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**Gene expression of nutrient-sensing molecules
in I cells of CCK reporter male mice**

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receptor

4041 words

22   **Abbreviations:**

23   CCK: Cholecystokinin

24   GIP: Glucose-dependent insulintropic polypeptide / gastric inhibitory polypeptide

25   GLP-1: Glucagon-like peptide-1

26   FFAR: Free fatty acid receptor

27   GPR: G protein-coupled receptor

28   FATP: Fatty acid transport protein

29   CD36: Cluster of differentiation 36

30   SGLT1: Sodium-glucose cotransporter 1

31   GLUT: Glucose transporter

32   TGR5: Transmembrane GPR 5

33   PEPT1: Peptide transporter 1

34   CASR: Calcium-sensing receptor

35

Abstract

Cholecystokinin (CCK) is secreted from enteroendocrine I cells in response to fat, carbohydrate, and protein ingestion. Gene expression of nutrient-sensing molecules in I cells remains unclear, primarily due to the difficulty in distinguishing I cells from intestinal epithelial cells *in vivo*. In this study, we generated CCK reporter male mice in which the red fluorescence protein tdTomato (Tomato) is produced by activation of the native murine *Cck* promoter. Fluorescence microscopy revealed the presence of Tomato-positive cells in upper small intestine (SI), lower SI, and colon. Flow cytometer analysis revealed that Tomato-positive cells among epithelial cells of upper SI, lower SI, and colon occurred at the rate of 0.95%, 0.54%, and 0.06%, respectively. In upper SI and lower SI, expression levels of *Cck* mRNA were higher in Tomato-positive cells than those in Tomato-negative cells. The fatty acid receptors *Gpr120*, *Gpr40*, and *Gpr43* and the oleoylethanolamide receptor *Gpr119* were highly expressed in Tomato-positive cells isolated from SI, but were not found in Tomato-positive cells from colon. The glucose and fructose transporters *Sglt1*, *Glut2*, and *Glut5* were expressed in both Tomato-positive cells and -negative cells, but these expression levels tended to be decreased in Tomato-positive cells from upper SI to colon. The peptide transporter *Pept1* and receptor *Gpr93* were expressed in both Tomato-positive cells and -negative cells, whereas *Casr* was expressed only in Tomato-positive cells isolated from SI. Thus, this transgenic mouse reveals that I cell number and gene expression in I cells vary according to region in the gastrointestinal tract.

57   **Introduction**

58   Gut hormones are released from enteroendocrine cells in response to nutrients, and
59   play an important role in food intake, nutrient absorption, energy accumulation and glucose
60   homeostasis. For example, ghrelin secreted from X/A-like cells expressed in the stomach
61   increases food intake and body weight (Nakazato *et al.* 2001); peptide YY (PYY) and the
62   incretin glucagon-like peptide-1 (GLP-1) released from enteroendocrine L cells inhibit food
63   intake and reduce body weight (Davis *et al.* 1998, Batterham *et al.* 2002). In addition,
64   glucose-dependent insulintropic polypeptide / gastric inhibitory polypeptide (GIP) is an
65   incretin secreted from enteroendocrine K cells, and plays an important role in obesity and
66   insulin resistance under high-fat diet (HFD)-fed condition (Harada *et al.* 2008, Nasteska *et*
67   *al.* 2014, Joo *et al.* 2017, Shimazu-Kuwahara *et al.* 2017).

68   Cholecystokinin (CCK) is a gut hormone secreted from enteroendocrine I cells in small
69   intestine and colon (Fakhry *et al.* 2017), and activates the nucleus of the solitary tract
70   through the vagus nerve system to suppress appetite and food intake (Whited *et al.* 2006).
71   CCK-producing cells are expressed in the central nervous system, and directly inhibit food
72   intake (D'Agostino *et al.* 2016). The OLETF rat, which has a deletion in the *CCK1 receptor*
73   gene, shows hyperphagia and obesity (Otsuki *et al.* 1995, Tachibana *et al.* 1996). On the
74   other hand, CCK induces secretion of bile and pancreatic lipase, which are involved in fat
75   digestion and absorption (Rehfeld 2004). HFD-fed *Cck*-knockout mice demonstrate that
76   inhibition of CCK signaling alleviates body weight gain and insulin resistance under HFD-fed
77   condition (Lo *et al.* 2011). We previously reported that CCK has an important role in oil-

induced secretion of GIP, which is involved in body weight gain and insulin resistance (Sankoda *et al.* 2017). This finding shows that CCK is involved in obesity and insulin resistance under HFD-fed condition. Thus, regulation of CCK signaling or CCK secretion is a potential therapeutic target for obesity and insulin resistance.

CCK is secreted from I cells by nutrient ingestion; fat and protein strongly stimulate CCK secretion in comparison with glucose (Green *et al.* 1989, Pilichiewicz *et al.* 2007, Hutchison *et al.* 2015). Some nutrient-sensing molecules have been identified. Glucose transporters such as sodium-glucose cotransporter 1 (SGLT1) and glucose transporter 2 (GLUT2) are associated with GLP-1 and GIP secretion after glucose loading (Mace *et al.* 2012, Gorboulev *et al.* 2012). Furthermore, free fatty acid receptors (FFARs) and fatty acid transporters (FATPs) play an important role in free fatty acid sensing in gut hormone-producing cells (Poreba *et al.* 2012, Lu *et al.* 2018). Some amino acid transporters and receptors are involved in GLP-1 secretion (Diakogiannaki *et al.* 2013). In contrast, it remains unclear whether these molecules are expressed in I cells, primarily due to the difficulty in isolating them from intestinal epithelial cells. In this study, we generated CCK reporter male mice in which the red fluorescence protein (RFP) variant tdTomato (Tomato) as well as CCK is produced by activation of the native murine *Cck* promoter, and evaluated gene expression of the molecules associated with nutrient sensing in I cells expressed in the gastrointestinal (GI) tract.

Materials and Methods

99 **Animals**

100 *CCK-internal ribosome entry site (IRES)-Cre* knock-in (CCK-Cre) mice and Ai14 mice were
101 previously generated (JAX stock #012706, #007908) (Jackson Laboratory, Bar Harbor, Maine,
102 US) (Madisen *et al.* 2010, Taniguchi *et al.* 2011). CCK-Cre and Ai14 heterozygous (CCK-
103 Tomato) mice, which enabled visualization of I cells by Tomato fluorescence, were
104 generated by crossbreeding CCK-Cre homozygous mice and Ai14 homozygous mice. Ai14
105 heterozygous mice were used as control. Male mice at 8-13 weeks of age were used in flow
106 cytometer analysis and immunohistochemical analysis. We performed two cohorts to
107 evaluate the phenotype of CCK-Tomato mice. In one cohort, 8-week-old male mice were
108 weighed weekly for 20 weeks. Non-fasting blood samples were collected from the portal
109 vein of mice at 10 weeks of age, and plasma CCK concentrations were measured by CCK
110 fluorescent enzyme immunoassay (EIA) kit (FEK-069-04) (Phoenix Pharmaceuticals Inc.,
111 Burlingame, CA, US). In the other cohort, male mice at 19 weeks of age were used. Oral
112 glucose tolerance tests (OGTTs) and oral corn oil tolerance tests (OCTTs) were performed
113 after a 16-hour fasting period. Mice were administrated glucose of 6g/kg body weight for
114 OGTTs and corn oil of 10mL/kg body weight for OCTTs. Blood glucose levels were measured
115 at 0, 15 (for OGTTs), 30, 60, and 120 minutes after oral glucose or oil administration by the
116 glucose oxidase method (Sanwa Kagaku Kenkyusho, Nagoya, Japan). 60 µl blood samples
117 were collected from peripheral blood vessels at 15 or 30 minutes after oral glucose or oil
118 administration, and plasma insulin (Shibayagi, Shibukawa, Japan) and CCK levels (Phoenix
119 Pharmaceuticals Inc.) were measured by EIA kit, respectively. Energy expenditure and

locomotor activity were measured by ARCO 2000 (ARCO System, Chiba, Japan) every 5 minutes over 24 hours with free access to water and diet (Kanemaru *et al.* 2020). Animal care and procedures were approved by Kyoto University Animal Care Committee (MedKyo15298).

Immunohistochemistry

Stomach, upper small intestine (upper SI), lower small intestine (lower SI), and colon were collected from CCK-Tomato mice and fixed by 4% paraformaldehyde. The protocol of immunohistochemistry was previously described (Ikeguchi *et al.* 2018). Anti-CCK antibody (CCK8-MO-167-2, 1:1000) (Frontier Institute Co., Ltd., Hokkaido, Japan), anti-RFP antibody (600-401-379, 1:1000) (Rockland Immunochemicals Inc., Limerick, PA, US), and secondary antibodies (Abcam, Cambridge, UK) were used. Images were taken using a fluorescence microscope FSX100 (Olympus Corporation, Tokyo, Japan).

Isolation of Tomato-positive and -negative cells by flow cytometry

The protocol to isolate fluorescence protein-producing cells from murine intestinal epithelium was described previously (Suzuki *et al.* 2013). The small intestine was divided in half, and the oral and rectal portions were defined as upper SI and lower SI, respectively. The collected intestinal epithelial cells were filtered through a 40 µm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, US), and phosphate buffered salts (PBS) containing 4', 6-diamidino-2-phenylindole (DAPI) (Dojindo Molecular Technologies, Inc.,

Kumamoto, Japan) was added. After excluding DAPI-positive cells as dead cells or doublets, Tomato-positive cells and -negative cells were collected using FACS Aria III cell sorter (Becton, Dickinson and Company). The number of Tomato-positive cells was also calculated as Tomato-positive cells / intestinal epithelial cells (%).

Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNAs of sorted Tomato-positive and -negative cells were extracted with a PicoPure RNA Isolation Kit (Applied Biosystems, California, CA, US). For cDNA synthesis of sorted 2,000 Tomato-positive and -negative cells, RNA was reverse-transcribed using SuperScript II Reverse Transcriptase and Oligo(dT)12-18 (Invitrogen, Carlsbad, CA, US). SYBR Green PCR Master Mix (Applied Biosystems) was prepared for the PCR run. The mRNA expression levels were measured by quantitative real-time PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). *Ppia* was used as the internal control. Each data point was analyzed by the comparative threshold cycle method ($\Delta\Delta C_t$ method). Primer pairs designed for evaluation of gene expression are as follows: *Glut2*, 5'-AATGGTCGCCTCATTCTTTG-3' and 5'-ATCAAGAGGGCTCCAGTCAA-3'; *Glut5*, 5'-TCATCTCTGTGTGGAAGTTG-3' and 5'-AGATCTGATCGGCGTAGTAG-3'; *Sglt1*, 5'-GTGCTGGGCTGGATATTTGT-3' and 5'-AGGCCCAAGGCTAGATTGAT-3'; *Pept1*, 5'-ATCATTGTGCTCATCGTGGC-3' and 5'-GTGCTTCAATCTCTGCTGGG-3'; *Gpr93*, 5'-GGTGCTGATGATAATGGTGCT-3' and 5'-GTAGCCAAAGGCCTGGTATTC-3'; *Casr*, 5'-GCATCAGGTATAACTTCCGTGG-3' and 5'-TTGGAGACGGTGTTACAGGTG-3'; *Gpr41*, 5'-

162 TTCTTGCAGCCACACTGCTC-3' and 5'-GCCCACCACATGGGACATAT-3'; *Gpr43*, 5'-
163 ACAGTGGAGGGGACCAAGAT-3' and 5'-GGGGACTCTCTACTCGGTGA-3'; *Gpr40*, 5'-
164 TTTGCGCTGGGCTTTCC-3' and 5'-GCTGGGAGTGAGTCGCAGTT-3'; *Gpr119*, 5'-
165 AGAAAGCGCCTATCACATCG-3' and 5'-CAACCTGCCTTTACCAGTTG-3'; *Cd36*, 5'-
166 CGCTTTCTGCGTATCGTCTG-3' and 5'-GATGCACGGGATCGTGTCT-3'; *Fatp1*, 5'-
167 TCTGTTCTGATTCTGTTCGG-3' and 5'-AAGATGCACGGGATCGTGTC-3'; *Fatp2*, 5'-
168 TCCTCCAAGATGTGCGGTACT-3' and 5'-TAGGTGAGCGTCTCGTCTCG-3'; *Fatp3*, 5'-
169 ATGACAGGGGAGCCTATTCG-3' and 5'-ATCCTTCAGCAGCTTGTCT-3'; *Fatp4*, 5'-
170 ACTGTTCTCCAAGCTAGTGCT-3' and 5'-GATGAAGACCCGGATGAAACG-3'; *Fatp5*, 5'-
171 CTACGCTGGCTGCATATAGATG-3' and 5'-CCACAAAGGTCTCTGGAGGAT-3', *Secretin*,
172 5'-AGCCCTTAGAGGACCAGCTC-3' and 5'-TGAACGATCAACAGCAGACC-3', *Glp-1*, 5'-
173 TGAAGACAAACGCCACTCAC-3' and 5'-TCATGACGTTTGGCAATGTT-3'. Others were
174 previously designed (Iwasaki *et al.* 2015, Sankoda *et al.* 2017).

175

176 **Statistical analysis**

177 Results are shown as dot plot or mean $\pm$ SEM. One or two data points of some results that
178 exceeded mean $\pm$ 2SD were excluded. Statistical significance was determined by Student's
179 t-test or one way analysis of variance with Tukey or Games-Howell test. *P* values < 0.05 were
180 considered statistically significant.

181

182 **Results**

Phenotype of CCK-Tomato mice

IRES and Cre recombinase were inserted downstream of the murine *CCK* locus in CCK-Cre mice (Taniguchi *et al.* 2011). With this construction, the promoter and coding region of both *Cck* genes were intact in CCK-Tomato mice. Body weight of the CCK-Tomato mice was similar to that of control mice during 9-29 weeks of age (Fig. 1A). There was no significant difference in non-fasting CCK levels between control and CCK-Tomato mice (Control mice 194.9±102.1mg/dl vs. CCK-Tomato mice 328.1±248.1mg/dl; P=0.10). There was no significant difference in food intake, energy expenditure and locomotor activity between CCK-Tomato mice and control mice (Fig. 1B and 1C). During OGTTs and OCTTs, blood glucose levels were not different between the two types of mice (Fig. 1D and 1E). Plasma insulin and CCK levels after glucose or corn oil administration were not different between the two groups. These results indicated that the *CCK-IRES-Cre* allele does not affect body weight gain, food intake, energy expenditure, locomotor activity, and glucose tolerance under 11% fat-containing diet-fed condition.

Number of I cells in CCK-Tomato mice

Under fluorescence microscopy, Tomato-positive cells were detected in upper SI, lower SI, and colon of CCK-Tomato mice, but not in stomach (data not shown). Immunohistochemical analysis showed that Tomato-expressing cells were identical to CCK-expressing cells in upper SI, lower SI, and colon of CCK-Tomato mice (Fig. 2A).

We then evaluated the number of Tomato-expressing cells in small intestine and colon by histological analysis. The length of villus and the number of Tomato-expressing cells in small intestine were greater than those in colon (Fig. 2B and 2C). The ratio of Tomato-expressing cell number / length of villus was significantly higher in upper SI and lower SI than that in colon (Fig. 2D). In addition, the number of Tomato-positive cells was calculated by flow cytometry system (Fig. 2E). Tomato-positive cells / epithelial cells in upper SI, lower SI, and colon was $0.95 \pm 0.30\%$, $0.54 \pm 0.14\%$, and $0.06 \pm 0.01\%$, respectively. Tomato-positive cell number was greater in upper SI and lower SI than that in colon, but the number significantly differed only between lower SI and colon. After purification of each 2,000 Tomato-positive and -negative cells, gene expression of *Cck* mRNA in the cells was evaluated (Fig. 2F). In upper SI, lower SI and colon, *Cck* mRNA expression was detected in Tomato-positive cells but not in Tomato-negative cells. In upper SI and lower SI, expression levels of *Cck* mRNA were higher in Tomato-positive cells than those in Tomato-negative cells. On the other hand, there was no significant difference in *Cck* mRNA expression levels between Tomato-positive and -negative cells in colon. *Cck* mRNA expression levels in Tomato-positive cells of upper SI and lower SI were significantly higher than those of colon.

Gene expression of molecules involved in fatty acid sensing in I cells

We then evaluated gene expression of G protein-coupled receptors (GPRs) and transporters for free fatty acid. In upper SI and lower SI, expression levels of the long-chain fatty acid (LCFA) receptors *Ffar4* (*Gpr120*) (Fig. 3A) and *Ffar1* (*Gpr40*) (Fig. 3B) and the

oleoylethanolamide (OEA) receptor *Gpr119* (Fig. 3C) mRNA were significantly higher in Tomato-positive cells than those in Tomato-negative cells. The expression levels did not differ between Tomato-positive and -negative cells in colon. In upper and lower SI, expression levels of the short-chain fatty acid (SCFA) receptor *Ffar2* (*Gpr43*) mRNA (Fig. 3D) were high in Tomato-positive cells compared to those in Tomato-negative cells, but expression levels of *Ffar3* (*Gpr41*) mRNA (Fig. 3E) did not differ between Tomato-positive and -negative cells. *Bile acid receptor transmembrane GPR 5* (*Tgr5*) mRNA was highly expressed in Tomato-positive cells compared to that in Tomato-negative cells in lower SI (Fig. 3F). Gene expression of the fatty acid transport protein (FATP) 1-5 and cluster of differentiation 36 (CD36) was also evaluated. *Fatp4* and *Cd36* mRNA expressions were detected in Tomato-positive cells, but the expression levels did not differ between Tomato-positive cells and -negative cells (data not shown). *Fatp1*, *Fatp2*, *Fatp3*, and *Fatp5* expressions were not detected in Tomato-positive or -negative cells (data not shown).

Gene expression of molecules involved in glucose, fructose, and amino acid sensing in I cells

In upper SI, expression levels of glucose transporters *Sglt1* (Fig. 4A) and *Glut2* (Fig. 4B) and fructose transporter *Glut5* (Fig. 4C) mRNA tended to be higher in Tomato-positive cells than those in Tomato-negative cells, but there was not a significant difference between the two groups. Expression levels of *Sglt1*, *Glut2*, and *Glut5* mRNA tended to be higher in

upper SI than those in lower SI. In colon, these expressions were not detected in Tomato-positive cells.

Gene expression levels of *peptide transporter 1 (Pept1)* (Fig. 4D) and *Gpr93* (Fig. 4E), which are protein metabolite-sensing molecules, did not differ between Tomato-positive and -negative cells in upper and lower SI. In colon, *Gpr93* mRNA was not detected in Tomato-positive or -negative cells, whereas *Pept1* mRNA was highly expressed in Tomato-negative cells compared to that in Tomato-positive cells. mRNA of the *calcium-sensing receptor (Casr)*, which is reported to be involved in amino acid-induced gut hormone secretion (Mace *et al.* 2012), was highly expressed in Tomato-positive cells of upper SI (Fig. 4F). On the other hand, *Casr* mRNA was not detected in Tomato-negative cells.

Gene expression of gut hormones in I cells

Some gut hormones are reported to be co-expressed in enteroendocrine cells (Egerod *et al* 2012, Habib *et al* 2012). In addition, nutrient-sensing molecules are reported to be expressed in glucose-dependent insulintropic polypeptide / gastric inhibitory polypeptide (GIP)-producing K cells and glucagon-like peptide-1 (GLP-1)-producing L cells and to be involved in nutrient-induced GIP and GLP-1 secretion (Iwasaki *et al* 2015, Reimann *et al* 2012). We therefore evaluated mRNA expression of other gut hormones in Tomato-positive cells and -negative cells. *Secretin* and *Gip* were found to be highly expressed in Tomato-positive cells of upper SI and lower SI (Fig. 5A and 5B). On the other hand, *Glp-1* was expressed in I cells of upper SI and colon (Fig. 5C). These three gut hormones were not

expressed in Tomato-negative cells of SI and colon. These results indicate that some gut hormone-producing cells overlap with I cells.

Discussion

In previous studies, characterization of I cells was done using purified cells from the intestine of transgenic (CCK-GFP Tg) mice expressing green fluorescence protein (GFP) under control of a *Cck* promoter derived from a BAC clone (Liou *et al.* 2011). Although expression of some molecules associated with nutrient sensing in I cells has been reported, these analyses focused on I cells expressed in small intestine. We have established CCK-Tomato mice in which Tomato is expressed under endogenous and native *Cck* promoter; the present study is the first to report I cell number and the expression of CCK and various molecules associated with nutrient sensing in I cells of each part of the GI tract.

I cells in the GI tract have previously been evaluated by immunohistochemistry with anti-CCK antibodies. I cells are distributed throughout the small intestine and large intestine, but the number of I cells in the small intestine is greater (Fakhry *et al.* 2017). Our findings regarding I cell number in the GI tract of CCK-Tomato mice by immunohistochemistry with anti-RFP antibodies are consistent with previous studies. However, CCK-Tomato mice enabled evaluation of not only I cell number in the GI tract, but also *Cck* gene expression in isolated I cells. Using the flow cytometry system, I cells among epithelial cells of upper SI, lower SI, and colon were found to occur at the rate of $0.95 \pm 0.30\%$, $0.54 \pm 0.14\%$, and 0.06

± 0.01%, respectively. A majority of the I cells were detected in upper SI, and their frequency decreased toward the distal part of the GI tract. The expression levels of *Cck* mRNA in isolated I cells from upper SI were highest in the GI tract, and decreased toward the distal part of the GI tract. These results indicate that I cells in upper SI are the main contributor to CCK secretion in response to various nutrients.

Fat ingestion strongly stimulates CCK secretion (Green *et al.* 1989). GPR40, GPR120, and GPR119 are receptors activated by the nutrients LCFAs and OEA. These receptors are reported to be expressed in incretin-producing cells and to be involved in incretin secretion (Iwasaki *et al.* 2015, Sankoda *et al.* 2019). Bile, which is composed of bile acids, is important for fat digestion and absorption, and is reported to induce CCK secretion in the fasting state (Meyer-Gerspach *et al.* 2013). TGR5, a bile acid receptor, is expressed in L cells and is involved in GLP-1 secretion (Brighton *et al.* 2015). Previous studies using the mouse intestinal cell line STC-1 and *GPR40*- or *GPR120*-knockout mice showed that GPR40 and GPR120 are involved in CCK secretion in response to LCFAs and fat (Tanaka *et al.* 2008, Sankoda *et al.* 2017). On the other hand, it remains unclear whether GPR119 and TGR5 are involved in CCK secretion, although these receptors are expressed in I cells of the small intestine of CCK-GFP Tg mice (Sykaras *et al.* 2012). In our study, *Gpr40*, *Gpr120*, and *Gpr119* were found to be expressed mainly in Tomato-positive cells of upper and lower SI and were not detected in the cells of colon. *Tgr5* was highly expressed in Tomato-positive cells of lower SI. These results indicate that these receptors may well be involved in CCK secretion upon fat ingestion. Indeed, some LCFA transporters are expressed in various tissues

including intestine. We evaluated gene expression of *Fatp1-5* and *Cd36* mRNA in Tomato-positive cells and -negative cells, and found that *Fatp4* and *Cd36* are expressed in Tomato-positive cells as well as -negative cells. Our data is consistent with the previous report showing that FATP4 and CD36 are involved in GLP-1 and CCK secretion, respectively (Poreba *et al.* 2012, Sundaresan *et al.* 2013), and go further to suggest their role in CCK secretion upon fat ingestion.

Glucose and fructose are known to induce CCK secretion (Kuhre *et al.* 2014); the glucose transporters SGLT1 and GLUT2 and fructose transporter GLUT5 are expressed in enteroendocrine cells and are involved in glucose and fructose-induced gut hormone secretion, respectively (Reimann *et al.* 2008, Parker *et al.* 2009, Mace *et al.* 2012, Gorboulev *et al.* 2012). The previous study using small intestine of CCK-GFP Tg mice revealed that SGLT1 is expressed in I cells as well as other intestinal epithelial cells (Kaelberer *et al.* 2018), but the expression of GLUT2 and GLUT5 in I cells was not examined. In the present study, *Sglt1*, *Glut2*, and *Glut5* were found to be expressed in both Tomato-positive cells and -negative cells in upper SI, lower SI, and colon, and the expression levels tended to be higher in upper SI than those in lower SI and colon. These results revealed that the expression patterns in Tomato-positive and -negative cells in SI and colon are similar among the three transporters.

Peptide transporter PEPT1, peptone receptor GPR93, and amino acid-sensing receptor CASR have been identified as protein metabolite-sensing molecules associated with gut hormone secretion (Feng *et al.* 2010, Mace *et al.* 2012, Diakogiannaki *et al.* 2013).

In vitro and *in vivo* studies have shown that GPR93 and CASR are involved in amino acid-induced CCK secretion from I cells (Choi *et al.* 2007, Liou *et al.* 2011); PEPT1, GPR93, and CASR have been reported to be expressed in I cells of small intestine of CCK-GFP Tg mice (Liou *et al.* 2011). In the present study, these molecules were found to be expressed in Tomato-positive cells of upper and lower SI; *Pept1* and *Gpr93* were found to be expressed in both Tomato-positive and -negative cells. On the other hand, *Casr* was expressed in Tomato-positive cells but not in Tomato-negative cells, indicating that *Casr* is specifically expressed in I cells. In colon, expression levels of *Pept1* mRNA were significantly higher in Tomato-negative cells than those in Tomato-positive cells. As PEPT1 is reported to play an important role in the regulation of water absorption into intestinal epithelium (Wuensch *et al.* 2013), the molecule might be highly expressed in intestinal epithelial cells detected as Tomato-negative cells.

CCK plays a key physiological role in fat absorption and regulation of energy intake. Thus, regulation of nutrient-sensing molecule-mediated CCK secretion might represent possible novel therapeutic approaches to obesity and type 2 diabetes. Analysis using CCK-Tomato mice revealed that I cells are broadly distributed throughout the GI tract, and that the various molecules involved in nutrient sensing are abundantly expressed in I cells.

Declaration of interests

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Pharma Ltd. All other authors have nothing to disclose.

Author contribution statement

T.K. and N.H. planned the study, researched data, contributed to discussion, wrote, reviewed
and edited the manuscript. E.I-O., A.S., T.H., X.L., T.Y., and S.Y. researched data. N.I. planned
the study, contributed to discussion, and edited the manuscript. All authors approved the
final version of the manuscript.

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373

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Figure legends

Figure 1. Phenotype of CCK-Tomato mice

(A) Body weight, (B) food consumption, (C) energy expenditure, and locomotor activity (n=5-6). Blood glucose levels and plasma insulin and CCK levels during (D) OGTTs and (E) OCTTs (n=6). Control mice (white circles) and CCK-Tomato mice (black circles). #P<0.05, ##P<0.01 vs plasma glucose levels at 0 min, n.s.; not significant.

Figure 2. Localization of I cells in the GI tract of CCK-Tomato mice.

(A) Immunohistochemical images of the upper SI, lower SI, and colon in CCK-Tomato mice. Red: Tomato-expressing cells, Green: CCK-expressing cells, Yellow: merged image. (B) Length of villus and (C) number of Tomato-expressing cells in 50 villi and crypts were measured by immunohistochemistry (n=6). (D) Tomato-expressing cells were quantified as the number of Tomato-expressing cells / length of mucous membrane (n=6). (E) The levels of red fluorescence in isolated epithelial cells were evaluated and Tomato-positive cells were counted from the data of flow cytometry analysis (n=6). (F) Expression of *Cck* mRNA in I cells (n=6-7). #P<0.05 and ##P<0.01 vs. Tomato negative cells, *P<0.05, n.s.; not significant.

Figure 3. Expressions of FFARs and TGR5 mRNA and in I cells.

Data are shown as relative expression to that of *Ppia* expression in parallel in the same samples (A: *Gpr120*, B: *Gpr40*, C: *Gpr119*, D: *Gpr43*, E: *Gpr41*, F: *Tgr5*) (n=6-8). #P<0.05 and ##P<0.01 vs. Tomato-negative cells, *P<0.05 and **P<0.01, n.s.; not significant.

Figure 4. Expressions of glucose transporters, amino acid transporter, and amino acid receptors mRNA in I cells.

Data are shown as relative expression to that of *Ppia* expression in parallel in the same samples (A: *Sglt1*, B: *Glut2*, C: *Glut5*, D: *Pept1*, E: *Gpr93*, F: *Casr*) (n=6-8). #P<0.05 and ##P<0.01 vs. Tomato-negative cells, *P<0.05 and **P<0.01, n.s.; not significant.

Figure 5. Expressions of gut hormones mRNA in I cells.

Expression levels of (A) *Secretin* mRNA, (B) *Gip* mRNA, and (C) *Glp-1* mRNA in Tomato-positive and -negative cells. Data are shown as relative expression to that of *Ppia* expression in parallel in the same samples (n=6-8). #P<0.05 and ##P<0.01 vs. Tomato-negative cells, *P<0.05 and **P<0.01, n.s.; not significant.

Fig. 1

Fig. 2

A

B

C

D

E

Tomato-positive cells / Intestinal epithelial cells (%)

F

Fig. 3

Fig. 4

Fig. 5