INSL5 is a novel marker for human enteroendocrine cells of the large intestine and neuroendocrine tumours

THATCHAWAN THANASUPAWAT[1], KATRIN HAMMJE[2], IBRAHIM ADHAM[3], JEAN-ERIC GHIA[4,5], MARC R. DEL BIGIO[1,6], JERRY KRCEK[1,7], CUONG HOANG-VU[2*], THOMAS KLONISCH[1,7,8*] and SABINE HOMBACH-KLONISCH[1,9*]

[1]Department of Human Anatomy and Cell Science, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0J9, Canada; [2]Clinics of General, Visceral and Vascular Surgery, Martin Luther University (MLU), Halle-Wittenberg, Germany; [3]Institute of Human Genetics, University of Göttingen, Göttingen, Germany; Departments of [4]Internal Medicine, [5]Immunology, [6]Pathology, [7]Surgery, [8]Medical Microbiology and Infectious Diseases, [9]Obstetrics, Gynecology and Reproductive Medicine, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0J9, Canada

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Abstract. We report for the first time the distribution of human INSL5 and its cognate leucine rich G-protein coupled receptor RXFP4 in the large intestine and in neuroendocrine/carcinoid tissues. Immunoreactive INSL5 was uniquely expressed by enteroendocrine cells (EECs) located within the colonic mucosa, whereas colonocytes were immunopositive for RXFP4. INSL5$^+$ and RXFP4$^+$ cells were also detected in human neuroendocrine/carcinoid tissues. We employed a recently described Insl5 knockout mouse model and 2 mouse models of induced colitis to address the relevance of Insl5 in EEC development and in acute inflammation of the colon. We identified INSL5 as a specific marker for synaptophysin$^+$ EECs in the mucosa of the normal human and mouse colon. Insl5 was not essential for the development of mouse synaptophysin$^+$ EECs. The mouse models of chemically induced colitis (dextran sulfate sodium and dinitrobenzene-sulfonic acid) failed to show changes in the numbers of Insl5$^+$ EECs at inflammatory sites during the acute phase of colitis. In conclusion, we showed that INSL5 is a novel marker of colorectal EECs and provide first evidence for the presence of a potentially autocrine/paracrine INSL5-RXFP4 signaling system in the normal human and mouse colon and in rare human neuroendocrine tumours.

Correspondence to: Dr Sabine Hombach-Klonisch, Department of Human Anatomy and Cell Science, Faculty of Medicine, University of Manitoba, 130 Basic Medical Sciences Building, 130-745 Bannatyne Ave., Winnipeg, MB R3E 0J9, Canada
E-mail: hombach@cc.umanitoba.ca

*Contributed equally

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Introduction

The insulin superfamily comprises insulin, insulin-like growth factors (IGF-1 and -2), relaxin and the insulin-like (INSL) peptides 3, 4, 5, 6 and 7 (1). Similar to the other INSL members, INSL5 is composed of a B- and A-chain connected by 2 disulfide bonds. The *Insl5* gene is expressed in various tissues, including the gastrointestinal tract (2-4). INSL5 is the ligand for the orphan receptor RXFR4/GPCR142/GPR100 and this receptor-ligand interaction results in the inhibition of intracellular cAMP levels (3,5,6). INSL5 shares high sequence homology with INSL7/relaxin 3. The latter engages in low affinity receptor interactions with RXFP4 but preferentially binds to the orphan receptor RXFR3/GPCR135 (3,7). The biological consequences of the INSL5-RXFP4 activated signaling pathway remain largely elusive.

Enterochromaffin cells constitute the most abundant enteroendocrine cells (EECs) of the GI tract and are found in the highest number (>70%) in the small intestine, with a frequency decreasing to around 40% in the large intestine. Despite representing only a small minority (<1%) of dispersed cells within the large epithelial cell population, overall EECs constitute one of the largest endocrine systems in the body. EECs alone and in bidirectional interaction with the enteric nervous system have important roles in gut motility, intestinal transit, absorption of nutrients and energy homeostasis. Their role as an important component in the gut-brain, gut-pancreas and immuno-endocrine axis has initiated a new and exciting area of endocrine research (8,9). EECs have high turnover rates of 4-6 days and are scattered throughout the entire gastrointestinal mucosa (10). Currently, at least 15 different EEC types can be distinguished based on the ultrastructural characteristics of their secretory granules and the diverse number of hormones produced (11). EECs are endoderm-derived (12) and develop from the same pluripotent stem cells as the other 3 epithelial cell lineages which include the absorptive enterocytes, Paneth cells and goblet cells (13). Studies in transgenic mice have

identified a cascade of basic helix-loop-helix transcription factors involved in EEC differentiation, among them neurogenin-3, the downstream target of Math-1, which is essential for the commitment of progenitor cells to the EEC lineage. Notch signaling initiates lateral inhibition, thus, preventing adjacent cells to differentiate into EECs (14,15). Serving as general EEC markers, chromogranin A and synaptophysin are used in diagnostic pathology to identify an entero-/neuroendocrine cell component in intestinal tumours (16). Different sections of the gastrointestinal tract contain distinct EEC populations, with the richest diversity of EECs in the small intestine. The types of EECs found in the large intestine are less complex and lacks EEC secreting secretin (S-cells), gastric inhibitory polypeptide, cholecystokinin, motilin (M-cells) and neurotensin (N-cells) (11,17).

Clinical and animal data show an involvement of EEC populations in gastrointestinal disorders stretching from inflammatory bowel disease (IBD) to neuroendocrine tumours (NETs; also named carcinoids). Patients suffering from IBD have a higher incidence of developing NETs (10,18). Carcinoids are rare and slow-growing tumours derived from EEC populations representing approximately 0.5% of all malignancies (19-22). Even small-sized NETs can display unexpectedly high aggressiveness in the metastasis rate and these patients have a significantly reduced 5-year survival rate (20,23,24).

In the present study, we demonstrated the tissue localization of human INSL5 and its cognate receptor RXFP4 in the normal large intestinal tract, thus, providing first evidence for a potential autocrine/paracrine signaling role of the INSL5-RXFP4 ligand receptor in the human large intestine and NET/carcinoid tumours.

Materials and methods

Human tissues. Formalin-fixed human normal colorectal tissues (n=10) embedded in paraffin blocks were cut into 5-μm sections for histology and immunohistochemistry. Three cryopreserved invasive carcinoid tumour tissues (n=3) derived from the stomach (male) and rectum (female) and 1 lymph node metastasis of a carcinoid cancer (male) composed of chromogranin A^+ and cytokeratin KL-1^+ carcinoid cells of unknown primary origin were obtained. Tissues were cryosectioned (8 μm) and processed for immunofluorescence. All tissues were collected at the Department of Surgery, University of Halle, Germany, by surgical resection for clinical indications. This study was approved by the ethics committee of the Martin Luther University, Faculty of Medicine, Germany and all patients gave written consent.

Animal models. We used dextran sulfate sodium (DSS)-induced acute colitis (25), a lymphocyte-independent model, by oral administration of DSS and 2,4 dinitrobenzene-sulfonic acid (DNBS), a lymphocyte-dependent model (26), by intracolonic administration. Male C57BL/6 (7-9 weeks old) were purchased from Charles River suppliers and maintained in the animal care facility at the University of Manitoba under specific pathogen-free (SPF) conditions. Mice were housed under standard conditions for a minimum of 1 week before experimentation. DSS [molecular weight (MW), 40 kDa; ICN Biomedicals, Inc., Soho, OH, USA] was added to the drinking water at a final concentration of 5% (wt/vol) for 5 days (25,27). Controls were all time-matched and consisted of mice that received normal drinking water only. Mean DSS consumption was noted/cage each day. Five or 3 days after the beginning of the DSS or DNBS treatments, the mice were sacrificed, the abdominal cavity was opened and the colon was located. Formalin-fixed colon segments were paraffin-embedded and 3-μm sections were stained with hematoxylin and eosin (H&E). Colonic damage was scored based on a published scoring system that considers architectural derangements, goblet cell depletion, oedema/ulceration and degree of inflammatory cell infiltrate (28). All experiments were approved by the University of Manitoba Animal Ethics Committee and were conducted under the Canadian guidelines for animal research. *Insl5*-deficient mice (29) were housed under normal conditions at an ambient temperature in a 12-h light/12-h dark cycle, and mice had free access to standard mouse chow and tap water. The collection of mouse tissues was approved by the Institutional Animal Care and Use Committee of the University of Göttingen.

Immunodetection in tissues. Paraffin-embedded tissue sections (5 μm) were deparaffinized and washed in 0.1% Tween-20 in Tris-buffered saline at pH 7.4 (TBS/T). Sections were incubated with 3% hydrogen peroxide in methanol for 20 min in the dark to quench endogenous peroxidase. Sections were pretreated with 20 μg/ml pepsin (Sigma-Aldrich, St. Louis, MO, USA) in 0.01 M HCl for 10 min at 37˚C before performing the antigen retrieval step by boiling tissue sections in citrate buffer for 4 min, and incubating sections at 90˚C for 35 min before cooling down to room temperature (RT) for 30 min. After washing with TBS/T, tissue sections were incubated for 1 h at RT in 10% goat normal serum (Sigma-Aldrich) in TBS/T to block non-specific binding sites. The tissue sections were incubated with primary antibody against INSL5 (1:100; Sigma-Aldrich), synaptophysin (1:200; Thermo Scientific, Waltham, MA, USA) and chromogranin A (1:100; Abcam, Cambridge, MA, USA) overnight at 4˚C. The pretreatment step was not necessary for synaptophysin and chromogranin A. As a negative control, the primary antibody was replaced with rabbit isotype IgG1 control at the same concentration. After washing with TBS/T, the tissue sections were incubated with 1:200 biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h at RT, followed by a 30-min incubation with streptavidin conjugated to horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories). Specific immunostain was developed with DAB substrate (Thermo Scientific). Tissue sections were counterstained with hematoxylin and embedded prior to bright field imaging with a Zeiss A2 microscope (Zeiss, Jena, Germany).

Cryosections (8 μm) of human colon and carcinoid tissues were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at RT, washed twice in PBS for 10 min and treated for 1 min with permeabilization buffer (1.7 mM EGTA, 5 mM PIPES, 1% Triton X-100 pH 6,7). Tissues were incubated with the primary antibody against INSL5 (1:50) in Dako S2022 blocking buffer overnight at 4˚C. For the detection of INSL5, a goat anti-rabbit secondary antiserum conjugated to TRITC (1:100; Dako, Hamburg, Germany) was incubated for 1 h prior to 3x10 min washes in PBS. For the

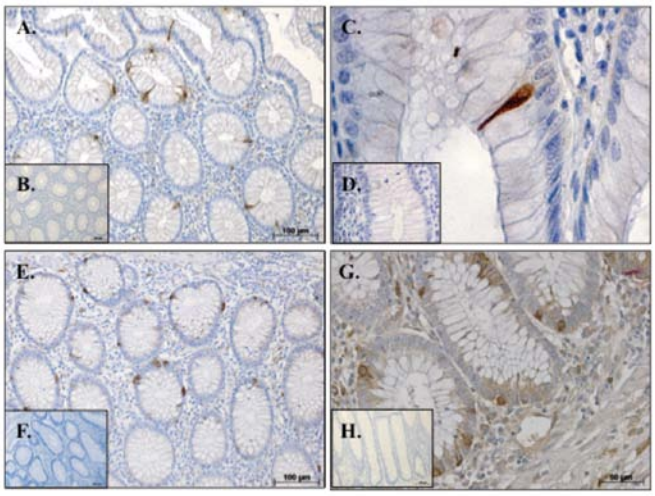

Figure 1. (A and C) Representative images of the immunohistochemical detection of human INSL5$^+$ in individual enteroendocrine cells located within the colon mucosa. (E) Detection of immunoreactive synaptophysin in colonic enteroendocrine cells. (G) Immunodetection of chromogranin A in the normal human colon. (B, D, F and H) For negative controls, the specific immune serum was replaced by a specific-specific non-immune immunoglobulin of the same isotype and revealed no specific immunostaining. Magnifications: A, B, E, F and H, x200; C, D and G, x400.

co-localization of INSL5 and synaptophysin, we performed double immunofluorescence and used a rhodamine-labeled synaptophysin antiserum (1:50; Acris, San Diego, CA, USA). Primary antibodies were replaced with rabbit isotype IgG1 control to serve as the negative control. Nuclei were stained for 2 min with Hoechst stain at 1:100 and washed twice in PBS (Sigma-Aldrich). Stained sections were embedded in fluorescent mounting medium (Dako S3023) and viewed with a Zeiss Axioplan 2 fluorescence microscope. Images were captured with an AxioCam Zeiss digital camera at x40 magnification. The same buffer conditions were used for the fluorescence detection of RXFP4. A goat anti-GPR100/RXFP4 antibody (ab79155; Abcam) was used at 1:500, and specific binding was detected with a goat anti-rabbit secondary antiserum conjugated to TRITC (1:100; Dako).

For co-localization of mouse Insl5 and synaptophysin, mouse tissue sections of the descending colon and rectum (5 μm) were incubated overnight at 4˚C with a primary rabbit antiserum against mouse Insl5 (1:200; Sigma-Aldrich) and a sheep antiserum against synaptophysin (ab72242, 1:100; Abcam). As a negative control, the primary antisera were replaced with a species-specific IgG isotype control at the same concentration (Abcam). After washing, tissue sections were incubated with 1:200 FITC-labeled anti-rabbit IgG and rhodamine-labeled anti-sheep IgG (Vector Laboratories) for 1 h at RT. Specific immunostaining was detected by immunofluorescence imaging with a Zeiss A2 microscope (Zeiss).

Results

INSL5 and RXFP4 in the normal colorectal epithelium. We observed the presence of individual INSL5 immunoreactive cells within the mucosa of normal human colorectal tissue (Fig. 1A and B). To further characterize these INSL5$^+$ intra-epithelial cells, we immunostained serial sections for the EEC markers synaptophysin and chromogranin A. Intra-epithelial cells of similar appearance to the INSL5$^+$ cells were also immunopositive for synaptophysin (Fig. 1E) and chromogranin A (Fig. 1G). Double immunofluorescent staining on cryo-sectioned human normal colon tissues demonstrated the co-localization of INSL5 and synaptophysin within the same intra-epithelial cells (Fig. 2A-D). We aimed to ascertain whether the expression of INSL5 was required for the presence of synaptophysin$^+$ EECs within the large intestine. Employing double immunofluorescent staining on cryosections of normal mouse colon tissues revealed the co-localization of Insl5 and synaptophysin in EECs (Fig. 2I-L). We also employed a newly derived mouse model in which we deleted the *Insl5* gene resulting in a complete loss of *Insl5* expression (RNA and protein) in mouse tissues, including colorectal tissues (29). In agreement with our recent mouse data, we failed to detect the Insl5 peptide but did observe immunoreactive synaptophysin in mouse colonic EECs of *Insl5*-deficient mice indicating that in our mouse model the Insl5 peptide is not an essential requirement for the formation of synaptophysin$^+$ EECs in colorectal tissues (29). We were unable to co-localize INSL5 and chromogranin A since the available primary antisera had been generated in the same species and precluded the specific detection of both markers with different sets of labelled secondary antisera.

Insl5 in a mouse model of inflammatory bowel disease (IBD). IBD, consisting of Crohn's disease (CD) and ulcerative colitis (UC), are characterized by a chronic relapsing and remitting course as a result of intestinal inflammation (30). Mucosal changes in IBD as characterized by ulcerative or infection-induced lesions are accompanied by an alteration of EECs (31-33). In the experimental acute models of colitis induced by DNBS and DSS mimicking CD and UC, respectively, EC hyperplasia has been described. We employed both mouse models to study the early dynamic changes provoked by induced inflammatory responses on the expression of INSL5 in EECs in the large intestine. Histological analysis of the large intestine revealed the frequent presence of submucosal inflammatory sites in both IBD models and we detected the presence of immunoreactive INSL5 and synaptophysin in the overlaying epithelial cells. However, we failed to observe differences in either the frequency and/or staining intensity of EECs at these inflammatory sites when compared to the adjacent colon without obvious signs of inflammation or colon tissues of untreated mice (Fig. 1A and C). This suggested that during the acute phase of inflammation there was no obvious change in the number of INSL5$^+$/synaptophysin$^+$ EECs within the mucosal layer.

INSL5 and RXFP4 in human neuroendocrine/carcinoid tumours. Members of the INSL-like family of peptides and their receptors have specific functions in cancer (34). Immunofluorescent analysis revealed the presence of INSL5$^+$/synaptophysin$^+$ cells in normal colon tissue (Fig. 2A-D) and both markers also were co-localized in cells of neuroendocrine/carcinoid tumours (Fig. 2E-H). Immunofluorescence for RXFP4$^+$ cells was also detected in neuroendocrine/carcinoid tumours (Fig. 3A) and normal colon tissue (Fig. 3B). Secreted INSL5 can bind with high affinity to

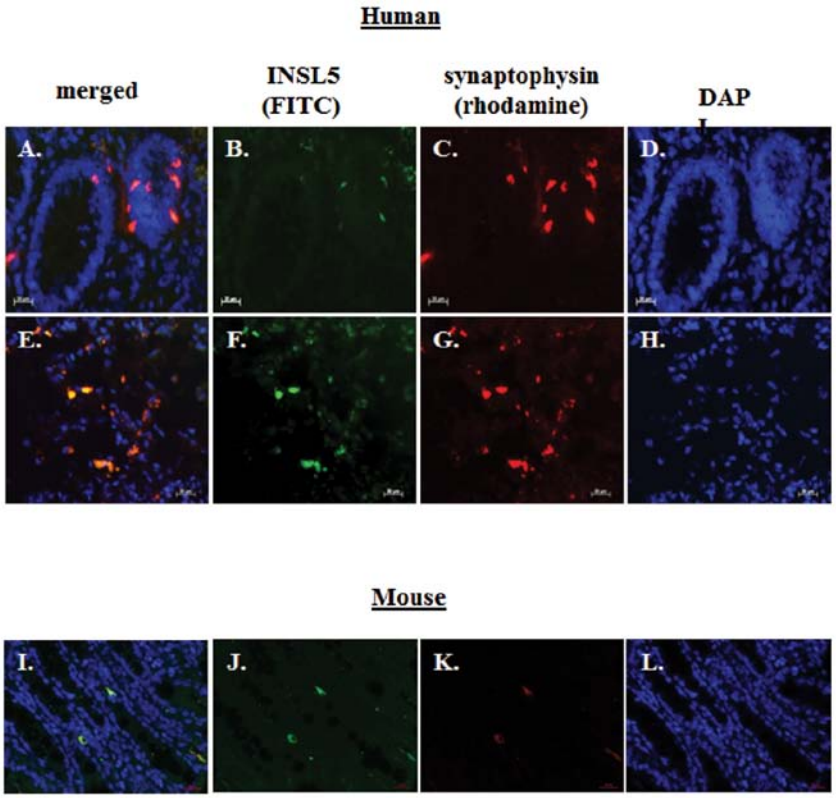

Figure 2. (A-D) In the normal human colon, double immunofluorescence is shown for the localization of (FITC; B) INSL5 and (rhodamine; C) synaptophysin in EECs. (D) DAPI was used as a nuclear stain. (A) Merged images demonstrated the same EECs to co-express INSL5 and synaptophysin. (E-H) Detection in human neuroendocrine/carcinoid tumour tissues of (FITC; F) INSL5 and (rhodamine; G) synaptophysin. (H) DAPI was used as a nuclear stain. (E) Merged images demonstrated the co-localization of INSL5 and synaptophysin in human carcinoid cells. (I-L) In the normal mouse colon, (FITC; J) INSL5 and (rhodamine; K) synaptophysin were also co-localized as noted in the (I) merged image. (L) DAPI was used as a nuclear stain. The corresponding negative controls for both human and mouse colon tissues failed to show any staining (data not shown). Magnifications: A-L, x400.

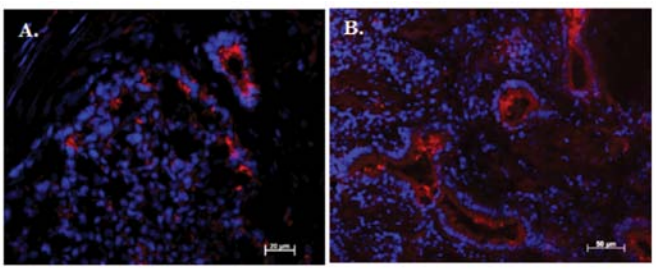

Figure 3. Immunofluorescence detection of the INSL5 receptor RXFP4 (rhodamine) in (A) human NET tissues and (B) normal human colon shown in comparison. (A) DAPI was used to visualize cell nuclei. RXFP4-expressing tumour cells were detected in carcinoid tissues. Corresponding negative controls did not show any specific staining (data not shown). Magnifications: A, x400, B, x200.

its G protein-coupled receptor RXFP4 (3) and the proximity of INSL5-expressing EECs and RXFP4$^+$ colonocytes suggest the presence of a novel autocrine/paracrine INSL5-RXFP4 ligand receptor system within the mucosa of the human large intestine and in NETs.

Discussion

INSL5 is among the most recently identified member of the insulin-like peptides and has been shown to bind with high affinity to the class-A neuropeptide-like $G_{i/o}$ protein-coupled receptor RXFP4/GPCR-142 (2-4). Transcripts for both INSL5 and RXFP4 have been detected in the human colon, and increasing copy numbers for INSL5 were reported in the mid- and distal-colon (2,3). In the present study we identified human INSL5 as a novel marker of an EEC population within the mucosa of the normal colon. We recently employed a novel *Insl5*-deficient mouse model and showed a depletion of these Insl5-expressing mucosal EECs without apparent alterations to the remaining colon mucosa in these mice (29). Our data confirm and extend a previous report on the high lacZ activity in discrete EEC populations present exclusively in the colorectal mucosa of a mouse model that had the Insl5 gene replaced by an *in vivo* lacZ reporter system (35). The human large intestine contains EECs specifically staining for synaptophysin, a major integral glycoprotein and component of small synaptic-like microvesicles (SLMV) that is also present in chromaffin cells of the adrenal medulla, endocrine cells of the pancreas and neuroendocrine cells of carcinoid tumours (16,36-38). Here we demonstrated by immunofluorescent imaging the co-expression of INSL5 in EECs immunopositive for synaptophysin. The EEC lineage derives from the same pluripotent stem cells, similar to the other 3 lineages of the intestinal epithelium: absorptive enterocytes, goblet and Paneth cells (13). To determine whether the presence of INSL5 is essential for EEC development in the large intestine, we utilized mice with a deletion of the *Insl5* gene (29). Despite

the lack of Insl5 and similar to wild-type mice, the colonic mucosa of *Insl5*-deficient mice contained synaptophysin$^+$ EECs. Thus, we concluded that Insl5 was not essential for the development of synaptophysin$^+$ EECs or the development of other colonic epithelial cell types. Intriguingly, INSL5$^+$ EECs were embedded within an epithelium largely composed of colonocytes immunopositive for the INSL5 receptor RXFP4, suggesting a potential intraepithelial autocrine/paracrine biological role for the INSL5-RXFP4 ligand-receptor system in the colon. Little is known about the biological roles of either INSL5 or its receptor RXFP4. However, reports on the presence of both INSL5 and RXFP4 in multiple peripheral tissues including the colon, placenta, heart, spleen, brain, prostate, kidney, bone marrow and liver implicate the involvement of this ligand receptor system in a broad spectrum of biological functions (3,4). *In vitro* experiments revealed that INSL5 is capable of activating RXFP4 at EC_{50} values as low as 1.2 nM and this resulted in Ca^{2+} mobilization in HEK-293 cells expressing RXFP4 and Gα16 protein (3). *Insl5*-deficient mice displayed impaired fertility in males and females as a result of a marked reduction in sperm motility and alterations in the estrus cycle. Intriguingly, *Insl5*-deficient mice also had impaired glucose homeostasis and elevated serum glucose levels in aging mice (29). This glucose intolerance resulted from reduced insulin secretion and coincided with markedly reduced average islet size and β-cell mass in *Insl5*-deficient mice. This suggests a novel signaling role of the Insl5-RXFP4 system by affecting the balance of pancreatic α-cells to β-cells in favor of the former cell type, resulting in altered regulation of insulin secretion and β-cell homeostasis. It is clear from these data that the INSL5-RXFP4 pathway is emerging as a novel autocrine/paracrine mucosal and systemic regulatory signaling system with intriguing potential to impact on important physiological/metabolic functions within the neurogastrointestinal endocrine network.

The large intestine is a site of inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) and is associated with changes in gut sensation, motility and secretion. Patients suffering from IBD also have a higher risk of developing neuroendocrine tumours (NETs) (18). IBD is associated with alterations in the number of EECs, and complete lack of mucosal EECs (anendocrinosis) as a result of a point mutation in Ngn3, the key transcription factor for enteroendocrine lineage differentiation, causes life-threatening malabsorptive congenital diarrhea syndrome (39-41). Increased levels of EECs have been reported in human Crohn's ileitis (42) and in animal models of colitis (43-45). To address whether INSL5$^+$ EECs may be affected by acute IBD, we chose 2 mouse models of dextran sodium sulphate (DSS)- and dinitrobenzenesulphonic acid (DNBS)-induced colitis. DSS induced IBD symptoms resembling UC and DNBS induced Crohn's-like pathology 5 days after the application of these compounds. The presence of mucosal INSL5$^+$ EECs was confirmed at inflammatory sites but their number remained unchanged when compared to normal colon in the untreated animals. Thus, the acute phase of DSS- and DNBS-induced IBD did not coincide with changes in the number of Insl5$^+$ EECs at the inflammatory sites. The Insl5-immunopositive EECs behaved similar to enteroendocrine cell populations reported to be immunopositive for cholecystokinin, glucagon-like peptide 2, glucose-dependent insulinotropic peptide and peptide YY which also showed no significant numerical change in experimental colitis (45). This is in contrast to increased numbers of neurotensin, somatostatin and serotonin immunopositive EECs observed in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in guinea pigs and DSS- and DNBS-induced colitis in mice (45,46). Additional studies will reveal whether the number of Insl5-immunopositive EECs is affected during the chronic phase of IBD.

Here, we provide the first report on the presence of human INSL5 and RXFP4 in carcinoid tumours. All neuroendocrine/carcinoid tumours showed aggressive invasive behavior and were immunopositive for the neuroendocrine markers synaptophysin and/or chromogranin A. Carcinoid cells co-expressed immunopositive INSL5 and synaptophysin and the presence of RXFP4 suggests a possible autocrine/paracrine INSL5-RXFP4 signaling system active within human carcinoid tissues. Recent evidence from relaxin 2, INSL3 and their receptors RXFP1 and RXFP2, respectively, have shown that members of the relaxin-like peptide family engage in similar local signaling systems to promote growth, survival and migration/invasion of tumour cells (34,47-49). The characterization of essential structural and signaling parameters of the INSL5-RXFP4 interaction (50,51) may provide the basis to explore molecular mechanisms engaged by the INSL5-RXFP4 system in cancer.

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