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INSL5 activates multiple signalling pathways and regulates GLP-1 secretion in NCI-H716 cells

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Abstract

Insulin-like peptide 5 (INSL5) is a newly discovered gut hormone expressed in colonic enteroendocrine L-cells but little is known about its biological function. Here, we show using RT-qPCR and *in situ* hybridisation that *Ins5* mRNA is highly expressed in the mouse colonic mucosa, colocalised with proglucagon immunoreactivity. In comparison, mRNA for RXFP4 (the cognate receptor for INSL5) is expressed in various mouse tissues, including the intestinal tract. We show that the human enteroendocrine L-cell model NCI-H716 cell line, and goblet-like colorectal cell lines SW1463 and LS513 endogenously express *RXFP4*. Stimulation of NCI-H716 cells with INSL5 produced phosphorylation of ERK1/2 (Thr²⁰²/Tyr²⁰⁴), AKT (Thr³⁰⁸ and Ser⁴⁷³) and S6RP (Ser^{235/236}) and inhibited cAMP production but did not stimulate Ca²⁺ release. Acute INSL5 treatment had no effect on GLP-1 secretion mediated by carbachol or insulin, but modestly inhibited forskolin-stimulated GLP-1 secretion in NCI-H716 cells. However, chronic INSL5 pre-treatment (18 h) increased basal GLP-1 secretion and prevented the inhibitory effect of acute INSL5 administration. LS513 cells were found to be unresponsive to INSL5 despite expressing *RXFP4*. Another enteroendocrine L-cell model, mouse GLUTag cells did not express detectable levels of *Rxfrp4* and were unresponsive to INSL5. This study provides novel insights into possible autocrine/paracrine roles of INSL5 in the intestinal tract.

Key Words

- ▶ INSL5
- ▶ RXFP4
- ▶ NCI-H716
- ▶ cell signalling
- ▶ GLP-1

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Introduction

Insulin-like peptide 5 (INSL5) is a novel gastrointestinal (GI) hormone identified in colonic enteroendocrine L-cells along with glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) (Grosse et al. 2014). The cognate receptor for INSL5 is the relaxin family peptide receptor 4 (RXFP4), a G protein-coupled receptor (GPCR) that couples to inhibitory G $\alpha_{i/o}$ proteins (Liu et al. 2003, 2005) and activates a number of signalling pathways including phosphorylation of extracellular signal-regulated protein

kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinases (p38MAPK), protein kinase B (AKT) and S6 ribosomal protein (S6RP) (Ang et al. 2017). INSL5 activates RXFP4 but not the other relaxin family peptide receptors, RXFP1–3 (Halls et al. 2015). Previous transcriptomic and *in situ* hybridisation studies indicated that, like *INSL5*, *RXFP4* is predominantly expressed in the GI tract i.e. colon and rectum (Yue et al. 2014, Uhlén et al. 2015), and is localised in regions that resemble the colonic

submucosal and myenteric plexuses (Grosse et al. 2014). Furthermore, RXFP4 immunoreactivity has been reported in normal human mucosa and human neuroendocrine tumours (NETs) (Mashima et al. 2013, Thanasupawat et al. 2013) arising from enteroendocrine cells (Gunawardene et al. 2011).

The physiological role of INSL5 is still unclear, but it was reported to be an orexigenic peptide upregulated during periods of energy deprivation and suggested to act via the enteric nervous system (Grosse et al. 2014). Most notably, exogenous administration of INSL5 stimulated feeding in wild type but not in *Rxfp4*-deficient mice. In addition, INSL5 appears to play a role in glucose metabolism with suggested actions on pancreatic β -cell insulin secretion (Burnicka-Turek et al. 2012, Luo et al. 2015) and hepatic glucose production (Lee et al. 2016). Furthermore, INSL5 reportedly stimulates ERK1/2 phosphorylation and promotes GLP-1 secretion in murine L-cell model GLUTag cell line (Luo et al. 2015), and *RXFP4* is expressed in a number of mucin 2 (*MUC2*)-expressing, goblet-like human colorectal carcinoma cell lines (Mouradov et al. 2014). These findings suggest that INSL5 secreted from colonic L-cells is likely to act in an autocrine/paracrine manner on locally expressed RXFP4 to regulate gut functions such as peptide secretion from enteroendocrine cells and/or mucin secretion from goblet cells.

This study aims to elucidate the expression patterns of *Insl5* and *Rxfp4* in various mouse tissues and investigate whether INSL5 can stimulate signalling in enteroendocrine L-cells and/or goblet cells using various cell culture models, which would provide evidence for an autocrine/paracrine action of this gut hormone. We confirm that *Insl5* is a product of L-cells in the colon while *Rxfp4* expression was detected at low levels in various tissues, including the intestinal tract. In addition, we report that the human enteroendocrine L-cell model NCI-H716 cell line, and goblet-like colorectal cell lines LS513 and SW1463, endogenously express *RXFP4*. Importantly, in NCI-H716 cells, INSL5 stimulates signalling and regulates GLP-1 secretion, suggesting a potential novel biological role for INSL5 in mediating L-cell function.

Materials and methods

Mouse and tissue preparation

129SvEv wild-type (WT) and RXFP4-deficient (*Rxfp4*^{-/-}) mice were obtained from Dr Johannes Grosse (Takeda Cambridge Limited, Cambridge, UK) (Grosse et al. 2014). WT and *Rxfp4*^{-/-} mice were genotyped before use

(Transnetyx, Cordova, TN, USA). C57BL/6J mice were obtained from the Monash Animal Research Platform. All mice were bred and maintained at the Monash University (Parkville) animal housing facility. Tissues for RT-qPCR and *in situ* hybridisation experiments were from C57BL/6J and 129SvEv mice, respectively. The protocol used was approved by the Monash University Animal Ethics Committee (MIPS.2015.14) and conforms to the Australian Code for the Care and Use of Animals for Scientific Purposes. Male or female mice (12–16-week-old) were killed by CO₂ inhalation and cervical dislocation, and tissues of interest dissected and frozen for RNA extractions or ileum and colon dissected and fat removed, fixed overnight in 4% (w/v) paraformaldehyde and cryoprotected with 30% (w/v) sucrose in diethyl pyrocarbonate-treated phosphate buffered saline (DEPC-PBS) for tissue staining. Fixed tissues were frozen in cryomoulds in Tissue-Tek (OCT, VWR International, QLD, Australia) and stored at –80°C until sectioning.

Cell culture

Human NCI-H716, LS513 and SW1463 cells were purchased from American Type Culture Collection (Manassas, VA, USA). GLUTag cells (Brubaker et al. 1998) were a kind gift from Professor Fiona M. Gribble (University of Cambridge, UK) with permission from Prof. Daniel J Drucker (University of Toronto, Canada). NCI-H716 cells were grown in suspension and maintained in complete media consisting of RPMI-1640 (Thermo Fisher Scientific) with 5% foetal bovine serum (FBS). GLUTag cells were grown in high glucose DMEM (Thermo Fisher Scientific) with 10% (v/v) FBS, and LS513 and SW1463 cells in high glucose DMEM with 5% (v/v) FBS. Cells were incubated at 37°C in a humidified chamber containing 5% CO₂. Cells were seeded onto multi-well plates and grown in complete media for 48 h before experimentation. NCI-H716 cells were plated onto multi-well plates coated with Cultrex basement matrix extract (Trevigen, Gaithersburg, MD, USA) at 100 µg/mL to allow cell adhesion.

RNA isolation and cDNA synthesis

Total RNA was isolated from mouse tissues, and cell lines using RNeasy mini RNA purification kit (Qiagen) and DNase-treated using the Ambion DNA-free DNase kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesised from 500 ng RNA in a 10 µL reverse transcriptase reaction using iScript Reverse Transcription Supermix kit for RT-qPCR (Bio-Rad).

qPCR

Quantitative polymerase chain reaction (qPCR) was performed as described previously (Ang et al. 2017) using a Mastercycler ep Realplex machine (Eppendorf, Hamburg, Germany) with the following TaqMan primers and probes (Thermo Fisher Scientific): Mouse – *Ins15*: Mm04210319_m1, *Rxfp4*: Mm00731536_s1, *Gcg*: Mm00801714_m1, *Glp1r*: Mm00445292, *Actb*: Mm02619580_g1; Human – *INSL5*: Hs00193884_m1, *RXFP4*: Hs00704281_s1, *GCG*: Hs01031536_m1, *GLPIR*: Hs00157705_m1, *ACTB*: Hs01060665_g1. After denaturation at 95°C for 10 min, fluorescence was measured over 40 cycles (95°C for 15 s, 60°C for 2 min).

Generation of *in situ* hybridisation probes

Digoxigenin (DIG)-labelled *Ins15* and *Rxfp4* *in situ* hybridisation probes were generated by *in vitro* transcription (Supplementary methods, see section on Supplementary data given at the end of this article).

Combined *in situ* hybridisation and immunofluorescence

For detailed protocol, see Supplementary methods. Briefly, 12 µm sections of mouse colon and ileum were cut using a Leica CM3050 cryostat and mounted on Superfrost Plus slides (Thermo Fisher Scientific) and allowed to air-dry for an hour. Sections were hybridised with DIG-labelled *Ins15* or *Rxfp4* probe (1:100 each) overnight at 60°C, washed and incubated with horseradish peroxidase (POD, 1:500, Roche) conjugated anti-DIG antibody before tyramide signal amplification with FITC-tyramide. Following *in situ* hybridisation, sections were blocked (2% BSA, 0.1% (v/v) Tween-20 in PBS), incubated with rabbit anti-proglucagon antibody (1:100, ab8055, Abcam) overnight, washed with TBST and incubated with anti-rabbit Alexa594 secondary antibody (1:500, Thermo Fisher Scientific). Fluorescent wide-field images were acquired by tile-scanning using a Nikon Ti-E fluorescence microscope with a 20× objective and photo-stitched during acquisition in NIS-Elements software (Nikon Instruments).

Alphascreen protein phosphorylation

Phosphorylation of ERK1/2 (Thr²⁰²/Tyr²⁰⁴), AKT (Thr³⁰⁸ and Ser⁴⁷³) and S6RP (Ser^{235/236}) was measured using Alphascreen SureFire kits (Perkin Elmer). GLUTag and LS513 cells were seeded onto 96-well plates and

NCI-H716 cells were prepared in 24-well matrix-coated plates. On the day of experiment, cells were serum-starved for 4–6 h in low glucose (1 g/L) DMEM to reduce basal phosphorylation levels, and then stimulated with human INSL5 (100 nM) for up to 60 min in time course studies or with increasing concentrations of peptides (10^{−10}–10^{−6.5} M) in concentration-response studies at 5 min (p-ERK1/2), 15 min (p-AKT) and 30 min (p-S6RP). At the end of INSL5 stimulation, cells were lysed and protein phosphorylation was measured as described previously (Ang et al. 2017).

Immunoblotting

All primary and secondary antibodies were from Cell Signalling Technology. NCI-H716 cells were grown in a 12-well plate and stimulated with INSL5 (100 nM) for up to 60 min and lysed with 80 µL of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.001% bromophenol blue and 5% β-mercaptoethanol). Cell lysates were transferred to microcentrifuge tubes, sonicated for 10–15 s to reduce viscosity and boiled at 95°C for 5 min to denature proteins. Protein samples were loaded onto 10% polyacrylamide gels and separated at 100 V for 2 h and electrotransferred to a polyvinylidene difluoride membrane (0.45 µm, Bio-Rad). Membranes were blocked for 2 h with 5% BSA in TBST and incubated overnight at 4°C with phospho-ERK1/2 (1:1000; #9101) or phospho-AKT Ser⁴⁷³ (1:1000; #9271) rabbit primary antibodies. The following day, membranes were washed 5 times with TBST, incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000; #7074) at room temperature for 2 h and immunoblots detected with enhanced chemiluminescence (ECL) using ChemiDoc Imaging System (Bio-Rad). The membranes were then stripped with stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 µM β-mercaptoethanol) for 30 min at 60°C, washed 5 times with TBST, re-probed with rabbit primary antibodies against total ERK1/2 (1:1000; #9102) or total AKT (1:1000; #9272) and immunoblots detected as before using the ECL detection method.

Inhibition of cAMP accumulation

Inhibition of forskolin-stimulated cAMP accumulation in NCI-H716 cells was measured using a LANCE cAMP kit (Perkin Elmer). Cells were seeded in 96-well plates and on the day of experiment, incubated in HBSS (Thermo Fisher Scientific) supplemented with 0.5 mM IBMX, 5 mM HEPES and 0.1% BSA. INSL5 (10^{−11}–10^{−6.5} M) was added

for 15 min followed by stimulation with forskolin (3 μ M) for 30 min. Cells were lysed in ice-cold absolute ethanol and air-dried. Pellets containing cAMP were solubilised in 0.3% (v/v) Tween-20, 5 mM HEPES and 0.1% (w/v) BSA, and cAMP in the solute was measured according to the manufacturer's protocol.

Intracellular Ca²⁺ mobilisation

NCI-H716 cells were prepared in a 96-well plate, loaded with the Ca²⁺ indicator Fluo-4 AM and real-time intracellular Ca²⁺ mobilisation during INSL5 stimulation measured on a FlexStation (Molecular Devices, Sunnyvale, CA, USA) as previously described (Ang et al. 2017).

GLP-1 secretion

NCI-H716 cells were grown overnight in complete RPMI media in 96-well plates and serum-starved with vehicle (0.02% DMSO) or INSL5 (100 nM) 18 h prior to experimentation. On the day of experiment, cells were washed with PBS and incubated in secretion buffer (115 mM NaCl, 5 mM KCl, 24 mM NaHCO₃, 1 mM MgCl₂, 2.5 mM CaCl₂, 25 mM HEPES, 5 mM glucose) and vehicle (0.02% DMSO) or INSL5 (100 nM) added for 15 min, followed by vehicle (0.04% DMSO) or secretagogues (100 μ M carbachol, 100 nM insulin or 3 μ M forskolin) for 90 min (Supplementary Fig. 1). 50 μ M DPP4 inhibitor (DPP4-010, EMD Millipore) was included in the secretion buffer to prevent GLP-1 degradation. Media was collected and GLP-1 content was measured using an active GLP-1 HTRF kit (Cisbio, Codolet, France) according to the manufacturer's protocol.

Data and statistical analysis

For RT-qPCR, data were expressed as the relative abundance of the gene of interest relative to the reference gene, β -actin (*ACTB*), using the following equation:

$$2^{-\Delta Ct} \times 1000$$

where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{ACTB}}$.

For Alphascreen protein phosphorylation assays, data were expressed as a % of untreated (basal) response in the time course study or % vehicle response in the concentration-response study, for inhibition of cAMP accumulation as % forskolin (3 μ M) response, for calcium mobilisation as % basal fluorescence intensity prior to ligand addition, and for GLP-1 secretion assay as picomolar

concentration (pM). All graphs were plotted using Prism (v7.0) (GraphPad Software) and concentration-response curves fitted using a three-parameter Hill equation within Prism. INSL5 protein phosphorylation in the Alphascreen time course study was statistically compared to the untreated (basal) response using two-way ANOVA followed by Sidak's multiple comparisons, while the GLP-1 response stimulated by various ligands was statistically compared to the untreated (basal) response using one-way ANOVA followed by Sidak's multiple comparisons test.

Peptides and drugs

Lyophilized human INSL5 was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA) and reconstituted. Human insulin (I9278), carbachol (C4382) and forskolin (F6886) were from Sigma-Aldrich.

Results

Expression patterns of *Insl5* and *Rxfp4* in mouse tissues

Since metabolic phenotypes have been reported in mice with genetic ablation of *Insl5* (Burnicka-Turek et al. 2012) or *Rxfp4* (Grosse et al. 2014), we investigated mRNA expression patterns of *Insl5* and *Rxfp4* by RT-qPCR in the following metabolically relevant mouse tissues: skeletal muscle (hind limb), heart, pancreas, brown adipose tissue (BAT), inguinal adipose tissue (iWAT), epididymal adipose tissue (eWAT), ileum, colon and rectum. *Insl5* expression was the highest in colonic mucosa followed by submucosa but was undetectable in external muscle (Fig. 1). *Insl5* expression was undetectable in other regions that include ileum, skeletal muscle, heart, pancreas, BAT, iWAT and eWAT. By contrast, the proglucagon mRNA *Gcg* was highly expressed, as expected, in pancreas where glucagon is synthesised, and also found in ileum, colon and rectum, all regions where GLP-1 is produced (Fig. 1). Using combined *in situ* hybridisation and immunohistochemistry, *Insl5* mRNA was localised in mouse colon to discrete cells that were also immunopositive for the GLP-1 precursor proglucagon (Fig. 2), in agreement with previous studies (Grosse et al. 2014, Lee et al. 2016). In line with our RT-qPCR data, no *Insl5* mRNA was detected by *in situ* hybridisation in mouse ileum, whereas proglucagon immunoreactivity was still observed (Supplementary Fig. 2).

While *Insl5* expression was confined to the mouse colorectum, its receptor *Rxfp4* had a more general tissue expression pattern with transcripts detected at relatively low levels across all the tissues investigated. *Rxfp4*

Figure 1

Expression pattern of *InsI5* mRNA in various mouse tissues, as determined by RT-qPCR using Taqman probes. Bars represent mean $\pm$ S.E.M. of 3–6 samples, except $n=2$ for colonic external muscle.

expression was the highest in pancreas and was also detected in heart, skeletal muscle, rectum and all layers of ileum and colon (Fig. 3). In comparison, *Glp1r* was expressed abundantly in the pancreas, in line with known expression in insulin-producing β -cells (MacDonald et al. 2002). Consistent with the relatively poor tissue expression in mouse, we found the *Rxfp4* *in situ* hybridisation signal weak and extremely difficult to detect in the colon. There was a faint perinuclear *Rxfp4* signal that colocalised with proglucagon immunoreactivity in mouse colonic mucosa (Fig. 4), suggesting that *Rxfp4* may be discretely expressed in a subset of colonic cells such as enteroendocrine

Figure 3

Rxfp4 mRNA expression in various mouse tissues as determined by RT-qPCR using Taqman probes. Bars represent mean $\pm$ S.E.M. of 3–6 samples, except $n=2$ for colonic external muscle.

L-cells. Unlike previously reports, we were not able to detect RXFP4 signals in enteric neurons using the *in situ* hybridisation approach.

RXFP4 is expressed in human L-cell model NCI-H716 cell line and goblet-like colorectal cell lines LS513 and SW1463, but not in murine L-cell model GLUTag cell line

Our RT-qPCR data, together with transcriptomic data published by others (Yue et al. 2014, Uhlén et al. 2015), indicate that RXFP4 is expressed in the GI tract. Thus, we focused our attention on cell lines with GI origin to investigate whether they express RXFP4 and could signal in response to INSL5. Moreover, INSL5 was recently shown to promote phosphorylation of ERK1/2 and to stimulate GLP-1 secretion in GLUTag cells (Luo et al. 2015). GLUTag cells are a murine model of enteroendocrine L-cells originally derived from a colonic tumour developed from transgenic mice expressing SV40 large T antigen under the control of the proglucagon promoter (Drucker et al. 1994, Gil-Lozano & Brubaker 2015) and are widely used to study GLP-1 release in response to a variety of L-cell stimuli (Lan et al. 2012, Parker et al. 2012, Gagnon et al. 2015, Luo et al. 2015). Here, using RT-qPCR, *Rxfp4* mRNA was surprisingly undetectable in GLUTag cells. In contrast, *InsI5* and *Gcg* were readily detected, with *Gcg* expressed at a higher level than *InsI5* (Fig. 5A). This supports a previous microarray gene expression study of GLUTag cells that also found no *Rxfp4* expression (Trabelsi et al. 2015). In contrast, NCI-H716 cells, a commonly used human model of enteroendocrine L-cells (Reimer et al. 2001, Jang et al. 2007, Lauffer et al. 2009, Lim et al. 2009) originally derived from ascites fluid of a patient with caecal adenocarcinoma (Park et al. 1987, Gagnon & Brubaker 2015), expressed reasonably high levels of

Figure 2

In situ hybridisation using mouse *InsI5* anti-sense probe revealed colocalisation of *InsI5* and proglucagon immunoreactivity in mouse colon. Scale bar = 100 μ m; inset scale bar = 10 μ m.

Figure 4

Localisation of *Rxfp4* in mouse colon. Top panel: *In situ* hybridisation using *Rxfp4* anti-sense probe revealed a weak *Rxfp4* signal that colocalised with discrete (arrowhead) but not all (arrow) proglucagon immunoreactive cells of the colon of WT mouse. Bottom panel: *Rxfp4* was not detected in proglucagon immunoreactive cells (arrow) of the colon of *Rxfp4*^{-/-} mouse. Scale bar = 50 μm, inset scale bar = 10 μm.

RXFP4. NCI-H716 also expressed *GCG* abundantly but *INSL5* poorly (Fig. 5B). Two goblet-like cell lines, LS513 and SW1463, both derived from patients with Duke's type C mucin-secreting caecal tumour (Leibovitz et al. 1976, Suardet et al. 1992), also expressed reasonably high levels of *RXFP4*, whereas *INSL5* expression was undetectable in LS513 and poor in SW1463 (Fig. 5 C and D). LS513 cells have been used as models to demonstrate protective roles of mucins in epithelial cells (Gustafsson et al. 2013, Sheng et al. 2013, 2017), whereas SW1463 cells are regularly used as a cell model for colorectal cancer (Singh et al. 2012, Pinto et al. 2016, Emons et al. 2017).

INSL5 stimulates phosphorylation of ERK1/2, AKT and S6RP and inhibits cAMP production in NCI-H716 cells

Since NCI-H716 and LS513 endogenously expressed *RXFP4*, we performed pharmacological studies on these cells using the cognate ligand for human *RXFP4*, human *INSL5*. In a recombinant cell system stably expressing human *RXFP4*, *INSL5* promoted phosphorylation of ERK1/2, AKT and S6RP and inhibited cAMP production (Ang et al. 2017). ERK1/2 and AKT pathways are widely implicated in cellular proliferation, differentiation, growth and survival (Manning & Cantley 2007, Turjanski et al. 2007), while S6RP is involved in protein synthesis (Ruvinsky & Meyuhav 2006). In time course experiments, exposure of NCI-H716 cells to *INSL5* (100 nM) for up to 60 min produced phosphorylation of ERK1/2 (Thr²⁰²/Tyr²⁰⁴), AKT (Thr³⁰⁸ and Ser⁴⁷³) and S6RP Ser^{235/236}. Maximum ERK1/2 phosphorylation occurred at 5 min, AKT (Thr³⁰⁸ and Ser⁴⁷³) phosphorylation at 15 min and S6RP phosphorylation at 30 min (Fig. 6A, B, C and D). *INSL5* concentration-response relationships in NCI-H716 cells were constructed at time points corresponding to

maximum protein phosphorylation. *INSL5* (0.1–300 nM) caused concentration-dependent phosphorylation of ERK1/2 (Fig. 6E), AKT (Thr³⁰⁸ and Ser⁴⁷³; Fig. 6F and G) and S6RP (Fig. 6H) with pEC₅₀ values ranging from 7.9 to 8.4. In addition, immunoblots for ERK1/2 and AKT (Ser⁴⁷³) performed in NCI-H716 cells following *INSL5* stimulation (100 nM), showed similar phosphorylation patterns (Fig. 6I and J) to those observed in the Alphascreen time course assays. Exposure of NCI-H716 cells to human *INSL5* (0.1–300 nM) also produced concentration-dependent inhibition of cAMP accumulation in response to forskolin (3 μM) (pEC₅₀ = 8.0 ± 0.3; Fig. 6K) reflecting activation of Gα_{i/o}-coupled *RXFP4*. Since Ca²⁺ mobilisation is an important mechanism leading to GLP-1 secretion (for review see Reimann et al. 2012), the effect of *INSL5* on

Figure 5

RXFP4, *INSL5*, *GLP1R* and *GCG* gene expression as determined by RT-qPCR using TaqMan probes in (A) mouse GLUTag L-cells, (B) human NCI-H716 L-cells and goblet-like human colorectal cell lines (C) LS513 and (D) SW1463. Bars represent mean ± s.e.m. n.d., not determined. –RT: qPCR performed on RNA without reverse transcription. A full colour version of this figure is available at <https://doi.org/10.1530/JME-17-0152>.

Figure 6

Signalling pathways activated by INSL5 in NCI-H716 cells. NCI-H716 cells were stimulated with INSL5 (100 nM) at various time points or with increasing concentrations of INSL5 (0.1–300 nM) and assessed for protein phosphorylation using Alphascreen assays. INSL5 (100 nM) stimulated phosphorylation of (A, E) ERK1/2 ($n=5$), (B, F) AKT Ser⁴⁷³ ($n=5$), (C, G) AKT Thr³⁰⁸ ($n=5$) and (D, H) S6RP Ser^{235/236} ($n=5$) in a time- and concentration-dependent manner. Blue bars indicate phosphorylation stimulated by human insulin (100 nM). * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; **** $P < 0.0001$ vs control, two-way repeated measures ANOVA with Sidak's multiple comparisons test. (I, J) Representative immunoblots showing phosphorylation patterns of ERK1/2 and AKT Ser⁴⁷³ in NCI-H716 cells treated with INSL5 (100 nM). (K) Effect of INSL5 on cAMP production. (L) Lack of effect of INSL5 up to 100 nM on intracellular Ca²⁺ mobilisation ($n=3$). Green bar represents carbachol response (Cch, 30 μ M, positive control). Data points and bars represent mean $\pm$ s.e.m. of independent experiments. A full colour version of this figure is available at <https://doi.org/10.1530/JME-17-0152>.

intracellular Ca²⁺ mobilisation was also investigated. INSL5 treatment up to 100 nM failed to stimulate a detectable Ca²⁺ response, whereas the muscarinic acetylcholine receptor agonist carbachol (10⁻⁴ M) caused a robust Ca²⁺ response (Fig. 6L).

Intriguingly, we were unable to observe INSL5 signalling in LS513 cells (Supplementary Fig. 3A, B, C, D and E), even though *RXFP4* was clearly expressed in these

cells (Fig. 5C). In addition, we were unable to adequately perform further pharmacological studies in SW1463 due to the poor growth of this cell line. Consistent with a lack of *Rxfp4* expression in GLUTag cells, treatment with mouse INSL5 (100 nM) over 60 min, failed to produce phosphorylation of AKT (Thr³⁰⁸ or Ser⁴⁷³) or S6RP Ser^{235/236} (Supplementary Fig. 4) both signalling events associated with RXFP4 activation (Ang et al. 2017). In contrast,

insulin (100nM) stimulated a robust AKT and S6RP response in the same cells.

Effects of acute and chronic administration of INSL5 on GLP-1 secretion from NCI-H716 cells

NCI-H716 is a model of human enteroendocrine L-cells, and as such we investigated the effects of INSL5 on GLP-1 secretion (Fig. 7). In cells exposed to chronic (18 h) vehicle, acute INSL5 treatment (100nM) had no significant effect on basal, carbachol or insulin-stimulated GLP-1 secretion but significantly inhibited forskolin-stimulated GLP-1 secretion ($P < 0.01$). However, in cells exposed to chronic (18 h) INSL5, acute INSL5 treatment had no effect on basal secretion and failed to inhibit forskolin-stimulated GLP-1 secretion, likely due to RXFP4 desensitisation by chronic INSL5 pre-treatment (Ang et al. 2017) or possibly downregulation of signalling effectors. Interestingly, cells chronically pre-treated with INSL5 displayed an increase in basal GLP-1 secretion ($P < 0.001$). This was not due to increased synthesis of GLP-1 precursor peptide proglucagon, since proglucagon peptide content remained unchanged in these cells (Supplementary Fig. 5A) or by an increased number of cells (Supplementary Fig. 5B).

Figure 7

Effect of INSL5 on GLP-1 secretion from NCI-H716 cells. NCI-H716 cells were prepared as described in materials and methods. 18 h before the start of GLP-1 secretion, cells were chronically pre-incubated in serum-free media in the presence of vehicle (0.02% DMSO) or INSL5 (100 nM). On the day of experiment, cells were washed and acutely stimulated with vehicle (0.02% DMSO) or INSL5 (100 nM) for 15 min followed by co-treatment with either vehicle (Veh; 0.04% DMSO) or the GLP-1 secretagogues carbachol (Cch, 100 μ M), insulin (Ins, 100 nM) or forskolin (Fsk, 3 μ M) for 90 min, and media assayed for active GLP-1. ** $P < 0.01$; **** $P < 0.0001$ vs acute vehicle response in cells chronically pre-treated with vehicle. **** $P < 0.0001$ vs acute vehicle response in cells chronically pre-treated with INSL5. # $P < 0.01$ vs forskolin treatment alone; ns, non-significant. One-way repeated measures ANOVA with Sidak's multiple comparisons test. Bars represent mean $\pm$ s.e.m. of $n = 6$ independent experiments. A full colour version of this figure is available at <https://doi.org/10.1530/JME-17-0152>.

Discussion

In this study, we demonstrate that *Insl5* mRNA is expressed mainly in the mouse colonic mucosa in discrete cells that are also immunopositive for the GLP-1 precursor proglucagon, confirming *Insl5* expression in enteroendocrine L-cells (Grosse et al. 2014, Lee et al. 2016). Indeed, a similar co-expression pattern was observed in the human colon (Grosse et al. 2014) suggesting that INSL5 likely has important biological functions across different species. By comparison, we found *Rxfp4* expression in mouse tissues to be weaker, less region specific and more difficult to detect than *Insl5*. We were unable to detect *Rxfp4* in enteric neurons by *in situ* hybridisation, unlike previous reports (Grosse et al. 2014). The disparity may reflect the different hybridisation strategies employed, and different sensitivities between fluorescent (present study) and chromogenic detection methods. Other approaches such as RNAscope, *Rxfp4* Cre reporter mouse line or receptor autoradiography would be useful to clarify the expression and localisation of RXFP4 in native tissues.

Although expression of *Rxfp4* is detectable in mouse pancreas, the level is only 4% of that seen in NCI-H716 cells and less than 0.3% of that seen for *Glp1r* in pancreas. These data are consistent with a number of RNA sequencing studies on both mouse and human pancreatic islets. In 4–6 independent preparations of mouse freshly isolated primary α , β and δ cells, *Rxfp4* has FPKM values ranging from 0.0 to 0.26 (average 0.04) (Adriaenssens et al. 2016). These values are negligible compared to those for GPCRs known to be important in islet function, for example, an average FPKM of 207 for *Glp1r* and 9.0 for *Sstr3* in mouse purified β cells. Moreover, we previously showed that the mouse insulinoma β -cell line MIN6 do not express *Rxfp4* (Ang et al. 2017). Similarly, human whole pancreatic islets display no detectable *RXFP4* transcripts (Eizirik et al. 2012), and 11 preparations of human FACS-purified β -cells show negligible RPKM values ranging from 0.0 to 0.11 (Nica et al. 2013). An FPKM or RPKM value greater than 1.0 is considered significant in samples of RNA derived from homogeneous cell populations (Hebenstreit et al. 2011, Adriaenssens et al. 2016), and in the same human preparations, the RPKM values for *GLP1R* ranged from 25.7 to 73.2 and those for *SSTR3* ranged from 7.7 to 21.8. Thus, it is highly unlikely that pancreatic islets in mouse or human are a physiologically relevant target for INSL5 action.

Transcriptomic analyses by others have revealed a slightly different pattern of *RXFP4* expression between human and mouse tissues. Whereas in human, *RXFP4* is expressed in duodenum, small intestine, colon and rectum

but not in lung, in mouse *Rxfp4* is expressed in colon and lung but not in duodenum or small intestine (Yue et al. 2014, Uhlén et al. 2015) (Supplementary Fig. 6). It is also noteworthy that the RXFP4 amino acid sequence is highly conserved in human, monkey (97% homology), cow (85% homology) and pig (87% homology) but less so in mouse (74% homology), and *Rxfp4* is a pseudogene in rat (Chen et al. 2005). Thus, RXFP4 may have different and perhaps more evolved biological functions in humans compared to mice. This also highlights the importance of using a human model such as NCI-H716 cells when studying the biological functions of INSL5 acting on RXFP4.

The effects of INSL5 signalling were examined on GLP-1 release from NCI-H716 cells. In general, we found that the response window for GLP-1 secretion in NCI-H716 cells was small but detectable, in agreement with a recent study showing a weak but comparable GLP-1 response in the same cells (Kuhre et al. 2016). In the current study, acute INSL5 treatment had no significant effect on basal GLP-1 release or on release in response to the secretagogues carbachol or insulin, but INSL5 did inhibit GLP-1 release in response to the adenylyl cyclase activator forskolin. Inhibition of GLP-1 secretion by INSL5 is consistent with activation of $G_{\alpha_{i/o}}$ -coupled RXFP4, inhibition of adenylyl cyclase activity and a decrease in cAMP production (Ang et al. 2017), since cAMP is upstream of GLP-1 secretion in L-cells (Reimann et al. 2012). For instance, stimulation of either the $G_{\alpha_{i/o}}$ -coupled somatostatin receptor SSTR5 or the galanin receptor GALR1 inhibits GLP-1 secretion (Chisholm & Greenberg 2002, Psichas et al. 2016).

Physiologically, the INSL5-RXFP4 system may constitute an L-cell feedback mechanism that inhibits GLP-1 secretion at times when the hormone is not required i.e. during the fasting state and/or at the end of the fed-state when the glucose-lowering action of GLP-1 is no longer required. Consistent with this notion, plasma INSL5 levels peak during fasting and are suppressed following food intake (Grosse et al. 2014), a trend that opposes that of GLP-1 (Hornnes et al. 1980, Ørskov et al. 1996). The inhibitory action of INSL5 on GLP-1 secretion suggests a possible autocrine/paracrine mechanism resembling that of somatostatin (Chisholm & Greenberg, 2002). Nevertheless, the inhibition of GLP-1 secretion by INSL5 is modest in NCI-H716 cells (~30% inhibition of forskolin-induced GLP-1 release). Given that regulation of GLP-1 release in L-cells is complex and governed by multiple signalling mechanisms including membrane depolarisation and Ca^{2+} influx, as well as cAMP (Reimann et al. 2006), it is possible that the inhibitory effect of INSL5 on GLP-1 release in this system may be less significant than that observed in

a physiological setting. In addition, the mechanisms that control INSL5 release – whether by neural, endocrine, paracrine and/or autocrine pathways – are still unknown.

Interestingly, chronic INSL5 pre-treatment resulted in a somewhat unexpected increase in basal GLP-1 secretion that could not be explained by an increase in proglucagon content. One speculative mechanism for this observation is a phenomenon known as heterologous sensitisation of adenylyl cyclase by $G_{i/o}$ -coupled receptors (see review by Brust et al. 2015). Indeed, a number of $G_{i/o}$ -coupled receptors including the δ -opioid receptor, μ -opioid receptor and dopamine D_2 receptor display a paradoxical enhancement of basal and/or Gs-mediated cAMP production following prolonged agonist stimulation (Watts et al. 2001, Clark et al. 2004, Zhang et al. 2006). Therefore, it is possible that prolonged INSL5 stimulation could lead to heterologous sensitisation of adenylyl cyclase to produce enhanced cAMP signalling in NCI-H716 cells, and hence, a resultant increase in GLP-1 secretion. In addition, as demonstrated in this study, INSL5 activates ERK1/2 and AKT signalling pathways both of which have been implicated in stimulation of GLP-1 secretion from L-cells (Lim et al. 2009, Gagnon et al. 2015). Thus, we speculate that acute INSL5 treatment inhibits GLP-1 secretion by coupling to $G_{i/o}$ proteins, whereas chronic INSL5 treatment potentiates the secretory capacity of L-cells by sensitisation of adenylyl cyclase and/or activation of ERK1/2 and AKT signalling pathways, i.e. INSL5 appears to play a role in mediating both short- and long-term L-cell function.

Although transformed cell lines are relatively easy to grow and maintain compared to isolated primary cells, there are caveats to be considered when using them. NCI-H716 and GLUTag are derived from intestinal carcinoma cells that may not necessarily reflect primary L-cells (Kuhre et al. 2016). Indeed, both cell lines produce and secrete high levels of GLP-1 and GLP-2 but, unlike primary L-cells, both also produce somatostatin (SST) even though SST-producing and GLP-1-producing cells are thought to have arisen from different stages of enteroendocrine lineage commitment (Egerod et al. 2012, Kuhre et al. 2016). Furthermore, NCI-H716 cells reflect neither proximal nor distal primary L-cells since they are undifferentiated in nature (Park et al. 1987) and do not produce nor secrete CCK or PYY (Kuhre et al. 2016). In addition, regulation of the proglucagon gene in these cells differs from other systems (Cao et al. 2003). In spite of these limitations, NCI-H716 cells do express high levels of the proglucagon gene *GCG* as well as *RXFP4*, but *INSL5* poorly (Fig. 5B). A more physiologically relevant model, such as mixed primary

intestinal cell culture (Reimann *et al.* 2008) would be necessary to clarify the role of INSL5 in primary L-cells.

The current study revealed no *Rxfp4* expression in GLUTag cells and no INSL5-stimulated cell signalling, unlike a previous report (Luo *et al.* 2015). Discrepancies in gene expression between GLUTag cells in different laboratories may reflect genomic and transcriptomic variability, possibly arising from differences in culture methods and conditions (Frattini *et al.* 2015). Thus, in our hands, GLUTag cells were not an appropriate model to study the role of RXFP4 signalling on GLP-1 release. Furthermore, in our hands, LS513 cells were unresponsive to INSL5 treatment even though the cells did express *RXFP4*. It may simply be a case of the mRNA being expressed but not the protein for RXFP4. Another possible explanation is that gel-forming mucus secreted from these cells may actually form a physical barrier at the cell surface that prevented INSL5 access to membrane-bound RXFP4 or even adsorbs peptide but allows small molecules to diffuse across and activate their receptors. In addition, the lack of INSL5 response may be explained by a polarised RXFP4 localisation at the basolateral membrane of LS513 cells that is inaccessible under the current conventional culture method. Indeed, bile acids stimulate GLP-1 secretion predominantly by accessing the receptor GPAR1 located at the basolateral surface of L-cells (Brighton *et al.* 2015).

In vivo, *Ins15*^{−/−} mice exhibit mild impairment in glucose metabolism compared to WT mice as demonstrated in glucose tolerance tests (Burnicka-Turek *et al.* 2012, Lee *et al.* 2016). Interestingly, however, this metabolic phenotype is not observed in *Rxfp4*^{−/−} mice (Grosse *et al.* 2014). In humans, single nucleotide polymorphisms of *RXFP4* are associated with increased body mass index (BMI) and obesity (Munro *et al.* 2012), indicating that INSL5-RXFP4 may have important roles in controlling energy balance. Here, our results provide further evidence for a metabolic function of INSL5 by showing that this peptide activates multiple signalling pathways in the human L-cell model NCI-H716 cell line and functions to regulate GLP-1 secretion. Nevertheless, a previous study reported that plasma GLP-1 levels were unaltered in *Ins15*^{−/−} mice compared to WT mice at baseline and following oral glucose administration (Burnicka-Turek *et al.* 2012). However, these mice lacked the *Ins15* gene since birth and therefore in theory could have developed compensatory mechanisms on L-cell secretory function that substitute for the loss of *Ins15*. To this end, it would be useful to investigate the effect of exogenous INSL5 administration and also the effect of switching on *Ins15* gene expression e.g. in a tetracycline inducible system in a mouse model, on plasma GLP-1 levels.

In conclusion, we have shown that (1) *Ins15* is expressed in enteroendocrine L-cells in mouse colon and its receptor *Rxfp4* is expressed in the various tissues including the GI tract and (2) INSL5 activates multiple signalling pathways in the human L-cell model NCI-H716 cell line that expresses *RXFP4* and regulates GLP-1 release differentially depending on the duration of agonist exposure. Understanding of the underlying signal transduction mechanisms stimulated by INSL5 in a biologically relevant cell culture model is a critical step in elucidating the function of this novel gut hormone.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0152>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S Y A planned and performed the experiments and wrote the manuscript. B A E provided research ideas, advised on gene expression experiments and designed hybridisation probes. D P P assisted in dissection and provided advice on immunofluorescence. R B and J D provided assistance and advice for *in situ* hybridisation. R A D B and M K provided research and manuscript ideas. D S H managed animals, assisted in dissection and provided research and manuscript ideas. R J S conceived the study, provided research ideas and wrote the manuscript.

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