**Title page**

**Comparison of human and murine enteroendocrine cells by transcriptomic and peptidomic profiling**

**Short title: Enteroendocrine transcriptomics and peptidomics**

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**Abstract**

Enteroendocrine cells (EECs) produce hormones such as glucagon-like peptide-1 (GLP-1) and peptideYY (PYY) that regulate food absorption, insulin secretion and appetite. Based on the success of GLP-1 based therapies for type 2 diabetes and obesity, EECs are themselves the focus of drug discovery programmes to enhance gut hormone secretion. The aim of this study was to identify the transcriptome and peptidome of human EECs, and to provide a cross-species comparison between humans and mice. By RNA sequencing of human EECs purified by flow cytometry after cell fixation and staining, we present a first transcriptomic analysis of human EEC populations, and demonstrate strong correlation with murine counterparts. RNA sequencing was deep enough to enable identification of low abundance transcripts such as G-protein coupled receptors and ion channels, revealing expression in human EECs of GPCRs previously found to play roles in post-prandial nutrient detection. By liquid chromatography mass spectrometry (LC-MS) we profiled the gradients of peptide hormones along the human and mouse gut, including their sequences and post-translational modifications. The transcriptomic and peptidomic profiles of human and mouse EECs, and cross-species comparison, will be valuable tools for drug discovery programmes and for understanding human metabolism and the endocrine impacts of bariatric surgery.

**Keywords**

Gut hormones; GLP-1; enteroendocrine; PYY; GIP; bariatric surgery; transcriptome; peptidome; LC-MS; RNAseq

**Introduction**

Enteroendocrine cells (EEC) are specialised hormone secreting cells in the intestinal epithelium which monitor the quality and quantity of ingested foods. They produce at least 20 different hormones, mostly peptides, that act in concert to coordinate digestion, peripheral nutrient disposal and appetite through actions at local and distant target tissues. In the field of human metabolism, glucagon-like peptide-1 (GLP-1) and peptideYY (PYY) have raised particular interest because of their central and pancreatic actions controlling food intake and insulin secretion. GLP-1 based drugs are widely used for the treatment of type 2 diabetes and obesity, and new gut hormone based therapeutics are under development, aiming to mimic the unrivalled effectiveness of gastric bypass surgery on weight loss and type 2 diabetes resolution(1).

Recent years have witnessed substantial progress in our understanding of murine EEC physiology, facilitated by the generation of transgenic mice with fluorescently labelled EECs that enable cell identification and functional characterisation through a range of approaches including fluorescence-activated cell sorting (FACS), transcriptomics and live cell imaging(2-7). Our knowledge of human EECs, however, is limited by a lack of methods to identify and characterise this scattered cell population which only comprises ~1% of the intestinal epithelium(2). A number of G-protein coupled receptors (GPCRs) have been identified and characterised in murine EECs that represent promising candidates for therapeutic approaches to enhance endogenous gut hormone secretion, but tools to predict the translatability of these findings from mouse to humans would be a major advance in this field(8).

The objectives of this study were to generate transcriptomic profiles of human EECs and to compare mouse and human EECs at the transcriptomic and peptidomic levels. We sequenced EECs from human and mouse at a depth sufficient for the identification of low abundance transcripts including GPCRs and ion channels. By liquid chromatography / tandem mass spectrometry (LC-MS/MS), we mapped the exact sequences of different gut peptides produced along the GI tract in human and mouse.

**Methods**

*Ethics*

This study was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice. Human ethical approvals were given by Cambridge Central and South Research Ethics Committees (ref: 09/H0308/24, 16/EE/0338, 15/EE/0152) and the Inserm ethics committee and Agence de la biomédecine (ref: PFS16-004). Animal work was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and conducted following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body.

***Human tissue transcriptome***

*Sample collection*

Jejunal tissue was obtained from 11 human participants (Supplementary table 1). Samples of human jejunum discarded during surgery were collected during total gastrectomy for treatment or prophylaxis of gastric cancer, or Roux-en-Y gastric bypass for obesity. All were from the point of entero-enterostomy 50cm distal to the ligament of Treitz. Two matched samples of jejunum and terminal ileum were collected during organ procurement from transplant donors. Data were collected on age, gender and BMI, and participants stratified as lean vs obese (BMI>30kg/m2). Tissue samples from different regions of the human GI tract for LC-MS were obtained from Addenbrooke’s Human Research Tissue Bank and the Cambridge Biorepository for Translational Medicine.

Samples were immediately placed in cold Leibovitz’s L-15 media (Thermo Scientific, Waltham, MA, USA) and processed to the point of fixation or homogenised and stored at -70oC within 6 hours.

*Tissue preparation for FACS*

FACS and RNA extraction from fixed human cells followed a modified version of the MARIS protocol(9). Intestine was rinsed in cold phosphate buffered saline (PBS) and the muscular coat removed. Diced mucosa was digested twice in 0.1% w/v collagenase XI (Sigma-Aldrich, MO, USA) in Hanks’ Buffered Saline solution (HBSS) #9394 (Sigma-Aldrich) for 30 minutes each, shaking vigorously every 10 minutes. Supernatants were triturated, passed through a 50µm filter and centrifuged at 300g. Pellets were resuspended in PBS and fixed in 4% w/v paraformaldehyde (PFA) at 4oC for 20 minutes. PFA-fixed cells were washed twice in nuclease free 1% w/v bovine serum albumin (BSA) in PBS, and if a FACS facility was not immediately available, were suspended in 1% w/v BSA and 4% v/v RNAsin plus RNAse inhibitor (Promega, WI, USA) in PBS at 4oC overnight.

Cells were permeabilised with either a single 30 minute incubation with 0.1% v/v Triton x100 (Sigma-Aldrich) in 1% w/v BSA in PBS prior to antibody staining, or by the addition of 0.1% w/v Saponin (Sigma-Aldrich) to solutions in all steps from this point until after the first wash post-secondary antibody staining, with identical results.

Primary antibody staining was for one hour in 4% v/v RNAsin, 1% w/v BSA, 1% v/v goat anti-GLP-1 (Santa Cruz, Dallas, TX, USA; sc7782), 2% v/v rabbit anti-CHGA (Abcam, Cambridge, UK; Ab15160), 0.25% v/v rabbit anti-SCG2 (Abcam, Ab12241) in PBS at 4oC. Cells were then washed twice in 1% w/v BSA, 1% v/v RNAsin, and secondary antibody staining was for 30 minutes in 4% v/v RNAsin, 1% w/v BSA, 0.2% v/v donkey anti-goat Alexa 555, 0.2% v/v donkey anti-rabbit Alex 647 in PBS at 4oC. Cells were washed twice then suspended in 4% v/v RNAsin, 1% w/v BSA in PBS on ice for FACS.

*FACS*

Cell populations were sorted on a BD FACS ARIA III in the Cambridge NIHR BRC cell phenotyping hub or at Institut Cochin, Paris. Single cells positive for Alexa 647 but not Alexa 555 (i.e. CHGA/SCG2 +ve / GLP-1 –ve) were classified as GCG- enteroendocrine cells. Single cells positive for both Alexa 647 and Alexa 555 were classified as GCG+ enteroendocrine cells. At least 5000 cells were collected for each positive population. Twenty thousand double negative cells were collected as the negative (i.e. non-enteroendocrine) cell population. Cells were sorted into 2% v/v RNAsin in PBS at 4oC.

*RNA extraction*

RNA was extracted using the Ambion Recoverall Total nucleic acid isolation kit for FFPE (Ambion, CA, USA) with modifications to the protocol as below. The FACS sorted cell suspension was centrifuged at 3000g for 5 minutes at 4oC and the pellet resuspended in 200µl digestion buffer with 4µl protease and incubated at 50oC for 3 hours. The solution was then stored at -70oC for at least 12 hours prior to further extraction. After thawing, RNA was extracted using the manufacturer’s protocol (including a DNase step) with the exception of performing 2x 60µl elutions from the filter column in the final step.

The RNA solution was concentrated using a RNEasy Minelute cleanup kit (Qiagen, Hilden, Germany). RNA aliquots were diluted to 200µl with nuclease free water. The standard manufacturer’s protocol was followed with the exception that 700µl, not 500µl, of 100% ethanol was added to the solution in step two, to generate optimum binding conditions for the PFA fragmented RNA. RNA concentration and quality was analysed using an Agilent 2100 Bioanalyser (Agilent, CA, USA).

*Sequencing*

cDNA libraries were created using the Clontech SMARTer Stranded Total RNA-Seq Kit – Pico Input Mammalian v1 (Takara Bio, USA). RNA input quantity was 5ng and the non-fragmentation protocol was used. The standard manufacturer’s protocol was followed with the exception that 175µl of AMPure beads were used for the final bead purification to ensure recovery of the small fragments of RNA arising from PFA fixation. Sixteen PCR cycles were used for amplification.

50 base single-end sequencing was performed using an Illumina HiSEQ 4000 at the CRUK Cambridge Institute Genomics Core.

***Mouse transcriptome***

*Sample collection and preparation for FACS*

Female NeuroD1-Cre/EYFP (mixed background: 3-10 generations back-crossed with C57BL6) and GLU-Venus mice (C57BL6)(6; 7)aged 8-10 weeks were killed by cervical dislocation (n=3 each). Diced mucosa from the proximal 10cm of small intestine was digested twice in 0.1% w/v collagenase XI in HBSS at 37oC for 30 minutes each. Cells were pelleted by centrifugation at 300g for 5 minute, triturated and passed through a 50µm filter. Cells were stained with DAPI (1µg/ml) for 5 minutes at room temperature, washed twice and sorted in HBSS on a FACSJazz sorter at the Cambridge NIHR BRC cell phenotyping hub.

*FACS and RNA extraction*

All positive cells, and 20,000 negative cells were collected separately into aliquots of 500µl of buffer RLT+ (Qiagen), with 143mM β-mercaptoethanol. RNA was extracted using a RNeasy Micro plus kit (Qiagen) and quantified using an Agilent 2100 Bioanalyser

*Sequencing*

2ng of each RNA was used for cDNA amplification by SPIA amplification using the Ovation RNAseq system V2 kit (Nugen, CA, USA). 1g of cDNA was then fragmented to ~200bp by sonication (Diagenode, Liege, Belgium) and adaptors for the indexing were added using the Ovation Rapid DR multiplex 1-96 kit. Samples were pooled and concentrated together with a MinElute column (Qiagen) to reach a concentration of 10nM. Single end 50 base sequencing was performed at the CRUK Cambridge Institute Genomics Core with an Illumina Hiseq4000.

***RNAseq pipeline***

Quality control and trimming of adaptors was performed using FastQC(10). Human sequenced transcripts were mapped to the human genome (GRCh37) and raw counts generated using STAR v2.5.1. Mouse reads were aligned to the mouse genome (GRCm38) using TopHat 2.1.0 and raw counts generated using Cufflinks 2.2.1(11-13). Differential gene expression analysis was performed in RStudio using DESEQ2(14). Gene annotation was pulled from the Ensembl dataset held in BioMart(11). Receptor and ion channel lists were generated from the IUPHAR “targets and families” list(15). Graphical output used ggplot2 and pheatmap in RStudio(16).

***Comparative transcriptomics***

Mouse and human data sets were directly compared using only the 15,507 genes present in both datasets, not annotated as ribosomal, mitochondrial or small-nuclear and described with one-to-one homology according to the Ensembl mouse-human homology dataset(11). Genes with no one-to-one homology were analysed separately. Normalised CPM (counts per million) were generated from the respective DESEQ2 models and compared for the human GCG+ population versus the murine GLU-Venus population and the human GCG- population versus the murine NeuroD1 population. Linear models were generated of the log10 CPM of the human vs murine datasets by a total least squares strategy, 99% confidence intervals calculated and the outliers hand searched for relevant genes.

Examining just the human samples, DESEQ2 models were generated for the following sets of jejunum samples: GCG+ versus negative; GCG- versus negative; GCG+ versus GCG-; GCG+ lean versus obese; GCG- lean versus obese. Participant paired DESEQ2 analyses were also performed comparing GCG+ and GCG- populations from the jejunum and ileum of the two transplant donor participants, for whom there were matched jejunum and ileum samples. An adjusted P value of ≤0.1 was defined as the cut off for statistical significance when examining for differential gene expression between populations.

***Tissue homogenates***

Small pieces (~2x2mm, 15-45 mg) of mucosa from different regions of the human or mouse GI tract were homogenised in 250µl of 6M guanidine hydrochloride (Sigma-Aldrich) with Lyzing MatrixD (MPbio), in a FastPrep-24 for 4x40s at 6ms-1. Samples were stored at -70C before further processing. Proteins were precipitated by adding 80% acetonitrile in water then centrifuged at 12000g at 4oC for 5 minutes. The aqueous phase containing the peptides was collected, dried on a centrifugal vacuum concentrator and stored at -70oC before analysis

***Mass spectrometry***

Samples were extracted using a Waters HLB μElution solid‐phase extraction (SPE) plate (Waters, MA, USA) after being resuspended in 500L 0.1% v/v formic acid in water as described previously and analysed after reduction/alkylation(17). Human homogenates and mouse homogenates for interspecies comparison were analysed using nano-flow based separation and electrospray approaches on a Thermo Fisher Ultimate 3000 nano LC system coupled to a Q Exactive Plus Orbitrap mass spectrometer (ThermoScientific). High-flow separation for the longitudinal mouse analysis was as previously described(17). Downstream analysis was performed using Peaks 8.0 software (Waterloo, ON, Canada) against the human and the mouse Swissprot databases (downloaded 26th October 2017)(18), with a fixed cysteine carbamidomethylation and variable methionine oxidation, N-terminal acetylation and pyro-glutamate and C-terminal amidation modifications. Manual searches were performed for other modifications. Peptides of interest were quantified by measuring peak areas for selected m/z ranges and retention times corresponding to the peptide sequences and normalised by tissue weight.

**Results**

*Cell and RNA collection for transcriptomics*

Human intestinal tissue pieces were digested, paraformaldehyde (PFA)-fixed and stained for Chromogranin A (CHGA) and Secretogranin 2 (SCG2) as general markers for EECs, and for GLP-1 as a marker of the EEC-subpopulation known as L-cells. By flow cytometry (Supplementary Figure 1A-C), we collected pooled cell populations that were (i) positive for CHGA, SCG2 and GLP-1 (henceforth named GCG+), (ii) positive for CHGA and SCG2 but negative for GLP-1 (henceforth named GCG-), and (iii) negative for all 3 markers (i.e. non-endocrine lineage cells, henceforth named negative). The GCG+ (L-cell) population represented ~0.2% of all single cells examined, and the ratio of GCG+ to GCG- cells was ~1:5.

From mouse, we collected unfixed EEC populations from the upper small intestine of the mouse strain GLU-Venus (n=3) to identify *Gcg*-expressing L-cells (Supplementary Figure 1E-G)(7), and of NeuroD1-Cre/EYFP (6) (n=3) to identify the total EEC population (Supplementary Figure 1I-K). GLU-Venus positive cells represented ~0.2% of singlets, and NeuroD1 positive cells ~0.6% of singlets.

RNA extracted from the purified fixed human cell populations had measured RIN values of 2-3 (compared with >7 in the unfixed murine cells), with most RNA fragments being 25-500 bases in length (Supplementary Figure 1D). The low RIN values of the human samples reflect the simultaneous fragmentation of ribosomal RNAs, but we predicted that the fragments were of lengths suitable for RNA sequencing using random primers and a rRNA depletion protocol. After RNA sequencing, a mean of 77% of reads from the human samples mapped to the human genome, compared with 86% of reads mapping to the murine genome in the mouse samples. The mean number of reads mapping uniquely to exons was 3.5x106 (range 0.8x106 to 8.8x106) in the human samples, and 3.9x106 (range 2.0x106 to 5.7x106) in murine samples.

*Transcriptomics of human EEC populations*

In total we obtained individual RNA sequencing data from GCG+, GCG- and negative cells from each of 11 human jejunum samples and 2 human ileum samples. Principle component analysis (PCA) of the top 500 differentially expressed genes in the jejunal samples separated EECs (GCG+ and GCG-) from negative cells on the first component, and GCG+ cells from GCG- cells on the second component (Figure 1A). PCA did not demonstrate clustering of samples by BMI of the donor. A heatmap of the top 500 differentially expressed genes (Figure 1B) revealed clear transcriptomic differences between the GCG+, GCG- and negative cells(14). A comparison between jejunal and ileal cell populations from the 2 matched donors is shown in supplementary Figure 2.

Human jejunal EEC populations expressed a wide range of hormonal transcripts (Figure 2A,C,E). As GLP-1 antibodies were used to purify the GCG+ cells, we were not surprised that the hormonal transcript showing the strongest differential expression between GCG+ and GCG- cells was *GCG* itself (Figure 2C). Consistent with previous findings in mice(3; 19-21), human GCG+ cells also expressed a range of additional hormonal transcripts including *GIP* (glucose-dependent insulinotropic polypeptide)*,* *CCK* (cholecystokinin), *NTS* (neurotensin), *PYY* and *SCT* (secretin) as well as *MLN* (motilin) – a hormone produced by human but not mouse(3; 19-22). Compared with GCG+ cells, GCG- cells had higher expression of *SCT*, *CCK*, *NTS*, *MLN*, *GHRL* (ghrelin) and *SST* (somatostatin), as well as *TPH1*, the enzyme responsible for serotonin biosynthesis in enterochromaffin cells. EECs also expressed the putative gut hormones *UCN3* (urocortin 3), *PCSK1N* (ProSAAS) and *NPW* (neuropeptide W), as well as lower levels of RNAs encoding peptides not classically described as gut hormones such as *VGF*, *GHRH* and *ADM*.

Transcripts of ~50 GPCRs were either enriched in EECs compared with negative cells, or expressed at > 100 CPM in one or both EEC populations (Figure 2B,D,F). Multiple GPCRs previously implicated in post-prandial gut hormone secretion in mice were highly expressed in human EECs, including the fat-sensing receptors *FFAR1*, *FFAR2*, *FFAR3,* *FFAR4* and *GPR119,* the amino acid sensing receptors *CASR* and *GPR142,* the butyrate and isovalerate sensing *OR51E1*(23), and the bile acid receptor, *GPBAR1.* At least four orphan GPCRs were differentially expressed in human EECs: *GPR148*, *GPR160*, *GPR173* and *GPR179*, hinting to as yet undescribed pathways that may control gut hormone secretion.

Human EECs expressed transcripts for a range of ion channel subunits (Supplementary Figure 3), consistent with previous reports that murine L-cells and enterochromaffin cells are electrically active (24). Transcription factor profiling of human GCG+ and GCG- cells is shown in Supplementary Figure 3.

*Comparison of human vs mouse EEC transcriptomes*

To compare the L-cell populations between humans and mice, we compared human jejunal GCG+ cells with murine upper small intestinal GLU-Venus cells (Figure 3). Log-log plots of normalised gene expression indicated a strong correlation between the two species (R2=0.73; Figure 4). Notable genes more highly expressed in human than mouse L-cells included *GIP*, *CHGA*, *ASIC5*, *GIPR*, *GPR142, SCTR, PTH2R, CHRNA5* and *OPRK1*; whereas genes more highly expressed in mouse included *Gpr174*, *Gpr171, Ghr, Grpr, Ptger1, Cnr1, Insl5, Gpr22* and *Ghrl*.

A similar comparison was made between human jejunal GCG- cells, representing the wider EEC population (although depleted of L-cells) and murine NeuroD1-positive cells, which similarly revealed a strong inter-species correlation (R2=0.74; Figure 4). Data on transcription factors, GPCRs, ion channels and hormones that were EEC-enriched in either species but excluded from the comparative analysis because they did not have annotated 1:1 orthologues between mouse and human are given in Supplementary Table 2, and notably highlight MLN and GPR148 as EEC-enriched transcripts in human that are not found in mice.

*Peptidomic analysis by LC-MS*

Peptide extraction and LC-MS protocols were optimised for maximum peptide retrieval and identification from fresh intestinal mucosal samples (Supplementary Figure 4), and enabled reliable detection and sequencing of peptides up to 65 amino acids in length. In the first instance, we compared human jejunum with mouse mid-small intestine, and assigned peptides to their parental proteins. This method identified known EEC prohormones, granins and enteric neuronal signalling peptides as well as peptides derived from a variety of house-keeping proteins that likely reflected the occurrence of some tissue damage/degradation prior to homogenisation. Of the 463 and 705 different proteins matched in human and mouse respectively, 234 were common between the two species, and showed good correlation (R2 = 0.54, Figure 5A). To identify candidate EEC-derived peptides, we restricted the analysis to peptides originating from genes that in the transcriptomic analysis showed >4-fold higher expression in at least one EEC sample compared with the corresponding negative cells (Figure 5B). Known gut hormone genes and members of the chromogranin family were mostly found to be common between mouse and human, but motilin and ghrelin were detected in human but not mouse jejunum. To search for novel candidate peptide hormones, we also examined the transcriptomic data for unannotated transcripts that had a base mean value >100 and were >16 fold more highly expressed in EECs than control cells. This analysis identified MIR7-3HG(25), C1orf127 and C6orf141, but we were unable to detect corresponding peptides by LC-MS.

We performed a longitudinal LC-MS analysis of known bioactive peptides along the mouse and human GI tracts. Most EEC peptides were identifiable in their known bioactive forms, but as our method was not optimized for very small peptides like CCK-8, we used CCK21-44 as a surrogate for CCK production. Peptides have been depicted in separate heatmaps for mouse and human (Figure 6), separated according to their origin from EEC prohormones, granins and non-EECs (likely reflecting enteric neural peptides). We observed longitudinal peptide profiles that broadly mirror historical immuno-staining patterns(26) but additionally provide details of the exact peptide sequences and their post-translational modifications.

**Discussion**

By RNA sequencing of fixed, FACS-purified cells from the human gut, we report here the transcriptome of human L-cells and the wider EEC population, and a between-species comparison showing a strong similarity with matching cells from the mouse. LC-MS based peptidomic analysis of the human and mouse gut revealed longitudinal gradients of a range of EEC peptides, including details of their exact sequences and post-translational modifications.

To purify human EECs for transcriptomics, cells were first fixed, permeabilised and antibody-stained, and the RNA extraction and sequencing kits were only just technically capable of dealing with PFA-modified RNA. Nevertheless, the RNAseq-derived transcriptomes of human L-cells and GCG- EECs mapped robustly onto the corresponding data from freshly purified matching murine EEC populations and the read depth was sufficient for the detection of low abundance mRNAs such as those encoding receptors and ion channels. Although we analysed samples from 11 humans with varying BMI, we were unable to detect any effect of BMI on the EEC transcriptome. However, the study was not designed or powered to address the impact of BMI or diet on EECs, and further studies will be needed to examine this question.

GPCRs play key sensory roles in EECs and a number are under investigation as candidate drug targets for enhancing gut hormone secretion as a therapy for type 2 diabetes and obesity. The GPCR repertoire of human EECs largely mirrored their well-studied murine counterparts(8; 27-29), including expression of receptors for amino acids (*CASR*, *GPR142*), triglyceride digestion products (*FFAR1, FFAR4, GPR119*) and bile acids (GPBAR1), as well as for hormones such as somatostatin (*SSTR1, SSTR2, SSTR5*), GIP (*GIPR*) and arginine vasopressin (*AVPR1B*). Interestingly, *GPR142* was highly expressed in human EECs, supporting current studies looking to exploit its ability to stimulate both insulin and incretin hormone secretion(28).

Our optimised peptide extraction protocol combined with nano-LC-MS analysis enabled identification of the exact peptide sequences biosynthesised in human and mouse intestinal mucosa, including post-translational modifications, for peptides ranging from ~8-10 to 65 amino acids in length. From the proglucagon gene, for example, we detected multiple processed and pre-processed products, including GRPP, oxyntomodulin, GLP-17-36 amide, GLP-17-37, GLP-11-37, IP131-142, IP-GLP2 and GLP-2. Intact (pancreatic-type) glucagon was detected in samples from the mouse stomach, but was undetectable in the remainder of the intestine and colon from both species, conflicting with recent suggestions that the small intestine secretes intact glucagon(30), but consistent with our recent finding that post-prandial glucagon concentrations were not altered following gastric bypass surgery in lean subjects despite dramatic increases in GLP-1(31). LC-MS also identified some additional peptides encoded by EEC-enriched genes, including peptides derived from PCSK1N, chromogranins and secretogranins. Whether any of these have specific physiological roles or are simply inactive by-products of enzymatic processing of the contents of secretory vesicles, requires further evaluation.

Mapping of gut hormone production along the GI tract length has previously been performed by immuno-staining or extraction/immuno-assays for specific peptides(26; 32). Many antibody-based methods, however, do not distinguish whether a prohormone was processed or unprocessed, or post-translationally modified. Our LC-MS method provides a robust mirror of previous antibody based maps of the GI tract, whilst additionally assigning an exact peptide sequence to each identified peptide, clearly distinguishing e.g. oxyntomodulin from glucagon, and PYY1-36 from PYY3-36. Interestingly we identified acylated as well as non-acylated ghrelin from the human jejunum despite our previous finding that plasma acylated ghrelin levels were undetectable in humans after total gastrectomy(31). We were surprised to find high levels of PYY3-36 as well as PYY1-36 in tissue homogenates, suggesting that dipeptidyl-peptidases (DPP) are active within L-cells, although GLP-1(7-36amide) was much more abundant than GLP-1(9-36amide). Why GLP-1 but not PYY seems protected from DPP-mediated processing in L-cells, despite both peptides being located in the same vesicular pool (33), remains unclear.

Conclusions:

The methods we describe here for performing RNA sequencing of rare cell populations and LC-MS/MS based peptidomic analysis from human surgical tissue samples have wide potential applications beyond the study of the enteroendocrine system, including islet cell biology. Although comparison of the human and mouse EEC transcriptomes revealed strong global similarities between the two species, variation at the level of individual genes could have profound implications for the use of mouse as a model species in drug development programmes targeting specific receptors. Our mouse/human comparative datasets thus provide tools for assessing the validity of using mice as a model for investigating specific signalling pathways in humans, and the human EEC GPCR-ome can be used independently as a potential source of drug targets in the human enteroendocrine system. Longitudinal peptide mapping of the GI tract by LC-MS is a key step towards understanding the metabolic benefits of gastric bypass surgery, as most bariatric procedures result in a shift in the location of nutrient digestion and absorption to more distal regions of the small intestine, with consequent stimulation of the more distal EEC population and release of their distinct profiles of peptide hormones. The results presented here should guide strategies to mimic gastric bypass surgery using injectable peptide mimetics or by targeting EEC-enriched receptors to mobilise endogenous gut hormones, which are highly topical challenges for industry and academia alike.

**Author Contributions**

GR, PR, PL, RS, RH, FR and FG designed the study. Tissue handling and FACS were performed by GR, PR, PL and LG. cDNA library production and bioinformatics analysis were performed by GR, PR, PL, MM and BL with support from GY and DC. Mass spectrometry was by PL, RK, EM, SG and GR. NeuroD1 mice were generated by AL and HJL. The manuscript was written by GR, FR and FG. All authors have had editorial oversight of the final manuscript version.

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FMG and FR act as guarantors for this manuscript.

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**Conflicts of interest**

FMG is a paid consultant for, and PR has (subsequent to his work on this project) taken up full time employment with Kallyope, New York. The Gribble-Reimann lab hosts projects which receive funding from Medimmune (FMG / FR). There are no other conflicts of interest to declare.

**Data availability statement**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD009788 (10.6019/PXD009788) and PXD009796 (10.6019/PXD009796)(34). RNA-seq data has been deposited in the NCBI GEO repository (human – GSE114853, mouse -GSE114913).

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**Figures legends**

*Figure 1. Transcriptomic distinction between cell populations from human small intestine*

(A) Principal component analysis plot of all human samples (n=3 cell populations from each of 11 jejunal ~~and 2 ileal~~ tissue samples), differentiated by cell population ~~and anatomical site~~. (B) Pairwise analysis of key genes differentiating the cell populations from jejunum was performed using a DESEQ2 model, and the normalised results for the 500 most differentially expressed genes are presented in a heatmap (n=3 cell populations per each of 11 participants), y axis – cell population identified by coloured bar, x axis – genes.

*Figure 2. Transcripts enriched in human EECs*

Enrichment vs expression plots for human jejunum EEC populations. Enrichment is presented as the log2 fold difference between the cell populations indicated, and expression is presented as the log2 base mean normalised expression extracted from the DESEQ2 model. (A,B) GCG+ vs negative, (C,D) GCG- vs negative, (E,F) GCG+ vs GCG-. Hormones and granins are shown in A,C,E; receptors and ion channels are shown in B,D,F. Red – enriched (adjusted P<=.1 in DESEQ2 model) in GCG+; Blue – enriched in GCG-; Green – enriched in negative cells.

*Figure 3. Comparison between human and mouse L-cells*

Human versus mouse jejunal L cell gene expression (log10 normalised counts per million +1; n = 11 humans, n = 3 mice). (A) all genes with 1:1 homology between species, excluding mitochondrial, ribosomal and small nuclear transcripts (n=15,507). (B) Hormones. (C) GPCRs and ion channels. (D) Transcription factors. Dashed lines are linear regression and 99% confidence interval. Each dot represents normalised CPM+1 for one gene. Red – enriched (>4x fold change) and differentially expressed (adjusted P<=.1) for human GCG+ vs Negative cell populations, but not murine GLU-Venus vs Negative cell populations in relevant DESEQ2 model. Blue – enriched and differentially expressed for murine GLU-Venus vs Negative cell populations, but not human GCG+ vs Negative cell populations. Purple – enriched and differentially expressed in both murine GLU-Venus and human GCG+ cells versus relevant negative cell populations. Black – not enriched and differentially expressed in either human GCG+ or murine GLU-Venus cells versus relevant negative cell populations. All genes are labelled in B, and genes outside the 99% CI are labelled in C and D, with those not differentially expressed or enriched in either population listed along the axis.

*Figure 4. Comparison between human GCG- and mouse NeuroD1 cells*

Human versus mouse jejunal EEC (GCG-) gene expression (log10 normalised counts per million +1; n = 11 humans, n = 3 mice). (A) all genes with 1:1 homology between species, excluding mitochondrial, ribosomal and small nuclear transcripts (n=15,507). (B) Hormones. (C) GPCRs and ion channels. (D) Transcription factors. Dashed lines are linear regression and 99% confidence interval. Each dot represents normalised CPM+1 for one gene. Red – enriched (>4x fold change) and differentially expressed (adjusted P<=.1) for human GCG- vs Negative cell populations, but not murine NeuroD1 vs Negative cell populations in relevant DESEQ2 model. Blue – enriched and differentially expressed for murine NeuroD1 vs Negative cell populations, but not human GCG- vs Negative cell populations. Purple – enriched and differentially expressed in both murine NeuroD1 and human GCG- cells versus relevant negative cell populations. Black – not enriched and differentially expressed in either human GCG- or murine NeuroD1 cells versus relevant negative cell populations. All genes are labelled in B, and genes outside the 99% CI are labelled in C and D, with those not differentially expressed or enriched in either population listed along the axis.

*Figure 5. Comparison between human and mouse jejunum peptidome*

Tissue samples were analysed by nano LC-MS/MS and compared between human jejunum (n=4) and mouse mid-small intestine (n=4). Peptides were assigned to their parental proteins by Peaks software, and protein intensity was calculated by Peaks v8.0 for all proteins detected corresponding to genes with 1:1 homology between human and mouse for jejunum mucosal homogenates. (A) All proteins, (B) proteins from genes enriched in at least one of the species from the human and the mouse transcriptome datasets. Enrichment was defined as adjusted P<=.1, fold difference >4 and base mean expression >50 from DESEQ2 model. Colours indicate in which species the mRNA for the genes was enriched.

*Figure 6. Longitudinal profiles of gut peptides along the human and mouse gut*

Heatmap of gut peptide quantification normalised by tissue weight (log10 scale) along human (A) and mouse (B) GI tract. Sequential samples were taken at 5cm intervals from the stomach to the rectum in mice (n=4 for each location), and human biopsies were analysed from the stomach (n=5), duodenum (n=9), jejunum (n=2), ileum (n=4), proximal colon (n=3), sigmoid colon (n=5) and rectum (n=3). Blue indicates not detected in the sample. Rows: Samples ordered from proximal to distal and colour coded by their region of origin. Mouse regions: P12: stomach lesser curvature, P1 to P7: small intestine from proximal to distal, sampling every 5 cm, P8 to P10: large intestine (proximal, mid and distal). Columns: peptide (using the human name if different between human and mouse), classified by origin (purple: classical EEC hormone peptides; medium blue: peptides from granins; light blue: enteric neuron bioactive peptides).