate the gene level segregation of KRJ-I and BON cell lines, transcriptomes of each cell line were reduced to 3 PCs using the PCA technique. Transcriptomes of normal jejunum (GSE2109) were included for reference (fig. 1a). 52.8% of variance was represented by the 1st PC, 35.2% by the 2nd, and 7.8% by the 3rd; 95.8% of the variance was therefore captured by all 3 components. Distance of separation between clusters is indicative of degree of similarity on a gene level; a greater distance is equivalent to reduced similarity.

To assess the degree of gene expression differentiation between BON and KRJ-I, transcriptomes were compared to normal jejunum. 6,496 and 151 significantly upregulated genes ($p \leq 0.05$, FC ≥ 2) were identified in BON and KRJ-I, respectively. No genes were shared by the two cell lines (fig. 1b). Assessment of down-regulated genes identified 2,454 genes unique to the BON cell line and 2,614 genes shared by BON and KRJ-I. KRJ-I did not contain unique downregulated genes. Additionally, the expressions of all differentially expressed genes were found to be significantly different in the two cell lines when compared in a pair-wise fashion (coefficient of linearity, $R^2 = 0.24$) (fig. 1c).

Variability in Transcript Expression

The transcript levels of neoplastic EC cell housekeeping genes *(ALG9*, *TFCP2* and *ZNF410)* were measured using real-time PCR. The KRJ-I cell line was characterized by low CV values (CV $\leq$4.5%), while the BON cell line demonstrated CVs above 11.8% (table 1).

NE marker transcripts *CgA, NSE, Ki-67* and the EC cell-specific markers *Tph-1, substance P*, and *guanylin* were present in both KRJ-I and BON, while *Tph-2* and *DDC* were present only in BON (table 1). Both cell lines showed variability in NE marker transcript expression. However, a wide variability in *guanylin* (KRJ-I 159% vs. BON 100%) and *substance P* (BON 165% vs. KRJ-I 21%) was noted.

ADBR1, M1, M2, M4, sst2 and *LRP1* receptor transcripts were identified in KRJ-I and BON. However, *TGF-βR2* and *M3* were identified in BON only. Although variability in transcript expression was noted in both cell lines, the BON cell line was characterized by a greater variability in *ADBR2* (2-fold), *sst2* (5-fold), *M1* (4-fold), and *M2* (5-fold) compared to KRJ-I (table 1).

BON and KRJ-I 5-HT Secretory Profiles

Basal 5-HT Secretion

5-HT release during 60-min and 24-hour intervals was significantly lower ($p < 0.05$) in BON (8.0 ± 4.1 and 16.7 ± 8.2 ng/mg, respectively) than KRJ-I (14.3 ± 1.6 and 40.1 ± 16.3 ng/mg, respectively) (fig. 2).

Secretory Agonists and Antagonists

The KRJ-I cell line secreted 5-HT in response to isoproterenol (EC_{50} = 100 nM), noradrenaline (EC_{50} = 1.7 nM), and PACAP (EC_{50} = 0.3 nM). Secretion could be inhibited by cholecystokinin (IC_{50} = 430 nM), somatostatin (IC_{50} = 400 nM), acetylcholine (IC_{50} = 3.7 nM), and $GABA_A$ (IC_{50} = 2 nM). Gastrin and bombesin had no ef-

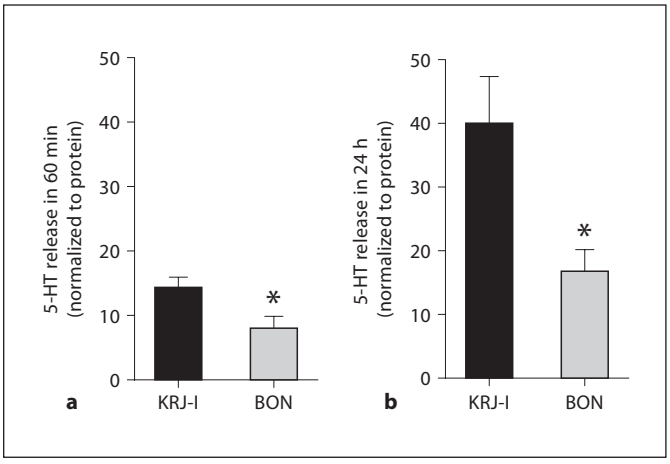


Fig. 2. Basal 5-HT secretion in KRJ-I and BON cell lines at 60 min and 24 h. After 60 min, 5-HT release in KRJ-I cell line was measured as 14.3 ± 1.6 ng/mg normalized to protein, while in BON 8.0 ± 4.1 normalized to protein (**a**). After 24 h, KRJ-I secretion reached 40.1 ± 16.3 normalized to protein and BON 5-HT secretion reached 16.7 ± 8.2 normalized to protein (**b**). During both intervals, BON 5-HT release was significantly lower than in KRJ-I (n = 4, * $p < 0.05$).

Table 1. Variability in housekeeping genes, NE markers and receptor transcripts

Transcript	KRJ-I, %	BON, %
Housekeeping genes		
ALG9	4.4	14.6
TFCP2	4.5	15.9
ZNF410	3.8	11.8
Neuroendocrine markers		
CgA	58	88
Tph-1	100	65
Tph-2	N/A	85
NSE	38	32
DDC	N/A	39
Substance P	21	165
Guanylin	159	100
Ki-67	38	14
Receptors		
ADBR2	53	116
SST2	21	106
LRP1	96	125
TGFβR2	N/A	17
M1	47	154
M2	33	154
M3	N/A	84
M4	73	46

The variability in ΔC_T was expressed as CV and was defined as the ratio of standard deviation to the mean.

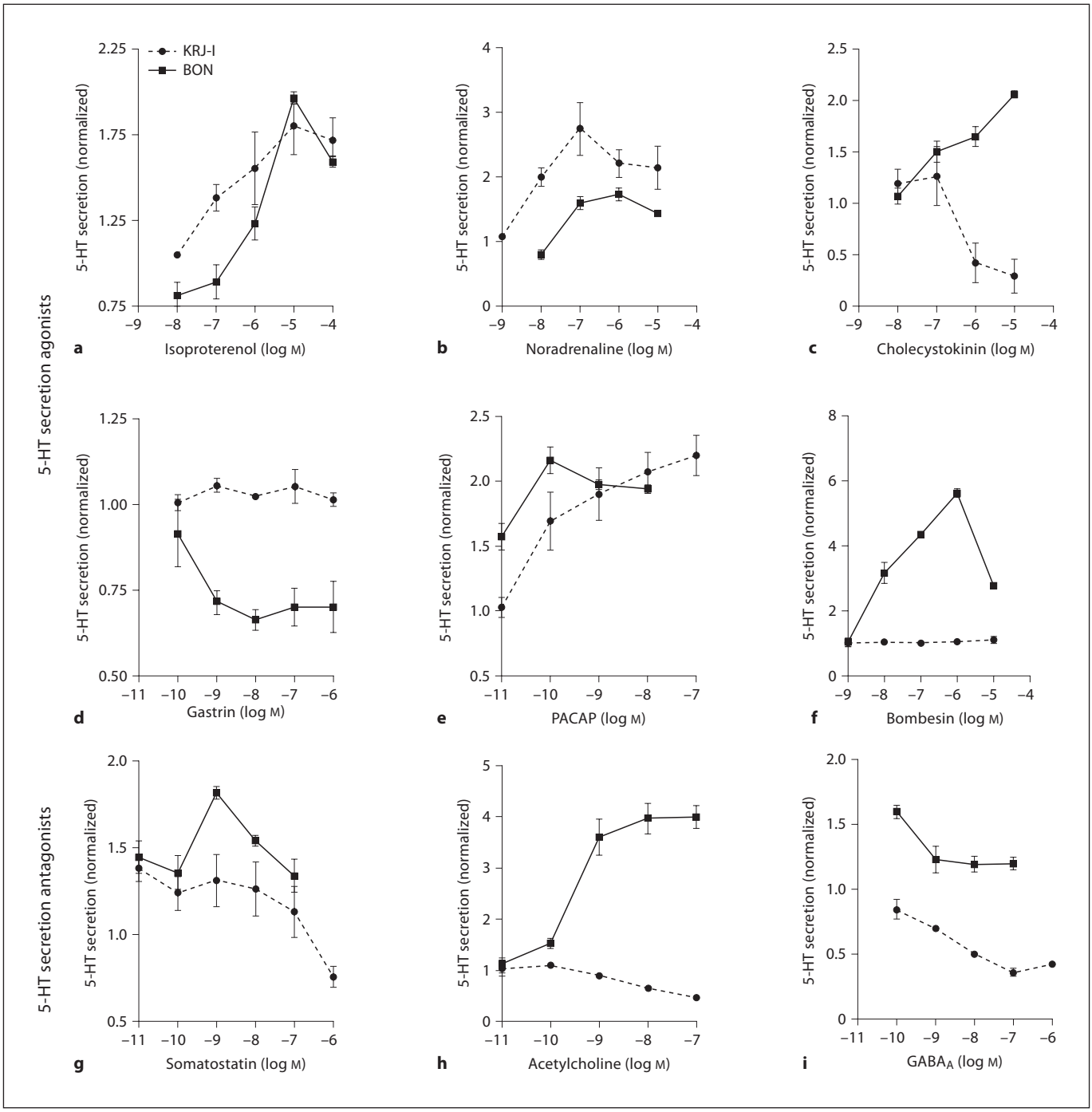


Fig. 3. 5-HT secretion: candidate agonists and antagonists. KRJ-I cell line responded with secretion to isoproterenol (EC_{50} = 100 nM), noradrenaline (EC_{50} = 1.7 nM), and PACAP (EC_{50} = 0.3 nM) (**a**, **b**, **e**). Secretion could be inhibited by cholecystokinin (IC_{50} = 430 nM), somatostatin (IC_{50} = 400 nM), acetylcholine (IC_{50} = 3.7 nM), and $GABA_A$ (IC_{50} = 2 nM) (**c**, **g**, **h**, **i**). Gastrin and bombesin had no effect on 5-HT secretion in this cell line. 5-HT secretion in the BON cell line was stimulated by isoproterenol (EC_{50} = 900 nM), noradrenaline (EC_{50} = 20 nM), cholecystokinin (EC_{50} = 130 nM), PACAP (EC_{50} = 0.12 nM), bombesin (EC_{50} = 15 nM), and acetylcholine (EC_{50} = 0.2 nM) (**a–c**, **e**, **f**, **h**). It was not significantly inhibited by gastrin or GABA but was by somatostatin (IC_{50} = 300 nM) (**d**, **g**, **i**).

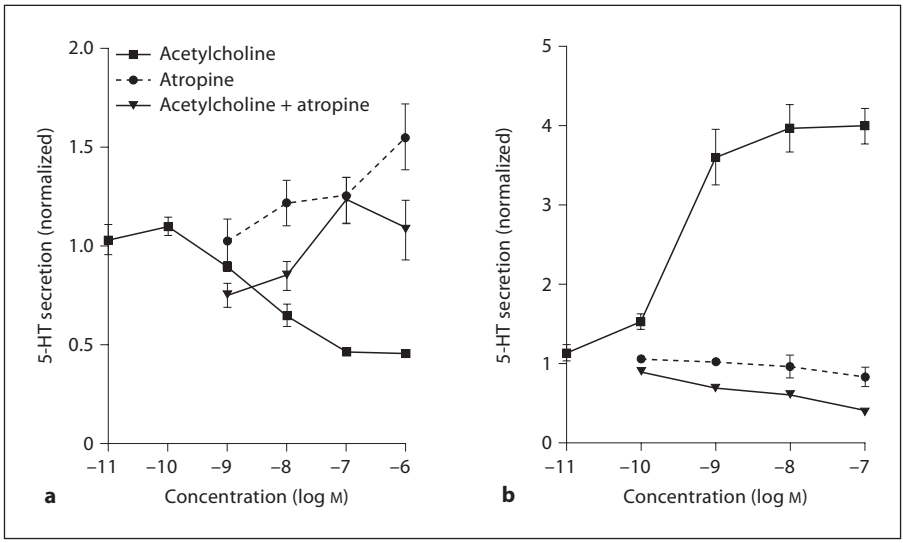


Fig. 4. Effects of acetylcholine, atropine, and acetylcholine + atropine on 5-HT secretion in KRJ-I and BON cell lines. 5-HT secretion in KRJ-I was inhibited by acetylcholine (IC_{50} = 3.7 nM). Atropine alone stimulated 5-HT release (EC_{50} = 13 nM) and acetylcholine + atropine reversed acetylcholine inhibited secretion (EC_{50} = 220 nM) (**a**). In the BON cell line, acetylcholine activated 5-HT secretion (EC_{50} = 0.2 nM). The effect could be reversed by atropine and acetylcholine + atropine (IC_{50} = 9.7 nM and IC_{50} = 1.1 nM, respectively) (**b**).

fect on 5-HT secretion in this cell line (fig. 3a–i). 5-HT secretion in the BON cell line was stimulated by isoproterenol (EC_{50} = 900 nM), noradrenaline (EC_{50} = 20 nM), cholecystokinin (EC_{50} = 130 nM), PACAP (EC_{50} = 0.12 nM), bombesin (EC_{50} = 15 nM), and acetylcholine (EC_{50} = 0.2 nM). It was inhibited by somatostatin (IC_{50} = 300 nM). GABA had no effect (fig. 3a–i).

To further define the effects of acetylcholine on 5-HT secretion in KRJ-I and BON cell lines and to determine if the effect could be reversed, cell lines were stimulated with acetylcholine, atropine, and acetylcholine + atropine. 5-HT secretion in KRJ-I was inhibited by acetylcholine (IC_{50} = 3.7 nM). Atropine alone stimulated 5-HT release (EC_{50} = 13 nM) and acetylcholine + atropine reversed acetylcholine-mediated inhibition (EC_{50} = 220 nM) (fig. 4a). In the BON cell line, acetylcholine activated 5-HT secretion (EC_{50} = 0.2 nM). The effect could be reversed by both atropine alone and in combination with acetylcholine (IC_{50} = 9.7 nM and IC_{50} = 1.1 nM, respectively) (fig. 4b). These results demonstrate very different muscarinic responses in the two cell lines: 5-HT secretion from the pancreatic-derived BON is stimulated while the intestinal-derived KRJ-I is inhibited. This is consistent with the substantially different regulatory mechanism of a pancreatic-derived cell system [42, 43].

BON and KRJ-I Proliferative Profiles

The KRJ-I cell line responded with proliferation to TGFα (EC_{50} = 0.63 ng/ml), TGFβ (EC_{50} = 0.63 ng/ml), CTGF (EC_{50} = 0.002 ng/ml), and the GHRH antagonist, MZ-4-147 (EC_{50} = 63 nM) (fig. 5b–d, f). Proliferation was marginally but not signficantly inhibited by the somatostatin/dopamine chimera BIM23A761 (IC_{50} = 3 × 10^{-3} nM) (fig. 5g), while EGF and somatostatin had no significant effect. Proliferation of the BON cell line was stimulated only by TGFα (EC_{50} = 10 ng/ml) (fig. 5c). TGFβ (IC_{50} = 0.16 ng/ml), MZ-4-147 (IC_{50} = 0.5 nM), and BIM23A761 (IC_{50} = 0.06 nM) inhibited proliferation (fig. 5d, f, g). EGF, CTGF and somatostatin had no significant effects.

Finally, the effects of cholecystokinin and gastrin on KRJ-I and BON cell proliferation were measured. Cholecystokinin inhibited KRJ-I proliferation (IC_{50} = 420 nM) but stimulated BON proliferation (EC_{50} = 130 nM) (fig. 6a). Gastrin had no effect on either cell line (fig. 6b).

Discussion

Molecular understanding of the EC cell-derived GI-NETs has been substantially hampered by the lack of an appropriate human neoplastic EC cell model. The NCI summit conference noted that the lack of appropriate cell lines and animal models was a key issue contributing to the limited advances in the field of NE cell biology [20]. In particular, there was no rapidly growing cell line or animal model suitable for the investigation of EC cell neoplasia, the commonest NE tumor. KRJ-I is the only validated rapidly dividing human EC cell NET model [31, 44]. However, to date, the BON cell line has been the most widely used NET cell line for investigation. The present study overall establishes that BON does not represent a

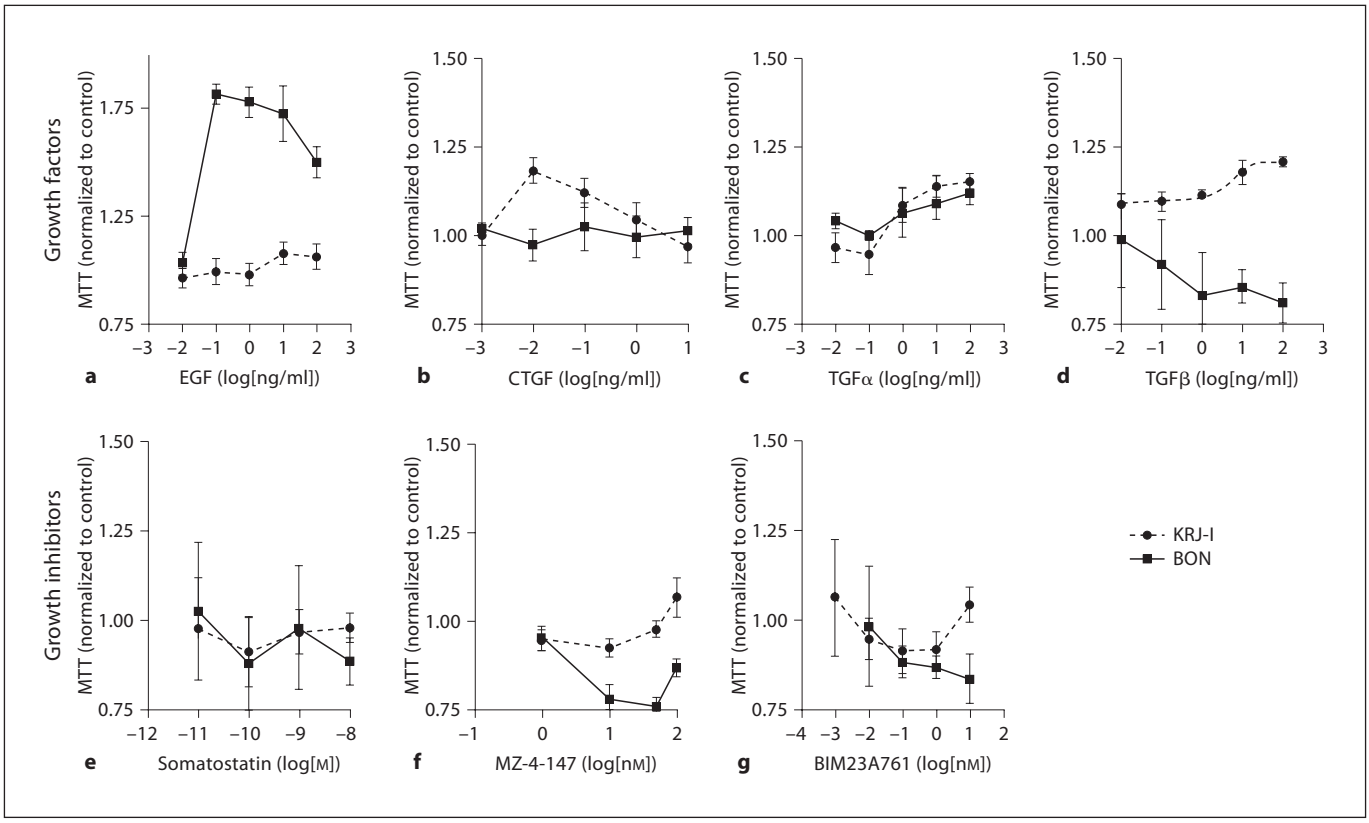


Fig. 5. Effects of growth factors and inhibitors on BON and KRJ-I cell lines. KRJ-I cell line responded with proliferation to CTGF (EC_{50} = 0.002 ng/ml), TGFα (EC_{50} = 0.63 ng/ml) and TGFβ (EC_{50} = 0.63 ng/ml) (**c**, **d**, **f**). Proliferation was inhibited by and BIM23A761 (IC_{50} = 3 × 10^{-3} nM) (**g**) while EGF and somatostatin had no effect. Proliferation of the BON cell line was stimulated by EGF and TGFα (EC_{50} = 15.8 and 10 ng/ml, respectively) (**a**, **c**). TGFβ (IC_{50} = 0.16 ng/ml) and MZ-4-147 (IC_{50} = 0.5 nM) all inhibited proliferation (**d**, **f**, **g**). CTGF and somatostatin had no significant effect.

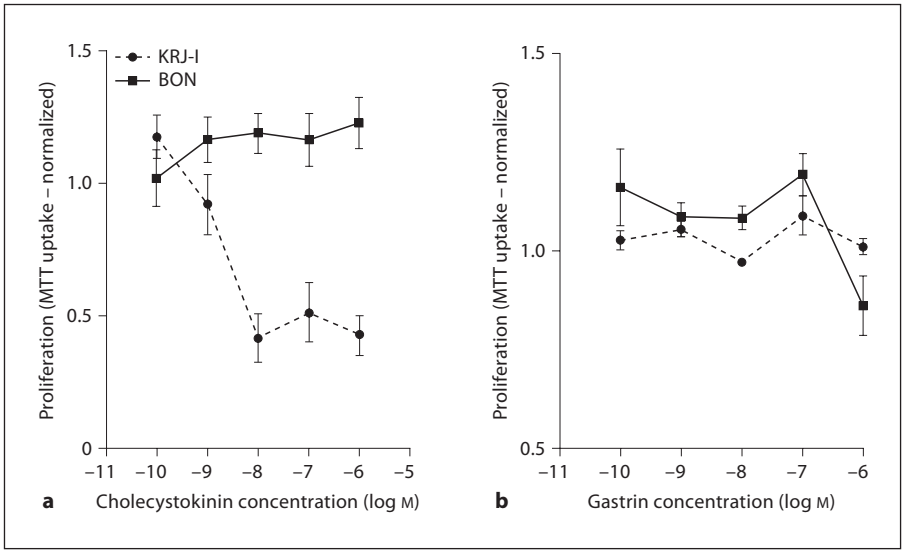


Fig. 6. Effects of cholecystokinin and gastrin on KRJ-I and BON cell proliferation. Cholecystokinin inhibited KRJ-I proliferation (IC_{50} = 420 nM), while stimulating the proliferation of the BON cell line (EC_{50} = 130 nM) (**a**). Gastrin had no effects in either cell line (**b**).

model of gut EC cell neoplasia: (1) BON and KRJ-I have substantially different transcriptome profiles; (2) transcript expressions of housekeeping genes, NE markers and receptors vary substantially over time in BON cell line compared to KRJ-I; (3) BON cell line has a differential 5-HT secretory profile exhibiting muscarinic-, cholecystokinin- and bombesin-mediated secretion and no GABAergic responses, and (4) the BON cell line and the EC cell-derived line, KRJ-I, respond differently to growth factors and proliferation inhibitors.

KRJ-I cells share a number of characteristics with normal human SI EC cells including expression of chromogranin A, 5-HT, Tph-1, substance P and guanylin [44]. As a neoplastic cell line, it exhibits predictable differences in proliferation (increased Ki-67 expression, rapid doubling-time) [31] to naïve (nontransformed) human EC cells. The broad commonality of receptor expression and secretory responses to a variety of neural and luminal stimuli [45] shared by the normal EC cell support the conclusion that KRJ-I is a transformed EC cell. Whole-genome analysis of BON and KRJ-I cell lines indicates that on a genomic level, the two are substantially different cell lines (table 2). For reference purposes, normal jejunum tissue was used to compare gene signatures of KRJ-I and BON cell lines and demonstrated that expression patterns were not reproducible and neoplastic transformation in each cell line may be associated with different alterations in gene expression. These differences may reflect the different tissue origins of each cell line but recapitulate several studies demonstrating the nonoverlapping nature of pancreatic NETs and GI-NET molecular alterations and transcriptomes [19, 21, 23].

Housekeeping genes are constitutively expressed to maintain cellular function [46]. As such, they should be resistant to regulative factors, and maintain constant RNA transcription. The housekeeping genes *ALG9*, *TFCP2* and *ZNF410* have previously been identified and characterized in the EC cell-derived KRJ-I cell line and their utility for transcriptional studies of GI-NETs has been validated [35]. Although all 3 NE housekeeping transcripts were identified in BON, the high variability (10%) in expression when measured through 7 days of continuous culture highlights that this cell line does not conform to a GI-NET profile.

Although both cell lines showed variability in NE marker transcripts over the 7-day period in culture, of note was the high variability in *substance P* and *guanylin* transcript expressions. Substance P is a tachykinin involved in GI motility, secretion, vascular permeability, and immune function [47]. Although some substance P is produced by the EC cells, the major source of tachykinins in the GI tract is the enteric nervous system, in which tachykinins are extensively colocalized with choline acetyltransferase [47]. In light of the diverse actions of substance P, the difference in transcript expression may represent a disparity in the functionality of substance P within BON and KRJ-I. With regard to KRJ-I, this may reflect the fact that this particular tumor is a 5-HT dominant secretor since it is well recognized that individual SI-NETs exhibit different profiles of peptide and amine secretion [1, 48]. Guanylin is a 15-amino-acid peptide that is secreted by the goblet cells in the colon. It is an intestinal modulator of water and electrolyte transport, and its augmented corelease with 5-HT may play a causal role in the symptomatic diarrhea experienced by GI-NET patients [48, 49]. Increased *guanylin* transcript expression

Table 2. Summary of differences between the EC cell-derived KRJ-I and the pancreatic BON cell line

Platform or agent	KRJ-I	BON
Transcriptome		
U133A Plus 2 Array (54,000 probes)	<10%, R^2 = 0.24, p = NS	
5-HT secretion		
Isoproterenol, nM	EC_{50}: 100	EC_{50}: 900
Noradrenaline, nM	EC_{50}: 1.7	EC_{50}: 20
PACAP, nM	EC_{50}: 0.03	EC_{50}: 0.12
Cholecystokinin, nM	IC_{50}: 400	EC_{50}: 130
Somatostatin, nM	IC_{50}: 400	IC_{50}: 300
Acetylcholine, nM	IC_{50}: 3.7	EC_{50}: 0.2
$GABA_A$, nM	IC_{50}: 2	–
Gastrin	–	–
Bombesin, nM	–	EC_{50}: 15
Proliferation		
CTGF, ng/ml	EC_{50}: 0.002	–
TGFα, ng/ml	EC_{50}: 0.63	EC_{50}: 10
EGF, ng/ml	–	EC_{50}: 15.8
TGFβ, ng/ml	EC_{50}: 0.63	IC_{50}: 0.16
SST	–	–
MZ-4-147, nM	–	IC_{50}: 0.5
BIM23A761, nM	–	IC_{50}: 0.06

The two cell lines exhibit overlap in <10% of their transcriptomes. Differences in receptor profiles or expression levels are reflected in very different efficacies in 5-HT secretory responses (β-adrenergic, cholecystokinin, gastrin-releasing peptide) or opposing effects (acetylcholine) on secretion. Proliferative responses were similarly identified to be different, dependent on receptor expression levels or signaling pathways. The absence of a BIM23A761 response in KRJ-I reflects the absence of dopamine 2 inhibitory receptors in this cell line.

in the KRJ-I cell line after 7 days is thus consistent with the secretory profile of this lesion and reflected in the patient's clinical presentation [31, 32]. Of note, the *guanylin* transcript declined in the BON cell line after 7 days, highlighting the variability in the transcriptome of this cell line.

Tryptophan hydroxylase (Tph) hydroxylates L-tryptophan to 5-hydroxy-L-tryptophan and represents the rate-limiting step in 5-HT synthesis, a key component of EC cell neoplasia [50]. There are two isoforms: Tph-1 is principally expressed in the periphery, and the recently identified Tph-2 which is exclusively expressed in neuronal cell types and is the predominant isoform in the central nervous system [51]. DOPA decarboxylase is an enzyme implicated in synthesizing dopamine and 5-HT. Dopamine is formed when DDC decarboxylates L-dihydroxyphenylalanine (L-DOPA) and 5-HT is formed when DDC decarboxylates 5-OH tryptophan [52]. DDC has been demonstrated to be a marker for both tumors of NE and non-NE origin, including non-small cell lung and colorectal carcinomas [53, 54].

Regulation of 5-HT synthesis – a key EC cell determinant – is provided by either the Tph-1 or Tph-2 enzyme isoform. The Tph-1 isoform is present in normal EC cells [44]. Similary, *Tph-1* but not *Tph-2* or *DDC* transcripts were detectable in KRJ-I, demonstrating that 5-HT synthesis is only regulated by the Tph-1 isoform, as in normal EC cells [44]. In contrast, both *Tph-1* and *Tph-2* transcripts as well as DDC are present in the BON cell system. Thus, 5-HT synthesis in this cell line does not occur through the classical Tph-1 pathway as it does in EC cells and EC cell neoplasia. In addition, the high variability (85% over a 7-day period in continuous culture) suggests that transcript expression for 5-HT synthesis in the BON cell line is unstable and fluctuates unpredictably during culture [15]. These differences in synthesis were reflected in secretion studies. Thus, basal 5-HT secretion was significantly higher (increased 2- to 3-fold) in KRJ-I than BON at both 60 min and 24 h. Midgut (EC cell) NETs usually secrete high levels of 5-HT in contrast to foregut NET which very rarely secrete 5-HT [55, 56]. The lower and inconsistent levels of 5-HT secretion from the BON cell line therefore argue that this cell system has not only different mechanisms of synthesis but also a variable, non-EC cell phenotype.

Defining functional receptors is important since not only do they define the mechanistic basis of an individual cell's function but they also provide potential diagnostic and therapeutic targets. In KRJ-I, we noted ADBR1, M2/4, sst2 and LRP1 transcripts. The existence of these catecholaminergic and cholinergic pathways is consistent with the physiology of EC cells since they release 5-HT when stimulated via β-adrenergic receptors and after vagal cholinergic stimulation [57]. With the exception of the inhibitory *M4* receptor, the BON cell line demonstrated a >2-fold variability in receptor transcript expressions (ADBR1, M1–4, and sst2), suggesting that receptor expression in this cell line is not reproducible in culture. Additionally, the stimulatory *M3* and *TGFβR2* receptor transcripts were only expressed in BON, suggesting substantial differences in muscarinic control and TGFβ-signaling pathways between KRJ-I and BON cell lines.

Isoproterenol, noradrenaline, and PACAP stimulated 5-HT release in both BON and KRJ-I cell lines, further supporting our proposal that neural regulation represents a common NE cell secretory mechanism (table 2). Thus, α- and β-adrenergic receptors and PACAP receptor stimulation appear to be potent activators of NE cell secretion in EC, ECL and G cells [44, 58, 59]. However, cholecystokinin stimulated 5-HT release in BON, but not KRJ-I, a phenomenon consistent with the effect of cholecystokinin on pancreatic endocrine and acinar cell secretion, e.g. β-cells [42, 43, 60]. In contrast, targeting the cholecystokinin-2 receptor with gastrin had no significant effect on secretion in KRJ-I or BON. This is consistent with previous studies identifying the lack of cholecystokinin-2 receptors in normal and neoplastic EC cells [30] and suggests that the provocative 'pentagastrin' test used to activate GI 'carcinoid' secretion probably occurs via an upstream, indirect, gastrin-initiated mechanism, rather than by gastrin itself. Of note was that bombesin and gastrin-releasing peptide stimulated BON cell 5-HT secretion, consistent with the pancreatic origin of the tumor [61]. KRJ-I, however, did not respond to gastrin-releasing peptide. Somatostatin inhibited 5-HT secretion in both BON and KRJ-I cell lines with similar efficacies (IC_{50}: 300–400 nM), but $GABA_A$ inhibited only KRJ-I secretion, confirming earlier results [44].

Muscarinic receptor activation with acetylcholine chloride, however, had opposing effects, stimulating secretion in BON (through M1/3 receptors) and inhibiting it in KRJ-I (through the predominant M2/4 receptors). These findings were confirmed by preincubating the cells with atropine, a competitive general antagonist for the muscarinic acetylcholine receptor, which reversed the effects of acetylcholine in both the BON and KRJ-I cell lines. These opposing responses are consistent with a cholinergic-stimulatory response characteristic of pancreatic endocrine/acinar cells, i.e. M3 receptor activation of insulin secretion from β-cells [42]. Intestinal EC cells,

in contrast, have inhibitory muscarinic receptors (M2/4) and respond to acetylcholine, as do KRJ-I cells, with inhibition of 5-HT secretion [44].

Growth factors and their receptors are expressed in GI-NETs and the tumor matrix and are considered key regulators of the neoplastic EC cell phenotype [62–65]. In particular, TGFβ and CTGF are considered regulators of SI-NET proliferation and its peri-tumoral and cardiac desmoplastic response [65–67]. Similarly, activation of somatostatin receptors may inhibit tumor growth [38, 68]. Contradictory effects were noted to TGFβ. Thus, BON cells responded with inhibition of proliferation consistent with expression of a functional TGFβR2 inhibitory pathway as previously noted in this cell line [27, 69]. In contrast, TGFβ stimulated KRJ-I proliferation, consistent with our previous observations of an altered-TGFβ-mediated regulatory pathway in SI-NETs [65, 66]. Further differences in growth regulation were noted by the observation that CTGF, a proliferative and profibrotic factor synthesized by GI-NETs [67, 70], stimulated KRJ-I but not BON cell proliferation. Fibrosis, identified in 40–60% of SI-NETs [1], is not a usual feature of pancreatic NETs. Proliferation in response to EGF and TGFα (growth factors that signal through the EGF receptor) was evident in BON cells. Of note is the observation that targeting the EGF receptor with gefitinib is only successful in BON cells [71] but not KRJ-I [31]. The different proliferative responses to TGFβ, CTGF and EGF lend further support to the contention that BON is not a neoplastic EC cell model.

Although somatostatin had no significant inhibitory effect on proliferation, BIM23A761 (a selective dopamine receptor 2 agonist) inhibited BON cells as did MZ-4-147 (a GHRH receptor antagonist), effects previously identified in foregut NETs (pituitary [72] and lung [37]). Substantially different proliferative mechanisms therefore govern KRJ-I and BON cell line proliferation.

This study presents evidence that the BON and KRJ-I cell lines differ widely at a number of levels, including transcriptome, receptor expression and secretory and proliferative responses (table 2). In particular, the minimal relationship at a genetic level is strongly indicative that the BON cell line is neither EC cell nor intestinal in origin and the secretory and proliferative responses to cholecystokinin and secretory responses to bombesin and acetylcholine further indicate that BON represents a modified pancreatic endocrine cell [73, 74]. The obvious similarities between KRJ-I and the normal EC cells in terms of receptor expression and secretory responses to a variety of neural and luminal stimuli as well as expression of common markers, e.g. Tph-1 [31, 45], strongly support its EC cell derivation.

The substantial differences identified between KRJ-I and BON strongly suggest the latter cell line is not an appropriate in vitro model for EC cell-derived luminal GI-NETs. Overall, it is evident that BON cells are likely derived from a pancreatic adenocarcinoma exhibiting NE cell transformation and not representative of an intestinal EC cell-derived tumor. Evidence for this is provided by studies with Notch [28] and the identification of a TGFβ:somatostatin autoinhibitory pathway [27]. Addition of Notch or alterations in TGFβ and somatostatin reverses the NE phenotype [27, 28] with a resultant transition to a mesenchymal phenotype [27]. This recapitulates the epithelial-to-mesenchymal transition noted in carcinomas [29], a feature not evident in NETs. Our conclusion is consistent with the consensus assessment of the National Cancer Institute NET Conference (Bethesda, Sept 23–25, 2007) [20]. BON may be of use as a model of pancreatic NETs, tumors which have only a limited relation to luminal GI-NETs as has been noted in their clinical, pathological and therapeutic response and behavior [1, 21]. In contrast, KRJ-I is an EC cell model and focused investigation of this cell line will enable delineation of the mechanistic basis of EC cell neoplasia, as well as facilitate identification of appropriate molecular targets for diagnostic and therapeutic evaluation.

References

1 Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A: The current status of gastroenteropancreatic neuroendocrine tumors. Lancet Oncol 2008;9:61–72.

2 Modlin IM, Lye KD, Kidd M: A 5-decade analysis of 13,715 carcinoid tumors. Cancer 2003;97:934–959.

3 Halfdanarson TR, Rabe KG, Rubin J, Petersen GM: Pancreatic neuroendocrine tumors (PNETs): incidence, prognosis and recent trend toward improved survival. Ann Oncol 2008.

4 Modlin IM, Kidd M, Latich I, Zikusoka MN, Shapiro MD: Current status of gastrointestinal carcinoids. Gastroenterology 2005;128:1717–1751.

5 The US National Cancer Institute, Surveillance Epidemiology and End Results (SEER) data base, 1973–2004, http://seer.cancer.gov/. 2007.

6 Spangeus A, Forsgren S, El-Salhy M: Effect of diabetic state on co-localization of substance P and serotonin in the gut in animal models. Histol Histopathol 2001;16:393–398.

7 Cetin Y, Kuhn M, Kulaksiz H, Adermann K, Bargsten G, Grube D, Forssmann WG: Enterochromaffin cells of the digestive system: cellular source of guanylin, a guanylate cyclase-activating peptide. Proc Natl Acad Sci USA 1994;91:2935–2939.
8 Quinn LA, Moore GE, Morgan RT, Woods LK: Cell lines from human colon carcinoma with unusual cell products, double minutes, and homogeneously staining regions. Cancer Res 1979;39:4914–4924.
9 Kolby L, Bernhardt P, Ahlman H, Wangberg B, Johanson V, Wigander A, Forssell-Aronsson E, Karlsson S, Ahren B, Stenman G, Nilsson O: A transplantable human carcinoid as model for somatostatin receptor-mediated and amine transporter-mediated radionuclide uptake. Am J Pathol 2001;158:745–755.
10 Van Buren G, 2nd, Rashid A, Yang AD, Abdalla EK, Gray MJ, Liu W, Somcio R, Fan F, Camp ER, Yao JC, Ellis LM: The development and characterization of a human midgut carcinoid cell line. Clin Cancer Res 2007; 13:4704–4712.
11 Evers BM, Townsend CM Jr, Upp JR, Allen E, Hurlbut SC, Kim SW, Rajaraman S, Singh P, Reubi JC, Thompson JC: Establishment and characterization of a human carcinoid in nude mice and effect of various agents on tumor growth. Gastroenterology 1991;101: 303–311.
12 Christofi FL, Kim M, Wunderlich JE, Xue J, Suntres Z, Cardounel A, Javed NH, Yu JG, Grants I, Cooke HJ: Endogenous adenosine differentially modulates 5-hydroxytryptamine release from a human enterochromaffin cell model. Gastroenterology 2004;127: 188–202.
13 Kim M, Cooke HJ, Javed NH, Carey HV, Christofi F, Raybould HE: D-glucose releases 5-hydroxytryptamine from human BON cells as a model of enterochromaffin cells. Gastroenterology 2001;121:1400–1406.
14 Jang HJ, Kokrashvili Z, Theodorakis MJ, Carlson OD, Kim BJ, Zhou J, Kim HH, Xu X, Chan SL, Juhaszova M, Bernier M, Mosinger B, Margolskee RF, Egan JM: Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. Proc Natl Acad Sci USA 2007;104:15069–15074.
15 von Mentzer B, Murata Y, Ahlstedt I, Lindstrom E, Martinez V: Functional CRF receptors in BON cells stimulate serotonin release. Biochem Pharmacol 2007;73:805–813.
16 Vikman S, Essand M, Cunningham JL, de la Torre M, Oberg K, Totterman TH, Giandomenico V: Gene expression in midgut carcinoid tumors: potential targets for immunotherapy. Acta Oncol 2005;44:32–40.
17 Ishizuka J, Beauchamp RD, Sato K, Townsend CM Jr, Thompson JC: Novel action of transforming growth factor beta 1 in functioning human pancreatic carcinoid cells. J Cell Physiol 1993;156:112–118.
18 Ishizuka J, Beauchamp RD, Townsend CM Jr, Greeley GH Jr, Thompson JC: Receptor-mediated autocrine growth-stimulatory effect of 5-hydroxytryptamine on cultured human pancreatic carcinoid cells. J Cell Physiol 1992;150:1–7.
19 Zikusoka MN, Kidd M, Eick G, Latich I, Modlin IM: The molecular genetics of gastroenteropancreatic neuroendocrine tumors. Cancer 2005;104:2292–2309.
20 Modlin I, Moss S, Chung D, Jensen R, Snyderwine E: Priorities for improving the management of gastropancreatic neuroendocrine tumors. J Natl Cancer Inst 2008;100: 1–8.
21 Duerr E, Mizukami Y, Ng A, Xavier R, Kikuchi H, Deshpande V, Warshaw AL, Glickman J, Kulke M, Chung D: Defining molecular classifications and targets in gastropancreatic neuroendocrine tumors through DNA microarray analysis. Endocr Relat Cancer 2008;15:243–246.
22 Kulke MH, Freed E, Chiang DY, Philips J, Zahrieh D, Glickman JN, Shivdasani RA: High-resolution analysis of genetic alterations in small bowel carcinoid tumors reveals areas of recurrent amplification and loss. Genes Chromosomes Cancer 2008;47: 591–603.
23 Rindi G, Kloppel G: Endocrine tumors of the gut and pancreas tumor biology and classification. Neuroendocrinology 2004;80(suppl 1):12–15.
24 Arany I, Rady P, Evers BM, Tyring SK, Townsend CM Jr: Analysis of multiple molecular changes in human endocrine tumours. Surg Oncol 1994;3:153–159.
25 Kulke MH, Lenz HJ, Meropol NJ, Posey J, Ryan DP, Picus J, Bergsland E, Stuart K, Tye L, Huang X, Li JZ, Baum CM, Fuchs CS: Activity of sunitinib in patients with advanced neuroendocrine tumors. J Clin Oncol 2008; 26:3403–3410.
26 Kloppel G, Perren A, Heitz PU: The gastroenteropancreatic neuroendocrine cell system and its tumors: the WHO classification. Ann N Y Acad Sci 2004;1014:13–27.
27 Leu FP, Nandi M, Niu C: The effect of transforming growth factor beta on human neuroendocrine tumor BON cell proliferation and differentiation is mediated through somatostatin signaling. Mol Cancer Res 2008; 6:1029–1042.
28 Nakakura EK, Sriuranpong VR, Kunnimalaiyaan M, Hsiao EC, Schuebel KE, Borges MW, Jin N, Collins BJ, Nelkin BD, Chen H, Ball DW: Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumor cells by notch signaling. J Clin Endocrinol Metab 2005;90:4350–4356.
29 Yang J, Weinberg RA: Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 2008; 14:818–829.
30 Modlin IM, Kidd M, Pfragner R, Eick GN, Champaneria MC: The functional characterization of normal and neoplastic human enterochromaffin cells. J Clin Endocrinol Metab 2006;91:2340–2348.
31 Kidd M, Eick GN, Modlin IM, Pfragner R, Champaneria MC, Murren J: Further delineation of the continuous human neoplastic enterochromaffin cell line, KRJ-I, and the inhibitory effects of lanreotide and rapamycin. J Mol Endocrinol 2007;38:181–192.
32 Pfragner R, Wirnsberger G, Niederle B, Behmel A, Rinner I, Mandl A, Wawrina F, Luo J, Adamiker D, Hoeger H, Ingolic E, Schauenstein K: Establishment of a continuous cell line from a human carcinoid of the small intestine (KRJ-I): characterization and effects of 5-azacytidine on proliferation. Int J Oncol 1996;8:513–520.
33 Parekh D, Ishizuka J, Townsend CM Jr, Haber B, Beauchamp RD, Karp G, Kim SW, Rajaraman S, Greeley G Jr, Thompson JC: Characterization of a human pancreatic carcinoid in vitro: morphology, amine and peptide storage, and secretion. Pancreas 1994;9: 83–90.
34 Kidd M, Modlin IM, Mane SM, Camp RL, Eick G, Latich I: The role of genetic markers – NAP1L1, MAGE-D2, and MTA1 – in defining small-intestinal carcinoid neoplasia. Ann Surg Oncol 2006;13:253–262.
35 Kidd M, Nadler B, Mane S, Eick G, Malfertheiner M, Champaneria M, Pfragner R, Modlin I: GeneChip, geNorm, and gastrointestinal tumors: novel reference genes for real-time PCR. Physiol Genomics 2007;30: 363–370.
36 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:RESEARCH0034.
37 Kidd M, Drozdov I, Joseph R, Pfragner R, Culler M, Modlin I: Differential cytotoxicity of novel somatostatin and dopamine chimeric compounds on bronchopulmonary and small intestinal neuroendocrine tumor cell lines. Cancer 2008;113:690–700.
38 Kidd M, Schally AV, Pfragner R, Malfertheiner MV, Modlin IM: Inhibition of proliferation of small intestinal and bronchopulmonary neuroendocrine cell lines by using peptide analogs targeting receptors. Cancer 2008;112:1404–1414.
39 Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
40 Partek: Partek® Genomics Suite™. St Louis, Partek, 2008.
41 Jolliffe IT: Principal Component Analysis. Berlin, Springer, 1986.

42 Gautam D, Han SJ, Duttaroy A, Mears D, Hamdan FF, Li JH, Cui Y, Jeon J, Wess J: Role of the M_3 muscarinic acetylcholine receptor in beta-cell function and glucose homeostasis. Diabetes Obes Metab 2007;9(suppl 2): 158–169.

43 Schmid SW, Modlin IM, Tang LH, Stoch A, Rhee S, Nathanson MH, Scheele GA, Gorelick FS: Telenzepine-sensitive muscarinic receptors on rat pancreatic acinar cells. Am J Physiol 1998;274:G734–G741.

44 Modlin IM, Kidd M, Eick G, Champaneria M: The functional characterization of normal and neoplastic EC cells. Journal of Clinical Endocrinology and Metabolism 2006;91: 2340–2348.

45 Kidd M, Modlin I, Gustafsson B, Drozdov I, Hauso O, Pfragner R: The luminal regulation of normal and neoplastic EC cell serotonin release is mediated by bile salts, amines, tastants and olfactants. Am J Physiol Gastrointest Liver Physiol 2008;295:G260–G272.

46 Koopmans KP, Brouwers AH, De Hooge MN, Van der Horst-Schrivers AN, Kema IP, Wolffenbuttel BH, De Vries EG, Jager PL: Carcinoid crisis after injection of $^{6-18}$F-fluorodihydroxyphenylalanine in a patient with metastatic carcinoid. J Nucl Med 2005;46: 1240–1243.

47 Holzer P, Holzer-Petsche U: Tachykinins in the gut. I. Expression, release and motor function. Pharmacol Ther 1997;73:173–217.

48 Kuhn M, Kulaksiz H, Cetin Y, Frank M, Nold R, Arnold R, Boker K, Bischoff SC, Manns MP, Forssmann WG: Circulating and tissue guanylin immunoreactivity in intestinal secretory diarrhoea. Eur J Clin Invest 1995;25: 899–905.

49 Cleary S, Phillips JK, Huynh TT, Pacak K, Fliedner S, Elkahloun AG, Munson P, Worrell RA, Eisenhofer G: Chromogranin A expression in phaeochromocytomas associated with von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2. Horm Metab Res 2007;39:876–883.

50 Wang L, Erlandsen H, Haavik J, Knappskog PM, Stevens RC: Three-dimensional structure of human tryptophan hydroxylase and its implications for the biosynthesis of the neurotransmitters serotonin and melatonin. Biochemistry 2002;41:12569–12574.

51 Jaffa AA, Usinger WR, McHenry MB, Jaffa MA, Lipstiz SR, Lackland D, Lopes-Virella M, Luttrell LM, Wilson PW: Connective tissue growth factor and susceptibility to renal and vascular disease risk in type 1 diabetes. J Clin Endocrinol Metab 2008;93:1893–1900.

52 Rosado de Christenson ML, Abbott GF, Kirejczyk WM, Galvin JR, Travis WD: Thoracic carcinoids: radiologic-pathologic correlation. Radiographics 1999;19:707–736.

53 Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, Schuller HM, Park JG: Expression of neuroendocrine cell markers L-DOPA decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 1988;48:4078–4082.

54 Oates T, Jensen D: The effects of training set size on decision tree complexity. Proc14th Int Conf on Machine Learning. Nashville, Morgan Kaufmann, 1997.

55 Davies AH, Larsson G, Ardill J, Friend E, Jones L, Falconi M, Bettini R, Koller M, Sezer O, Fleissner C, Taal B, Blazeby JM, Ramage JK: Development of a disease-specific quality of life questionnaire module for patients with gastrointestinal neuroendocrine tumours. Eur J Cancer 2006;42:477–484.

56 Orlefors H, Sundin A, Lu L, Oberg K, Langstrom B, Eriksson B, Bergstrom M: Carbidopa pretreatment improves image interpretation and visualisation of carcinoid tumours with ^{11}C-5-hydroxytryptophan positron emission tomography. Eur J Nucl Med Mol Imaging 2006;33:60–65.

57 Gronstad KO, Zinner MJ, Nilsson O, Dahlstrom A, Jaffe BM, Ahlman H: Vagal release of serotonin into gut lumen and portal circulation via separate control mechanisms. J Surg Res 1988;44:146–151.

58 Lindstrom E, Hakanson R: Neurohormonal regulation of secretion from isolated rat stomach ECL cells: a critical reappraisal. Regul Pept 2001;97:169–180.

59 Schubert ML, Peura DA: Control of gastric acid secretion in health and disease. Gastroenterology 2008;134:1842–1860.

60 Verspohl EJ, Ammon HP, Williams JA, Goldfine ID: Evidence that cholecystokinin interacts with specific receptors and regulates insulin release in isolated rat islets of Langerhans. Diabetes 1986;35:38–43.

61 Ahren B: The insulin response to gastric glucose is reduced in PAC1 and GRP receptor gene deleted mice. Nutr Metab Cardiovasc Dis 2006;16(suppl 1):S17–S21.

62 Chaudhry A, Oberg K, Gobl A, Heldin C, Funa K: Expression of transforming growth factors β1, β2, β3 in neuroendocrine tumors of the digestive system. Anticancer Res 1994; 14(5B):2085–2091.

63 Chaudhry A, Oberg K: Transforming growth factor alpha and epithelial growth factor receptor in neuroendocrine tumors of the digestive system. Diagn Oncol 1993;3:81–85.

64 Oberg K: Expression of growth factors and their receptors in neuroendocrine gut and pancreatic tumors, and prognostic factors for survival. Ann NY Acad Sci 1995;733:46–55.

65 Kidd M, Eick G, Shapiro MD, Camp RL, Mane SM, Modlin IM: Microsatellite instability and gene mutations in transforming growth factor-beta type II receptor are absent in small bowel carcinoid tumors. Cancer 2005;103:229–236.

66 Kidd M, Modlin IM, Pfragner R, Eick GN, Champaneria MC, Chan AK, Camp RL, Mane SM: Small bowel carcinoid (enterochromaffin cell) neoplasia exhibits transforming growth factor-β1-mediated regulatory abnormalities including up-regulation of C-Myc and MTA1. Cancer 2007;109: 2420–2431.

67 Kidd M, Modlin I, Shapiro M, Camp R, Mane S, Usinger W, Murren J: CTGF, intestinal stellate cells and carcinoid fibrogenesis. World J Gastroenterol 2007;13:5208–5216.

68 Guillermet-Guibert J, Lahlou H, Cordelier P, Bousquet C, Pyronnet S, Susini C: Physiology of somatostatin receptors. J Endocrinol Invest 2005;28:5–9.

69 Wimmel A, Wiedenmann B, Rosewicz S: Autocrine growth inhibition by transforming growth factor β1 (TGFβ1) in human neuroendocrine tumor cells. Gut 2003;52: 1308–1316.

70 Kidd M, Modlin IM, Eick GN, Camp RL, Mane SM: Role of CCN2/CTGF in the proliferation of *Mastomys* enterochromaffin-like cells and gastric carcinoid development. Am J Physiol Gastrointest Liver Physiol 2007; 292:G191–G200.

71 Hopfner M, Sutter AP, Gerst B, Zeitz M, Scherubl H: A novel approach in the treatment of neuroendocrine gastrointestinal tumours. Targeting the epidermal growth factor receptor by gefitinib (ZD1839). Br J Cancer 2003;89:1766–1775.

72 Jaquet P, Gunz G, Saveanu A, Dufour H, Taylor J, Dong J, Kim S, Moreau JP, Enjalbert A, Culler MD: Efficacy of chimeric molecules directed towards multiple somatostatin and dopamine receptors on inhibition of GH and prolactin secretion from GH-secreting pituitary adenomas classified as partially responsive to somatostatin analog therapy. Eur J Endocrinol 2005;153:135–141.

73 Kreiss C, Schwizer W, Erlacher U, Borovicka J, Lochner-Kuery C, Muller R, Jansen JB, Fried M: Role of antrum in regulation of pancreaticobiliary secretion in humans. Am J Physiol 1996;270:G844–G851.

74 Kuntz E, Pinget M, Damge P: Cholecystokinin octapeptide: a potential growth factor for pancreatic beta cells in diabetic rats. JOP 2004;5:464–475.