

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

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## 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*, *TGF $\beta$ 2*, 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  $\gamma$ -aminobutyric acid A (GABA<sub>A</sub>,  $IC_{50} = 2$  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<sub>A</sub>. KRJ-I responded with proliferation to connective tissue growth factor (CTGF,  $EC_{50} = 0.002$  ng/ml), transforming growth factor- $\alpha$  (TGF $\alpha$ ,  $EC_{50} = 0.63$  ng/ml) and transforming growth factor- $\beta$  (TGF $\beta$ ,  $EC_{50} = 0.63$  ng/ml). Epidermal growth factor (EGF) and somatostatin had no significant effect. BON cell proliferation was stimulated only by EGF and TGF $\alpha$  ( $EC_{50} = 15.8$  and 10 ng/ml). TGF $\beta$  ( $IC_{50} = 0.16$  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

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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.

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## 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- $\beta$  (TGF $\beta$ ) 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- $\alpha$  (TGF $\alpha$ ), connective tissue growth factor (CTGF), TGF $\beta$ ], 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<sub>2</sub>. 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  $\mu$ g/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  $\mu$ g 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  $\mu$ g 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  $1 \times 10^6$ ) 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 [ $\beta$ -1 adrenergic (ADBR1), muscarinic types 1–4 (M1–4), somatostatin type 2 (sst2), transforming growth factor type 2 (TGF $\beta$ R2), LRP1] were measured in KRJ-I 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/ $\mu$ l cDNA before the experiments; 1  $\mu$ l 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  $5 \times 10^4$  cells/well (n = 4), and maintained at 37°C in 5% CO<sub>2</sub>. 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.

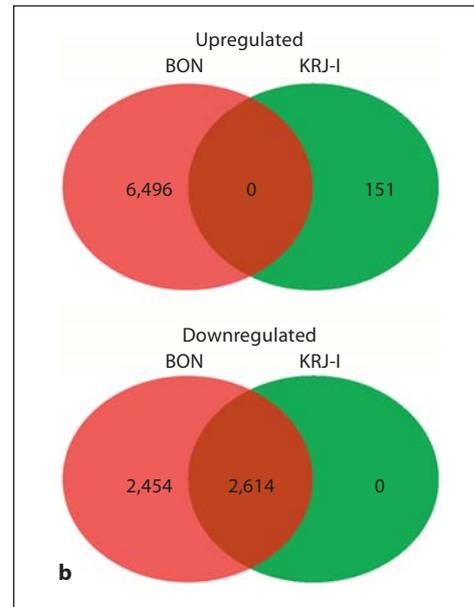
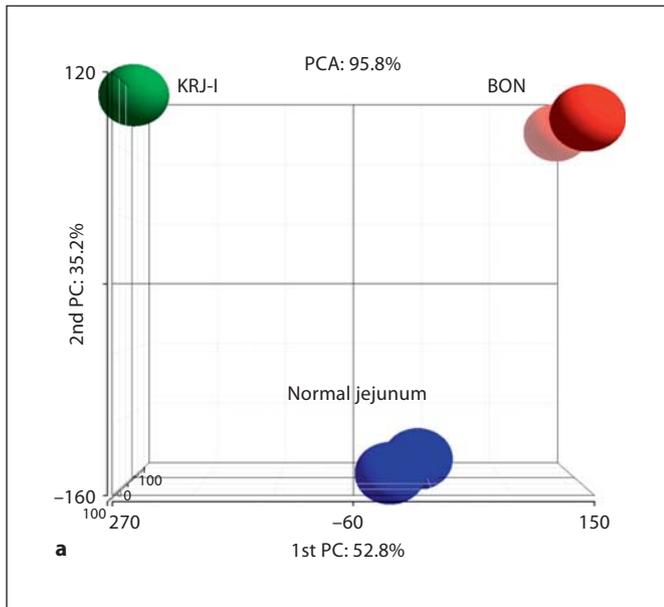
The effects of noradrenaline, isoproterenol (selective  $\beta$ -adrenergic receptor agonist), cholecystokinin, bombesin, pituitary adenylate cyclase (PACAP)-38, and acetylcholine chloride (muscarinic ligand),  $\gamma$ -aminobutyric acid (GABA) and somatostatin on 5-HT secretion were measured. The efficacy 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<sub>50</sub> or IC<sub>50</sub>).

### Proliferation Measurement

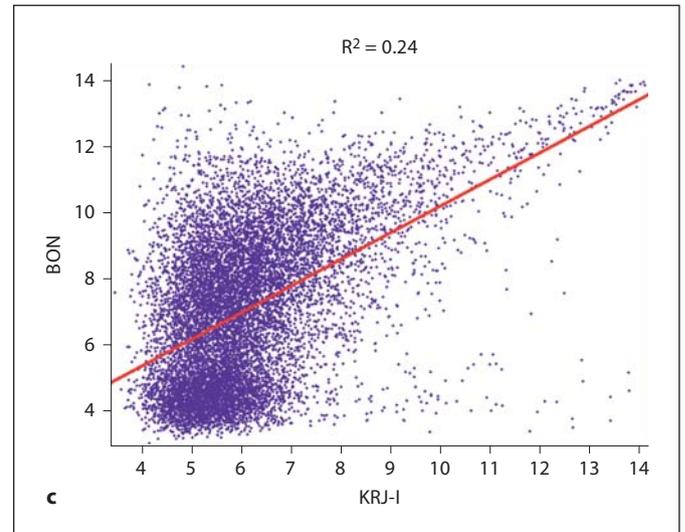
Effects of TGF $\alpha$ , TGF $\beta$ , EGF, CTGF, somatostatin, BIM23A761 (a chimeric somatostatin/dopamine agonist) [37], and MZ-4-147 (GHRH antagonist) [38] were evaluated. Additionally, the effects of cholecystokinin and gastrin on proliferation were evaluated. The methylthiazolyldiphenyl tetrazolium (MTT) assay for mitochondrial enzymatic activity was used to quantify proliferative responses [39]. Cells were seeded in 96-well plates at a density of  $5 \times 10^4$  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<sub>2</sub>. 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<sup>®</sup> 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 ( $p \leq 0.05$ ,  $FC \geq 2$ ) were identified in BON and KRJ-I, respectively. No genes were commonly upregulated in the two cell lines. 2,454 downregulated genes ( $p \leq 0.05$ ,  $FC \leq -2$ ) 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  $\leq 0.05$  and an absolute value of  $FC \geq 2.0$  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-dimensional 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  $\Delta C_T$  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 ( $p \leq 0.05$ ,  $FC \geq 2$ ) 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- $\beta$ 2* 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).

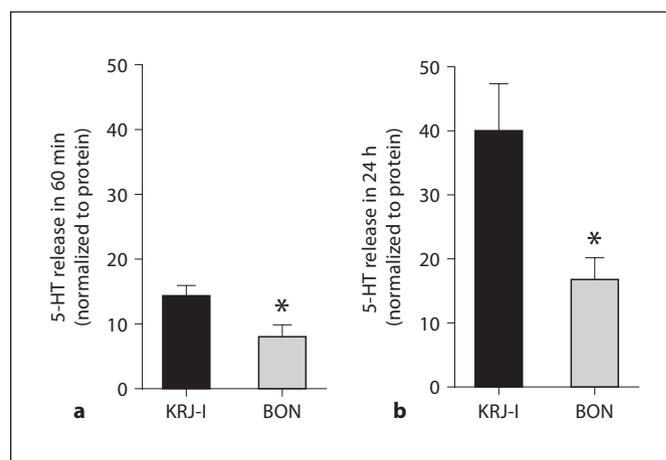
#### BON and KRJ-I 5-HT Secretory Profiles

##### Basal 5-HT Secretion

5-HT release during 60-min and 24-hour intervals was significantly lower ( $p < 0.05$ ) in BON ( $8.0 \pm 4.1$  and  $16.7 \pm 8.2$  ng/mg, respectively) than KRJ-I ( $14.3 \pm 1.6$  and  $40.1 \pm 16.3$  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 cholecystinin ( $IC_{50} = 430$  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-

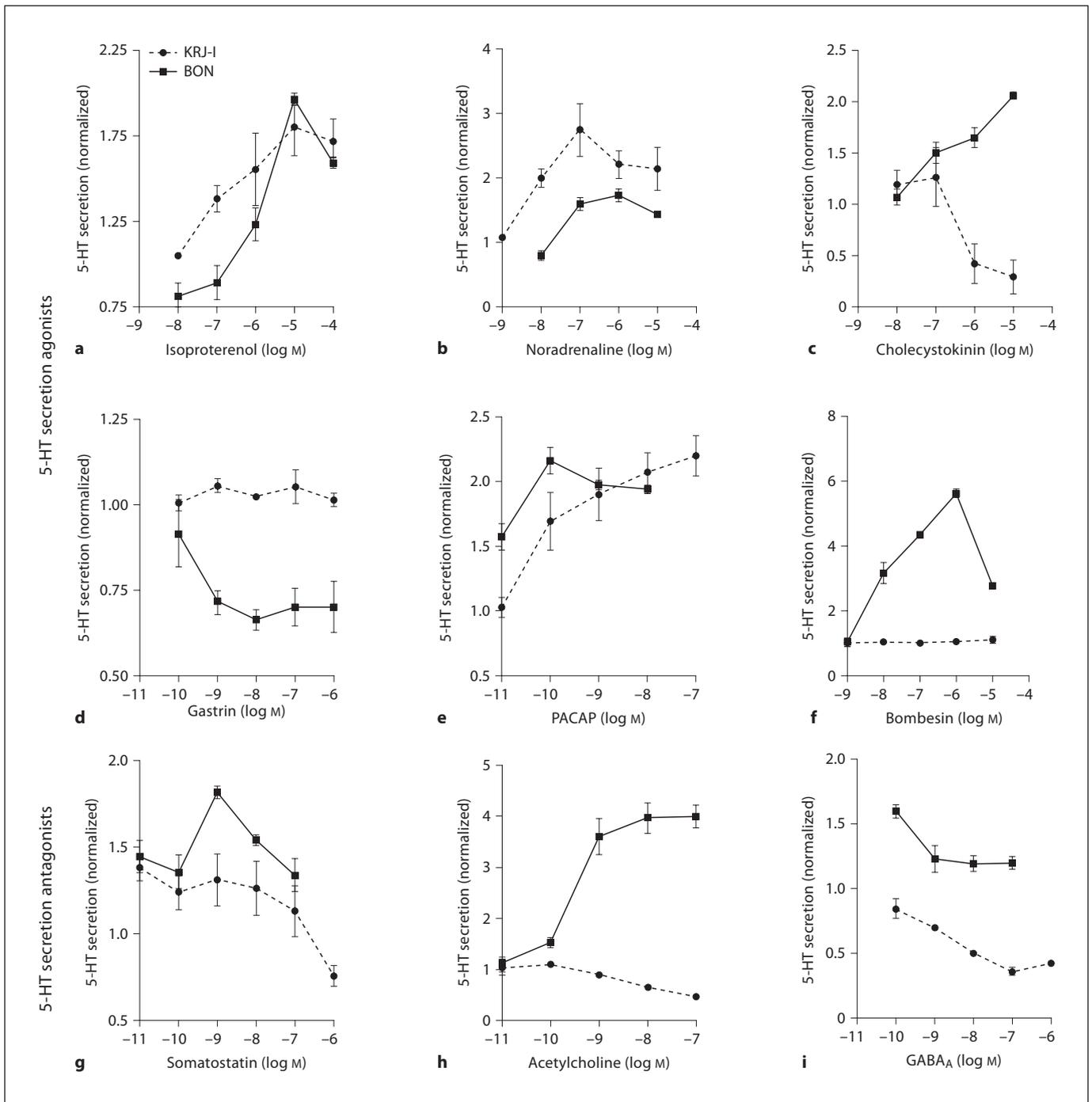


**Fig. 2.** Basal 5-HT secretion in KRJ-I and BON cell lines at 60 min and 24 h. After 60 min, 5-HT release in KRJ-I cell line was measured as  $14.3 \pm 1.6$  ng/mg normalized to protein, while in BON  $8.0 \pm 4.1$  normalized to protein (a). After 24 h, KRJ-I secretion reached  $40.1 \pm 16.3$  normalized to protein and BON 5-HT secretion reached  $16.7 \pm 8.2$  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		
<i>ALG9</i>	4.4	14.6
<i>TFCP2</i>	4.5	15.9
<i>ZNF410</i>	3.8	11.8
Neuroendocrine markers		
<i>CgA</i>	58	88
<i>Tph-1</i>	100	65
<i>Tph-2</i>	N/A	85
<i>NSE</i>	38	32
<i>DDC</i>	N/A	39
<i>Substance P</i>	21	165
<i>Guanylin</i>	159	100
<i>Ki-67</i>	38	14
Receptors		
<i>ADBR2</i>	53	116
<i>SST2</i>	21	106
<i>LRP1</i>	96	125
<i>TGF<math>\beta</math>2</i>	N/A	17
<i>M1</i>	47	154
<i>M2</i>	33	154
<i>M3</i>	N/A	84
<i>M4</i>	73	46

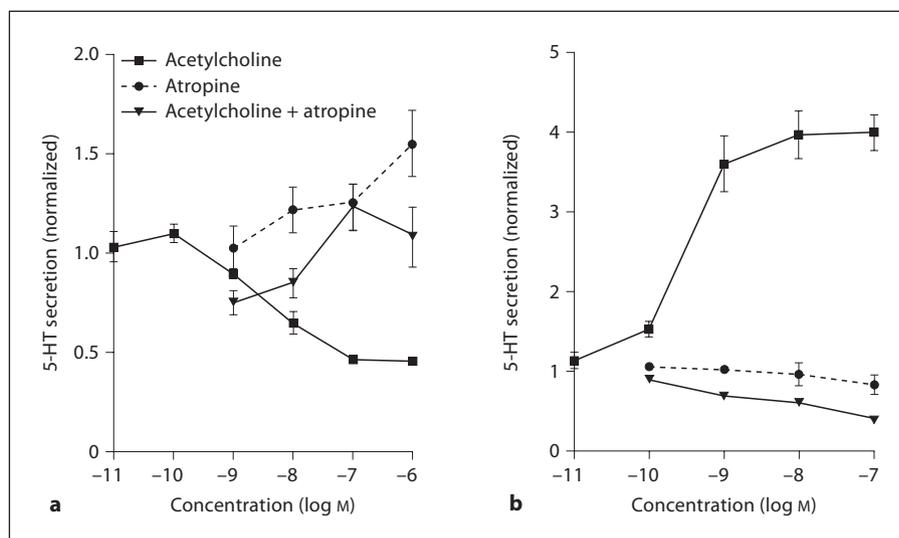
The variability in  $\Delta C_T$  was expressed as CV and was defined as the ratio of standard deviation to the mean.



**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 cholecystikinin ( $IC_{50} = 430$  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), cholecystikinin ( $EC_{50} = 130$  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), cholecystinin ( $EC_{50} = 130$  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

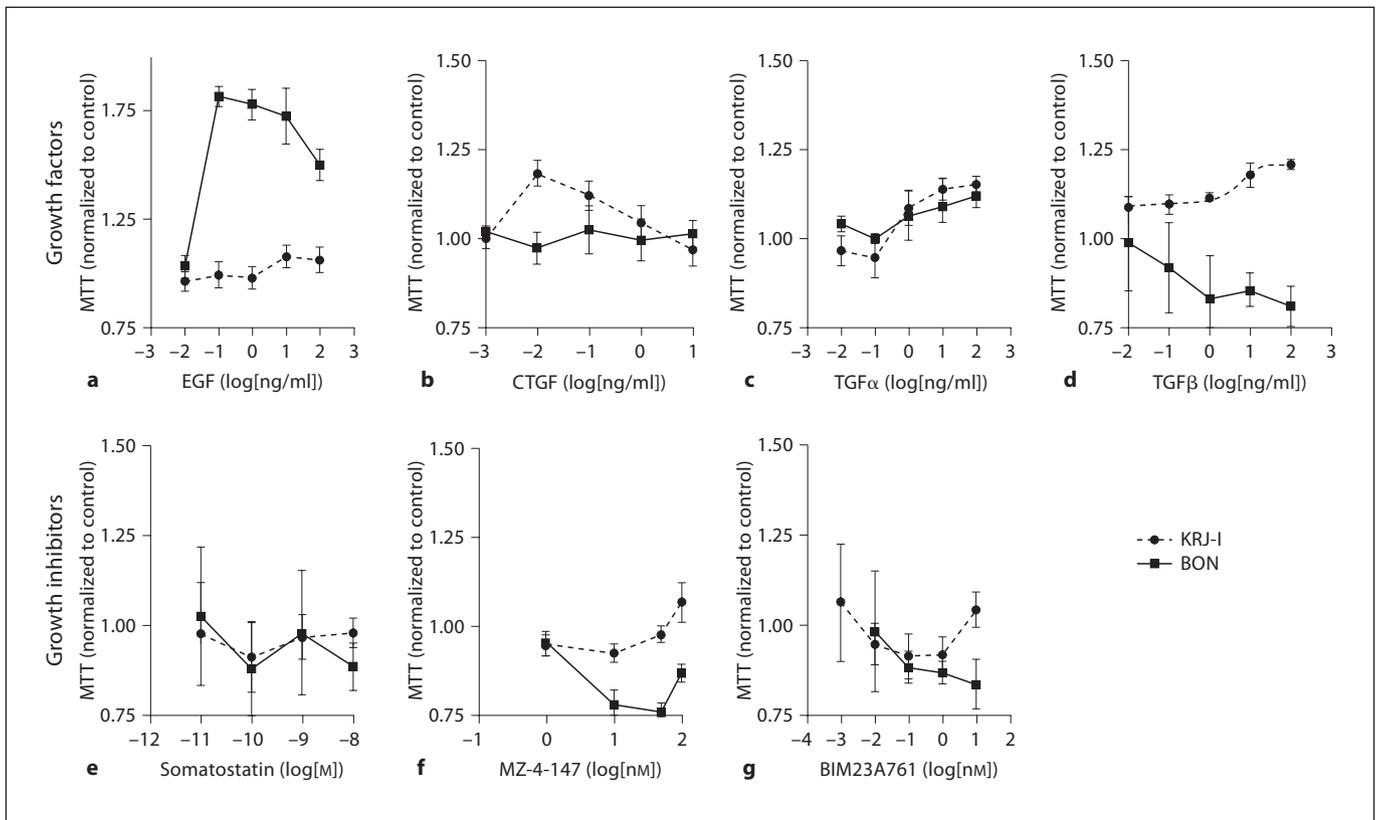
The KRJ-I cell line responded with proliferation to  $TGF\alpha$  ( $EC_{50} = 0.63$  ng/ml),  $TGF\beta$  ( $EC_{50} = 0.63$  ng/ml), CTGF ( $EC_{50} = 0.002$  ng/ml), and the GHRH antagonist, MZ-4-147 ( $EC_{50} = 63$  nM) (fig. 5b-d, f). Proliferation

was marginally but not significantly inhibited by the somatostatin/dopamine chimera BIM23A761 ( $IC_{50} = 3 \times 10^{-3}$  nM) (fig. 5g), while EGF and somatostatin had no significant effect. Proliferation of the BON cell line was stimulated only by  $TGF\alpha$  ( $EC_{50} = 10$  ng/ml) (fig. 5c).  $TGF\beta$  ( $IC_{50} = 0.16$  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 cholecystinin and gastrin on KRJ-I and BON cell proliferation were measured. Cholecystinin 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

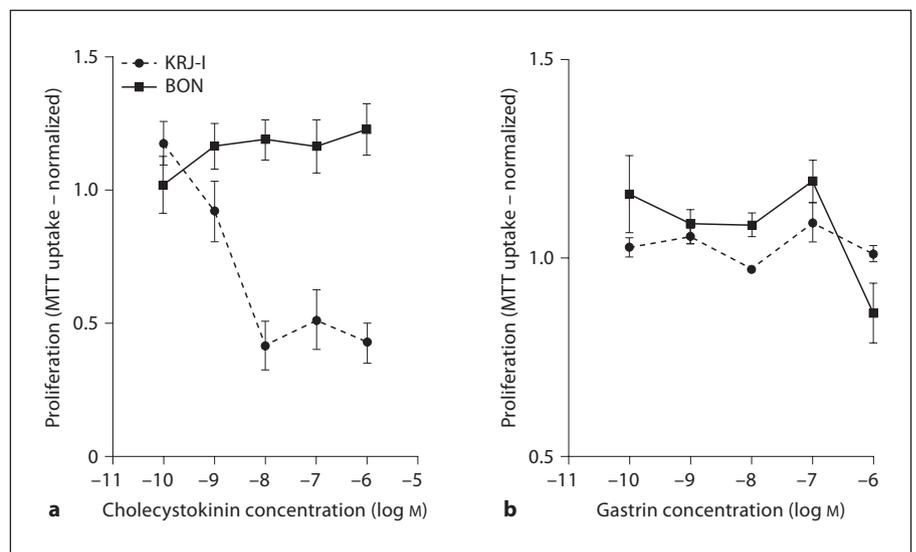
Molecular understanding of the EC cell-derived GINETs has been substantially hampered by the lack of an appropriate human neoplastic EC cell model. The NCI summit conference noted that the lack of appropriate cell lines and animal models was a key issue contributing to the limited advances in the field 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), TGF $\alpha$  ( $EC_{50} = 0.63$  ng/ml) and TGF $\beta$  ( $EC_{50} = 0.63$  ng/ml) (c, d, f). Proliferation was inhibited by and BIM23A761 ( $IC_{50} = 3 \times 10^{-3}$  nM) (g) while EGF and somato-

statin had no effect. Proliferation of the BON cell line was stimulated by EGF and TGF $\alpha$  ( $EC_{50} = 15.8$  and  $10$  ng/ml, respectively) (a, c). TGF $\beta$  ( $IC_{50} = 0.16$  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 cholecystikinin and gastrin on KRJ-I and BON cell proliferation. Cholecystikinin 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

**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 <sub>50</sub> : 100	EC <sub>50</sub> : 900
Noradrenaline, nM	EC <sub>50</sub> : 1.7	EC <sub>50</sub> : 20
PACAP, nM	EC <sub>50</sub> : 0.03	EC <sub>50</sub> : 0.12
Cholecystokinin, nM	IC <sub>50</sub> : 400	EC <sub>50</sub> : 130
Somatostatin, nM	IC <sub>50</sub> : 400	IC <sub>50</sub> : 300
Acetylcholine, nM	IC <sub>50</sub> : 3.7	EC <sub>50</sub> : 0.2
GABA <sub>A</sub> , nM	IC <sub>50</sub> : 2	–
Gastrin	–	–
Bombesin, nM	–	EC <sub>50</sub> : 15
Proliferation		
CTGF, ng/ml	EC <sub>50</sub> : 0.002	–
TGF $\alpha$ , ng/ml	EC <sub>50</sub> : 0.63	EC <sub>50</sub> : 10
EGF, ng/ml	–	EC <sub>50</sub> : 15.8
TGF $\beta$ , ng/ml	EC <sub>50</sub> : 0.63	IC <sub>50</sub> : 0.16
SST	–	–
MZ-4-147, nM	–	IC <sub>50</sub> : 0.5
BIM23A761, nM	–	IC <sub>50</sub> : 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 ( $\beta$ -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.

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

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]. Similarly, *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  $\beta$ -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 *TGF $\beta$ 2* receptor transcripts were only expressed in BON, suggesting substantial differences in muscarinic control and TGF $\beta$ -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,  $\alpha$ - and  $\beta$ -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.  $\beta$ -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<sub>A</sub> 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  $\beta$ -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, TGF $\beta$  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 TGF $\beta$ . Thus, BON cells responded with inhibition of proliferation consistent with expression of a functional TGF $\beta$ R2 inhibitory pathway as previously noted in this cell line [27, 69]. In contrast, TGF $\beta$  stimulated KRJ-I proliferation, consistent with our previous observations of an altered-TGF $\beta$ -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 TGF $\alpha$  (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 TGF $\beta$ , 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 expres-

sion 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 TGF $\beta$ :somatostatin autoinhibitory pathway [27]. Addition of Notch or alterations in TGF $\beta$  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.

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