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INSL5 may be a unique marker of colorectal endocrine cells and neuroendocrine tumors

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ABSTRACT

Insulin-like peptide 5 (INSL5) is a member of the insulin superfamily, and is a potent agonist for RXFP4. We have shown that INSL5 is expressed in enteroendocrine cells (EECs) along the colorectum with a gradient increase toward the rectum. RXFP4 is ubiquitously expressed along the digestive tract. INSL5-positive EECs have little immunoreactivity to chromogranin A (CgA) and might be a unique marker of colorectal EECs. CgA-positive EECs were distributed normally along the colorectum in INSL5 null mice, suggesting that INSL5 is not required for the development of CgA-positive EECs. Exogenous INSL5 did not affect the proliferation of human colon cancer cell lines, and chemically-induced colitis in INSL5 null mice did not show any significant changes in inflammation or mucosal healing compared to wild-type mice. In contrast, all of the rectal neuroendocrine tumors examined co-expressed INSL5 and RXFP4. INSL5 may be a unique marker of colorectal EECs, and INSL5–RXFP4 signaling might play a role in an autocrine/paracrine fashion in the colorectal epithelium and rectal neuroendocrine tumors.

1. Introduction

The human relaxin/insulin superfamily consists of insulin, insulin-like growth factor 1 and 2 (IGF1 and IGF2), relaxins-1 (H1 relaxin), -2 (H2 relaxin), insulin-like peptide 3–6 (INSL3–6), and relaxin-3/INSL7. Except for IGF1 and IGF2, which are single chain peptides, all members of the family consist of two chains (an A-chain and a B-chain) that are linked by two disulfide bonds with a third intramolecular disulfide within the A-chain, which is a signature structure of insulin-like molecules [1,2]. Relaxins have been shown to be multifunctional peptides involved in numerous physiological processes, including uterine relaxation, reproductive tissue growth, collagen remodeling in females, wound healing, cardiac protection and allergic responses [2].

INSL5 was first identified through database searches of expressed sequence tags for the presence of the B-chain Cys motif, which is conserved within the relaxin/insulin superfamily [3]. Human INSL5 mRNA has been detected in peripheral tissues, including the rectum, colon and uterus [3]. A quantitative RT-PCR study revealed the presence of INSL5 mRNA in a variety of human tissues, including the pituitary and at lower levels, the brain [4]. In mice, the highest expression of INSL5 mRNA is found in the colon [3] and kidneys [5]. INSL5 is also expressed in a population of cells in the mouse hypothalamus and pituitary, and it increases the internal [Ca^{2+}] by a mechanism that involves both Ca^{2+} influx and Ca^{2+} release from intracellular stores [6]. The high concentration of immunoreactive INSL5 in the hypothalamic–pituitary axis suggests that it has a neuroendocrine function in mice [6].

The G-protein-coupled relaxin family peptide receptors 4 (RXFP4), also known as G protein-coupled receptor 142 or GPR100, was discovered by searching the human genomic database using the RXFP3 sequence. Receptor–ligand binding assays revealed that INSL5 is the ligand for RXFP4, whereas relaxin-3/INSL7 binds to RXFP3 [4]. The Northern blot analyses conducted with RNA revealed RXFP4 expression in the heart, skeletal muscle, kidneys, liver and placenta [7]. Similar results were obtained by RT-PCR, with mRNA also detected in the human colon, thyroid, salivary glands, prostate, thymus, testes and brain [4,8]. Intriguingly, the genes for both INSL5 and its receptor are dysfunctional in the rat and dog genomes [4].

In the gastrointestinal mucosa, several types of enteroendocrine cells (EECs) are dispersed throughout the epithelial layer of the digestive tract. Unlike other endocrine systems, EECs have high turnover rates, with a lifespan on the order of 4–6 days [9]. EECs

Abbreviations: Ab, antibody; CgA, chromogranin A; DSS, dextran sulfate sodium; DMEM, Dulbecco's modified Eagle's medium; EEC, enteroendocrine cell; IGF, insulin-like growth factor; INSL5, insulin-like peptide 5; LDCV, large dense core vesicles; mTOR, mammalian target of rapamycin; NET, neuroendocrine tumor; NSE, neuron-specific enolase; PCR, polymerase chain reaction; RXFP, relaxin family peptide receptor; SLMV, synaptic-like microvesicles; WT, wild-type.

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produce and secrete multiple regulatory molecules, which play important roles in food intake, gut motility, intestinal transit, the absorption of nutrients, energy homeostasis, mucosal immunity and repair [9,10]. They act as sensors of luminal contents, either in a classical endocrine fashion, or by a paracrine effect on proximate cells.

There are at least 15 subtypes of EECs, based on the ultrastructural characteristics of their secretary granules and the diverse number of hormones produced [11]. As with extragastrointestinal endocrine systems, the co-expression, and presumably co-secretion, of more than one mediator is widely observed [9]. EECs present two regulated pathways of secretion characterized by large dense core vesicles (LDCV) and synaptic-like microvesicles (SLMV). EECs are recognized by the expression of several general markers, including the LDCV marker, chromogranin A (CgA), and the SLMV marker, synaptophysin, in addition to the cytosolic marker, neuron-specific enolase (NSE) [11]. The expression of different hormones identifies specific cell types.

Neuroendocrine tumors (NETs, also known as carcinoid tumors) are rare and slow-growing tumors derived from EEC populations, representing approximately 0.5% of all malignancies [12]. NETs consist of a spectrum of malignancies that can arise from neuroendocrine cells throughout the body [13]. Yao et al., reported that the incidence and prevalence of NETs has increased substantially over the past three decades at all primary sites and for all disease stages using the Surveillance, Epidemiology, and End Results (SEER) program (USA) [13].

In spite of numerous studies on INSL5, its biological function(s) remain largely elusive. Recently, Burnicka-Turek et al., has reported that INSL5-deficient mice displayed alterations in glucose homeostasis and impaired fertility [14]. However, the intense expression of INSL5 in the colorectum and its cognate receptor, RXFP4, strongly suggests that it has an autocrine/paracrine function in the peripheral digestive tract. The aim of this study was to examine the role of the INSL5–RXFP4 ligand–receptor system in the colorectum.

2. Materials and methods

2.1. Materials

The antibodies (Abs) used in the studies are listed in Supplementary Table 1.

2.2. Cell culture

Human colon cancer cell lines, $CaCO_2$ (RBRC-RCB0988) and LoVo (RBRC-RCB1639) [15] were purchased from RIKEN Cell Bank (Tsukuba, Japan). The COLO320DM (JCRB0225) cell line, derived from an untreated human colon carcinoid tumor [16], was purchased from the Health Science Research Resources Bank (Osaka, Japan). $CaCO_2$ cells and COLO320DM cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% and 10% fetal bovine serum (FBS), respectively, at 37 °C in a humidified environment of 95% air and 5% CO_2. LoVo cells were cultured in HamF12 medium containing 10% FBS. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Animal models

C57BL/6 INSL5 knock-out mice (*Insl5*$^{-/-}$ mice, targeted disruption of the gene) were obtained from the Jackson Laboratory (ME, USA). All mice were maintained in a specific pathogen-free (SPF) animal facility at Akita University. Eight- to sixteen-week-old mice were used in the study. The primer sequences used for genotyping are listed in Supplementary Table 2. All experiments using mice were approved by the Institutional Animal Care and Use Committee of Akita University.

2.4. Induction of dextran sulfate sodium (DSS)-induced colitis and grading of histologiccal changes

DSS [molecular weight, 36–50 kDa; MP Biomedicals (Solon, OH)] was added to the drinking water at a final concentration of 3% (wt./vol.) for 5 days (days 1–5) [17]. On day eight, the mice were sacrificed, and colorectal samples were extracted. The degree of inflammation on microscopic cross sections of the colorectum was graded semiquantitatively based on a scoring system that considers architectural derangement, goblet cell depletion, edema/ulceration and the degree of inflammatory cell infiltration [18]. All criteria were graded on a 0–3 scale (0, absent; 1, mild; 2, moderate; 3, severe). The four scores were added together to give a total score for each section of the colorectum.

2.5. Human tissues

All NET tissue samples (rectum ($n = 12$), stomach ($n = 3$) and duodenum ($n = 1$)) were collected from the Department of Gastroenterology, Akita University Hospital, by endoscopic resection for clinical indications. This study was reviewed and approved by the ethics committee of Akita University, Faculty of Medicine, and The Institute of Medical Science, The University of Tokyo. All patients gave their written consent for the use of their tissue specimens.

2.6. Northern blot analysis

Human MTN Blots and Human Digestive System 12-Lane MTN Blots were purchased from Clontech (Palo Atlo, CA). The blots were hybridized with a ^{32}P-labeled *Insl*5 and *Rxfp*4 cDNA probes. The blots were rehybridized with a ^{32}P-labeled β-*actin* cDNA fragment to confirm the equal loading of RNA.

2.7. Conventional reverse-transcription polymerase chain reaction (RT-PCR)

The total RNA was obtained using an RNeasy Mini kit (QIAGEN, Valencia, CA). First-strand complementary DNA was synthesized using the Superscript™ First-stranded Synthesis System for Reverse-Transcription Polymerase Chain Reaction (Invitrogen, Carlsbad, CA). The PCR primers and the conditions used in the study are listed in Supplementary Table 2.

2.8. Immunohistochemistry

After deparaffinization, heat-induced epitope retrieval and the quenching the endogenous peroxidase, the samples were immunostained sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary Abs and secondary Abs. Specific immunostaining was developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (DAKO).

For the co-localization of human INSL5 and CgA, NETs samples accompanying normal colorectal areas were used. Immunostaining proceeded similarly using a Cy3-conjugated anti-rabbit IgG (for INSL5) and an Alexa Fluor 488 anti-mouse IgG (for CgA) as the secondary Abs. As a negative control, the primary Ab was replaced with a species-specific IgG isotype control at the same concentration (DAKO).

2.9. Measurement of DNA synthesis

Human colon cancer cells were seeded at a density of 2–4 × 10^4 cells/ml in plastic 96-well plates. After serum starvation for 24 h, the cells were treated with recombinant human INSL5 (Phoenix Pharmaceuticals, Burlingame, USA) at the indicated concentrations for 48 h in media containing 0.1% FBS. BrdU was added for the last two hours of incubation, and DNA synthesis was determined using a BrdU incorporation assay kit (Roche Diagnostics, Mannheim, Germany).

2.10. Measurement of the blood glucose level

The blood glucose level was measured arbitrarily in blood samples (5–10 μl of each sample) collected from the tail vein using an Advantage Test StripS glucometer (Roche Molecular Biochemicals, Mannheim, Germany).

2.11. Statistical analysis

All data are presented as the means ± SD. The statistical significance of the values obtained was evaluated by Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Expression of INSL5 and RXFP4 in the colorectal epithelium

We observed immunoreactivity to INSL5 within the mucosal epithelium of WT murine colorectal tissue, whereas we could not detect any immunopositive cells in *Insl5*$^{-/-}$ mice. The INSL5-positive cells were restricted to the cells resembling EECs located within the epithelial cell layer (Fig. 1B). As pointed out in earlier studies using *Insl5* mRNA [3], the number of INSL5 positive cells appeared to be higher in the distal colon and rectum, compared to the proximal colon. We then counted the number of INSL5-positive cells, together with cells positive for CgA in *Insl5*$^{+/+}$, *Insl5*$^{+/-}$ and *Insl5*$^{-/-}$ mice. As shown in Fig. 1(C), we observed only a few INSL5-positive cells in the cecum and the number of immunopositive cells showed a gradual increase from the cecum to rectum in *Insl5*$^{+/+}$ and *Insl5*$^{+/-}$ mice. The number of positive cells in *Insl5*$^{+/-}$ colorectum was almost half of that in the WT mice, and no immunopositive cells could be detected in *Insl5*$^{-/-}$ colorectum. CgA-positive cells were distributed throughout the colorectum in *Insl5*$^{+/+}$, *Insl5*$^{+/-}$ and *Insl5*$^{-/-}$ mice. We could not find any differences between the groups, nor any relationship with the expression of INSL5, except that the number of CgA-positive cells was somewhat higher in the cecum of *Insl5*$^{+/-}$ mice.

Next, we examined the mRNA level of *Insl5* and its receptor, *Rxfp4*, in various human organs. The expression of *Insl5* was detected only in the rectum by the Northern blotting analysis. The mRNA of *Rxfp4* was ubiquitously expressed, and was highly expressed in the heart, muscle, pancreas and stomach (Fig. 1D). When we compared the *Insl5* mRNA levels by RT-PCR using human biopsy samples, the expression was increased from the proximal colon to rectum (Fig. 1E).

3.2. A few EECs co-expressed INSL5 and CgA

Since CgA is widely used in diagnostic histopathology as a general neuroendocrine marker, it is of importance to recognize its presence or absence under various conditions. We immunostained serial sections of WT mouse colorectal tissue for INSL5 and CgA. The INSL5-positive cells and CgA-positive cells showed a similar appearance in shape. We could not detect cells with co-localized staining, but some positive cells were located close together in the mouse colorectum (Fig. 2A, arrowheads). We then employed double immunostaining using human samples. When we counted 300 cells/sample ($n = 3$), we detected a few cells to co-express INSL5 and CgA (11.7 ± 1.5% in INSL5-positive cells, 7.8 ± 2.3% in CgA-positive cells, Fig. 2B, arrows), indicating that INSL5 may be a unique marker of colorectal EECs. We were unable to examine the co-localization using mouse samples, because the primary antisera generated in species other than rabbits is not good for immunofluorescent studies using mouse tissues.

3.3. INSL5 does not affect the proliferation of human colonic cell lines

RXFP4 was ubiquitously expressed along the digestive tract (Fig. 1D) and was expressed in human colonocytes [19]. We then examined whether INSL5 affects the proliferation of $CaCO_2$, LoVo and COLO320DM. The $CaCO_2$ and LoVo cell lines were derived from human colon adenocarcinoma and COLO320DM cells preserved their differentiated neuroendocrine characteristics [16]. All of the cell lines expressed mRNA for *Insl5* and *Rxfp4*, but exogenous INSL5 did not affect the proliferation of these cells (Supplementary Fig. 1).

3.4. INSL5 does not play a significant role in colitis or mucosal healing

Insl5$^{-/-}$ mice on a C57BL/6 genetic background appeared normal and were fertile. The *Insl5*$^{-/-}$ mice were indistinguishable from their WT littermates in appearance and gross behavior. DSS induced symptoms resembling ulcerative colitis five days after the application of the compound. We compared the changes in body weight, histological scores in acute colitis stage (day 8) and healing stage (day 28), and the number of INSL5- and CgA-positive cells between *Insl5*$^{+/+}$ and *Insl5*$^{-/-}$ mice. However, we could not detect any differences in any of the categories except for a lack of INSL5 immunoreactivity in the *Insl5*$^{-/-}$ mice (Supplementary Fig. 2).

3.5. Expression of INSL5, RXFP4 and neuroendocrine markers in human NETs

The INSL3–RXFP2 ligand–receptor system has been demonstrated to be a novel autocrine/paracrine effector influencing tumor progression and tissue invasiveness [20]. We therefore next examined the expression of INSL5 and RXFP4 in human NETs, along with other EEC markers (Fig. 3, table). All of the rectal NETs examined expressed both INSL5 and RXFP4. Among the rectal NETs, the CgA positive rate was 8.3%, that of synaptophysin was 58%, and that of NSE was 75%. INSL5 and CgA were co-expressed in only one case, where the immunoreactivity for CgA was strong and that of INSL5 was weak (Fig. 3, lower case). In most of the CgA-negative cases, the immunoreactivity of INSL5 was strong (Fig. 3, upper case). The rare co-localization of these two markers in human EECs suggests that INSL5 and CgA might be expressed reciprocally in the colorectal epithelium and NETs.

4. Discussion

As reported in previous studies [3,4], *Insl5* was highly expressed in the colorectum, and the expression of INSL5 showed a gradient increase from the proximal colon to the rectum (Fig. 1). The immunoreactivity for INSL5 was restricted to the intraepithelial cell population. The cognate receptor, *Rxfp4*, was ubiquitously expressed along the digestive tract, including in colonocytes (Fig. 1, [19]). These results suggest that INSL5 may play important roles not only in a classical endocrine fashion, but also in a specific autocrine/par-

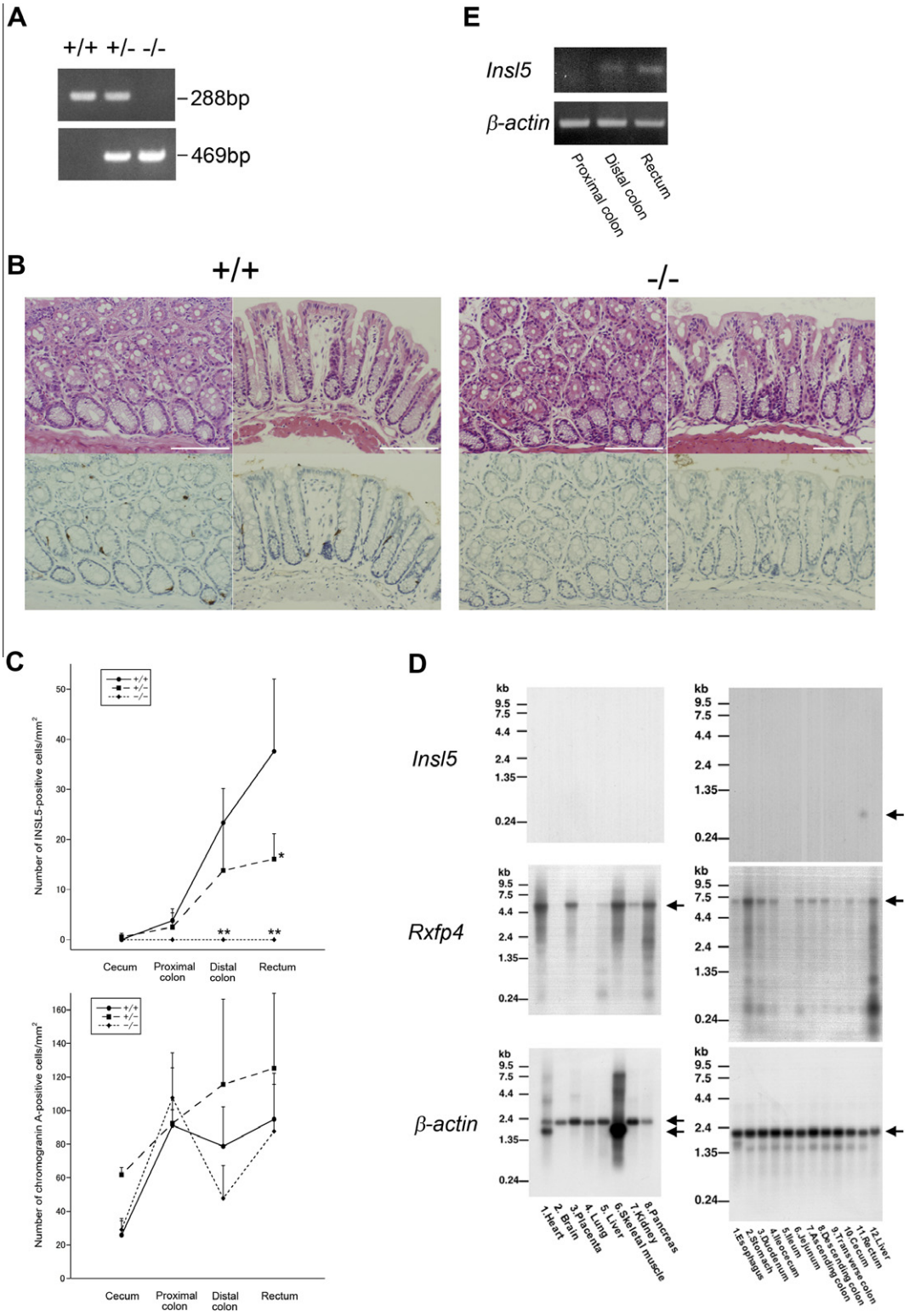

Fig. 1. Expression of INSL5, CgA and RXFP4 in the mouse and human colorectum. (A) Genotyping of *Insl5*$^{+/+}$, *Insl5*$^{+/-}$, and *Insl5*$^{-/-}$ mice. Genomic DNA was extracted from mouse tails, and PCR analyses were performed. The primers used are listed in Supplementary Table 2. (B) Representative hematoxylin and eosin staining and the immunohistochemical images (INSL5) of the colorectum from *Insl5*$^{+/+}$ and *Insl5*$^{-/-}$ mice. Bars, 100 μm. (C) The number of INSL5- and CgA-positive cells was counted along the colorectum (five fields (×200) for each section, $n = 4$ for each genotype). The area of the mucosal layer was measured using the ImageJ software program. The values are expressed as the means ± SD. The experiment was repeated twice independently, showing similar results. $^{*}p < 0.05$; $^{**}p < 0.01$, by analysis of variance. (D) The results of Northern blot analyses for *Insl5*, its cognate receptor, *Rxfp4*, and β-*actin* in various human tissue specimens are shown. (E) Semiquantitative RT-PCR for *Insl5* was performed using mRNA extracted from human biopsy samples of the proximal colon, distal colon, and rectum.

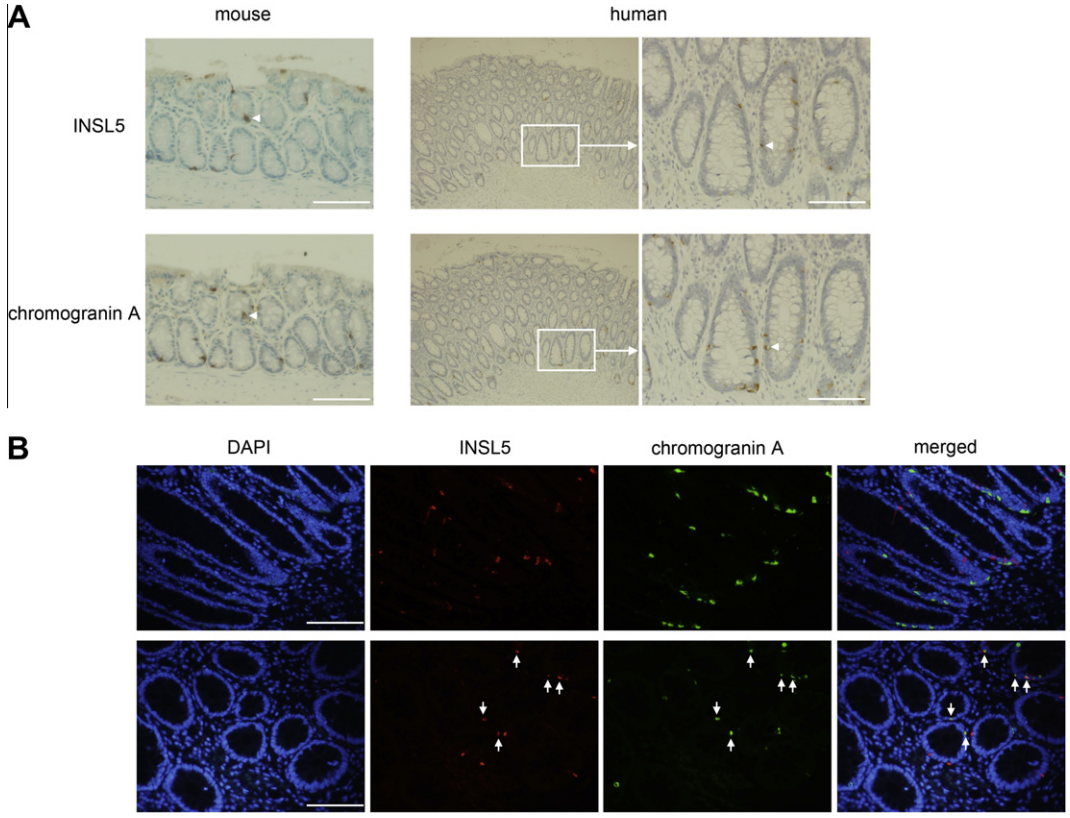

Fig. 2. INSL5 co-localizes rarely with CgA in colorectal EECs. (A) Serial sections of WT mouse colorectum were immunostained for INSL5 and CgA. We could not detect co-localization of these two markers, but some cells were located close together (arrowheads). (B) A double immunofluorescent study is shown using normal human colorectum. DAPI was used as a nuclear stain. Merged images demonstrated that INSL5 (Red, Cy3) and CgA (Green, Alexa Flour 488) were co-localized in a few human EECs (arrows). 300 cells/sample ($n = 3$) were counted. Bars, 100 μm.

Positive rate (%) of neuroendocrine markers in human NETs

	INSL5	RXFP4	chromogranin A	synaptophysin	NSE
Rectum (n=12)	100 (12/12)	100 (6/6)	8.3 (1/12)	58 (7/12)	75 (9/12)
Stomach (n=2)	50 (1/2)	100 (1/1)	100 (2/2)	100 (2/2)	50 (1/2)
Duodenum (n=1)	0 (0/1)	n.d.	100 (1/1)	100 (1/1)	100 (1/1)

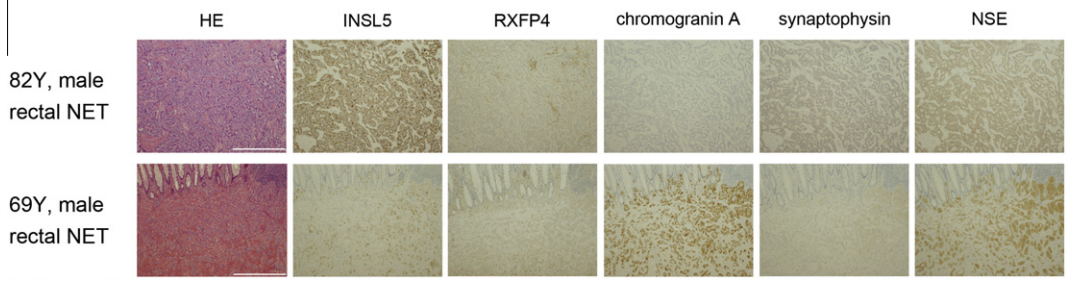

Fig. 3. The expressions of INSL5, RXFP4, CgA, synaptophysin and NSE in human NETs. Human rectal ($n = 12$), gastric ($n = 3$), and duodenal ($n = 1$) NETs were immunostained for the various markers. The positive rates of the markers are listed in the table. Representative immunohistochemical images of rectal NETs are shown in the upper panels. The only case, in which CgA and INSL5 were co-expressed, is shown in the lower panels. Bars, 500 μm.

acrine function in the colorectal epithelium. However, we could not detect any significant changes in the DNA synthesis of human colonic cell lines with the addition of recombinant INSL5. The severity of DSS-induced colitis and the mucosal healing in *Insl5*$^{-/-}$ mice were almost equivalent to those in WT mice.

EECs are located within an epithelial cell layer mainly consisting of colonocytes, which are immunopositive for RXFP4 [19], indicating a potential autocrine/paracrine role of the INSL5–RXFP4 ligand–receptor system in the colorectal epithelium. Liu et al., showed that INSL5 bound RXFP4 with a high affinity ($K_d = 2.5$ nM), activated it at EC_{50} values as low as 1.2 nM and stimulated Ca^{2+} mobilization in HEK293 cells expressing RXFP4 and Gα16 protein [4]. EECs are scattered as single cells throughout the colorectum, located within the crypts, and comprise ~1% of the epithelial cell population [9]. They secrete hormones that control physiological and homeostatic functions, particularly postprandial secretion

and gut motility [9]. A near complete absence of EECs in the mucosa (anendocrinosis) as a result of a point mutation in Ngn3, the key transcription factor for EEC lineage differentiation, causes life-threatening malabsorptive congenital diarrhea syndrome [21]. INSL5 is structurally closely related to relaxin3/INSL7. RXFP4 is activated mainly by INSL5 [4,22], but relaxin3/INSL7 can also activate it [8]. The lack of alterations observed in *Insl5*$^{-/-}$ mice may result from a compensatory effect of RXFP4 activation by relaxin3/INSL7. Relaxin3/INSL7 null mice also thrive and display normal health and well-being [23]. The failure to identify an altered phenotype in Relaxin3/INSL7 null mice has also been attributed to compensation by other INSL factors [1].

The immunofluorescent imaging findings showed INSL5-positive EECs rarely overlap with CgA-positive EECs. CgA belongs to a class of acidic proteins collectively named granins, which are widely distributed in a variety of normal tissues and in tumors of neuroendocrine and neuronal origin in vertebrates [24]. Despite the lack of INSL5, the colonic mucosa of *Insl5*$^{-/-}$ mice contained CgA-positive EECs, similar to WT mice. Therefore, INSL5 does not appear to affect the development of CgA-positive EECs or the architecture of the colonic epithelium. Thanasupawat et al., has recently reported that INSL5 co-localized with synaptophysin in EECs, and that INSL5 was not essential for the development of mouse synaptophysin-positive EECs [19].

Gut endocrine tumors consist mainly of cells that produce and secrete the same hormones that are expressed by the tissues from which they are derived. Thus, knowledge about the exact site of origin is often essential for establishing the nature of the respective tumor [24]. Funa et al., pointed out that foregut, midgut and rectal carcinoid tumors differ in their endocrine properties with regard to the expression of CgA and B [25]. It is intriguing that all of the rectal NETs examined expressed both INSL5 and RXFP4, and that INSL5 and CgA were expressed reciprocally in the colorectum and rectal NETs. Relaxin2–RXFP1 has been shown to facilitate the growth and metastasis of human prostate cancer [26,27]. INSL3–RXFP2 is a powerful and multifunctional promoter of tumor growth and angiogenesis in human thyroid cancer [20,28]. Thus, INSL5–RXFP4 signaling may play a role in rectal NETs.

Insl5$^{-/-}$ mice with a 129/Sv genetic background showed impaired glucose homeostasis and elevated serum glucose levels, but only in aging mice [14]. However, no significant differences in the glucose levels were observed in *Insl5*$^{-/-}$ (123.2 ± 14.0 mg/dl, $n = 50$), *Insl5*$^{+/-}$ (122.6 ± 11.0 mg/dl, $n = 45$) and WT (126.3 ± 12.3 mg/dl, $n = 32$) mice older than six-months of age. These differences may result from the different genetic background (C57BL/6 (our model) vs. 129/Sv [14]) or the different knock-out strategy (partial deletion of exon 2 (our model) vs. total deletion of exon 2 [14]).

Somatostatin analogues relieve symptoms resulting from hormone hypersecretion in functioning NETs, and may delay disease progression in selected patients [29,30]. Autocrine activation of the mammalian target of rapamycin (mTOR) signaling pathway, mediated through IGF1, has been implicated in the proliferation of pancreatic NETs. Everolimus inhibits mTOR, and has been shown to prolong the progression-free survival among patients with progressive advanced pancreatic NETs [31]. The multitargeted tyrosine kinase inhibitor, Sunitinib malate, also showed antitumor activity in patients with pancreatic NETs [32].

Therefore, clarifying the molecular signaling pathways in EECs and NETs is important for understanding the pathophysiology of the disease and for selecting or developing new strategies for their treatment. INSL5–RXFP4 signaling may play a role in an autocrine/paracrine fashion in the colorectum with effects other than those on cell proliferation, inflammation and mucosal healing, and INSL5 might represent a unique marker of colorectal EECs and NETs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.042.

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