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ORIGINAL ARTICLE

Dual regulatory role for phosphatase and tensin homolog in specification of intestinal endocrine cell subtypes

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Abstract

AIM: To investigate the impact of phosphatase and tensin homolog (Pten) in the specification of intestinal enteroendocrine subpopulations.

METHODS: Using the Cre/loxP system, a mouse with conditional intestinal epithelial *Pten* deficiency was generated. *Pten* mutant mice and controls were sacrificed and small intestines collected for immunofluorescence and quantitative real-time polymerase chain reaction. Blood was collected on 16 h fasted mice by cardiac puncture. Enzyme-linked immunosorbent assay was used to measure blood circulating ghrelin, somatostatin (SST) and glucose-dependent insulinotropic peptide (GIP) levels.

RESULTS: Results show an unexpected dual regulatory role for epithelial Pten signalling in the specification/differentiation of enteroendocrine cell subpopulations in the small intestine. Our data indicate that *Pten* positively regulates chromogranin A (CgA) expressing subpopulations, including cells expressing secretin, ghrelin, gastrin and cholecystokinin (CCK). In contrast, *Pten* negatively regulates the enteroendocrine subtype specification of non-expressing CgA cells such as GIP and SST expressing cells.

CONCLUSION: The present results demonstrate that *Pten* signalling favours the enteroendocrine progenitor to specify into cells expressing CgA including those producing CCK, gastrin and ghrelin.

Key words: Phosphatase and tensin homolog; Enteroendocrine cells; Intestinal epithelial cell specification; Chromogranin A

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INTRODUCTION

The phosphatase and tensin homolog (*PTEN*) tumour

suppressor gene is one of the most frequently mutated/deleted genes in various human cancers[1,2]. PTEN is a lipid and protein phosphatase. Its best-known substrate, the phosphatidylinositol 3,4,5-trisphosphate (PIP3), is a lipid second messenger mainly produced by class IA phosphatidylinositol 3-kinases (PI3Ks)[3]. PTEN dephosphorylates PIP3 to produce phosphatidylinositol 4,5-bisphosphate, which inhibits PI3K-dependent effectors such as the downstream kinases Akt and pyruvate dehydrogenase kinase 1. PI3Ks have been implicated in many signalling pathways that regulate cell survival, growth, proliferation, migration, phagocytosis, and metabolism[4]. PTEN has also been shown to regulate genomic stability[5,6], stem cell renewal[7,8], senescence[9] and cell differentiation[10-12].

The multiple cellular functions of PTEN suggest that this protein plays major roles in overall system homeostasis. Indeed, homozygous deletion of *Pten* in the mouse causes early embryonic lethality by embryonic day (E) 9.5, whereas *Pten* heterozygous mice ($Pten^{+/-}$) develop, over a period of time, various dysplasia and hyperplasia in organs such as the breast, thyroid, prostate and intestine[1,2,13]. As reviewed by Knobbe *et al*[14], *Pten* has also been conditionally deleted in many specific tissues. These models have established the tumour suppressive function of *Pten* but have also unravelled its important role in the maintenance of normal physiological functions in various tissues such as the immune system, skin, lung, liver, pancreas and hypothalamus[14].

In a previous study, we reported that Pten is important for intestinal homeostasis[10]. The villin-Cre system was used to specifically inactivate *Pten* in the mouse intestinal epithelium. *Pten* mutant mice developed an intestinalomegaly associated with an increase in epithelial cell proliferation. Histological analysis also demonstrated significant perturbation of the crypt-villus architecture, a marked increase in goblet cells and a decrease in enteroendocrine cells, suggesting a role for *Pten* in the commitment of the multipotential-secretory precursor cell[10].

Enteroendocrine cells are hormone-secreting epithelial cells that are scattered throughout the gastrointestinal epithelium and although they represent only 1% of the intestinal epithelium, taken together, they constitute the major endocrine organ of the body[15,16]. At least 10 different enteroendocrine cell types have been identified in the small intestine and are classified based on their main hormonal products[16,17]. The various hormones produced by these endocrine cells [ghrelin (GHR), gastrin-releasing peptide (GRP), glucose-dependent insulinotropic peptide (GIP), secretin (SCT), peptide YY (PYY), glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), cholecystokinin (CCK), neurotensin, serotonin, substance *P*, somatostatin (SST) and motilin] control important physiological functions, such as gastrointestinal motility, glycaemia, exocrine pancreatic secretion, biliary secretion, digestion, gut epithelial renewal and appetite[16,18,19]. Most enteroendocrine cell types secrete chromogranin A (CgA), a soluble glycoprotein stored with hormones and neuropeptides in secretory granules of endocrine cells. The important role of enteroendocrine cells in whole body homeostasis prompted us to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various enteroendocrine subpopulations. Using our Cre/loxP *Pten* conditional knock out mouse model[10], we report herein an unexpected dual regulatory role for epithelial Pten signalling in the specification of enteroendocrine cells. Our data indicate that Pten positively influences the determination and specification of CgA-expressing cell subpopulations in the small intestine including those expressing secretin, ghrelin, gastrin and CCK. Conversely, Pten limits determination and specification of non-expressing CgA endocrine cell subpopulations, including GIP and SST.

MATERIALS AND METHODS

Animals

BALB/c-$Pten^{fx/fx}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, United states). The C57BL/6 12.4KbVilCre transgenic line was provided by Dr. Deborah Gumucio (University of Michigan, Ann Arbor, MI, United states)[20]. Genomic DNA was isolated using the Spin Doctor genomic DNA kit from Gerard Biotech according to the manufacturer's protocol. Both mutations were genotyped following protocols already published[20] or as directed by The Jackson Laboratory. For this study, the BALB/c-$Pten^{fx/fx}$ mice mice were first crossed with the C57BL/6 12.4KbVilCre to generate F1-generation heterozygous animals. F1-generation heterozygous animals were then backcrossed with BALB/c-$Pten^{fx/fx}$ mice to produce F2-generation experimental animals. All experiments were conducted in F2-generation experimental animals. All mice were maintained on regular diet in the transgenic mouse facility at the Faculty of Medicine and Health Sciences of the Université de Sherbrooke. All experiments were approved by the animal research committee of the Faculty of Medicine and Health Sciences of the Université de Sherbrooke.

Tissue collection, tissue preparation, RNA extraction and gene expression analysis

Digestive tracts from 120-d-old $Pten^{\Delta IEC}$ mice and control littermates were fixed in 4% paraformaldehyde (PFA) overnight at 4 ℃, then dehydrated and embedded in paraffin. Sections of 5 μm were applied to Probe-On Plus slides (Fisher Scientific, Ottawa, ON, Canada) and kept at room temperature until used[10,21]. Total RNA was isolated and processed using the Totally RNA extraction kit (Ambion, Grand Island, NY, United states). Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR were performed as described previously[21]. Quantitative real-time PCR conditions were as follows: one cycle of 15 min at 95 ℃; 50 cycles at 95 ℃ for 15 s; 59 ℃ for 30 s and 72 ℃ for 30 s. The following forward and reverse primers were used: Hairy and enhancer of split 1 (NM_008235), 5'-TTCCAAGCTAGAGAAGGCAGA-3', 5'-GTTGATCTGGGT-

CATGCAGTT-3'; Atonal homolog 1 (NM_007500), 5'-GCTTCCTCTGGGGGTTACTC-3', 5'-ACAACGATCACCACAGACCA-3'; Neurogenin 3 (NM_009719), 5'-CGGATGACGCCAAACTTACAAAG-3' 5'-CACAAGAAGTCTGAGAACAACAG-3'; Growth factor independent 1 (NM_010278), 5'-TCCGAGTTCGAGGACTTTTG-3', 5'-CATGCATAGGGCTTGAAAGG-3'; Neurogenic differentiation 1 (NM_010894), 5'-AGCCACGGATCAATCTTCTCT-3', 5'-GACGTGCCTCTAATCGTGAAA-3'; Pancreatic and duodenal homeobox 1 (NM_008814), 5'-AACCCGAGGAAAACAAGAGG-3', 5'-TTCAACATCACTGCCAGCTC-3'; Forkhead box O1 (NM_019739), 5'-CCGGAGTTTAACCAGTCCAA-3', 5'-TGCTCATAAAGTCGGTGCTG-3'; Forkhead box a1 (NM_008259), 5'-CAAGGATGCCTCTCCACACTT-3', 5'-TGACCATGATGGCTCTCTGAA-3'; Forkhead box a2 (NM_010446), 5'-GAGCACCATTACGCCTTCAAC-3', 5'-GGCCTTGAGGTCCATTTTGT-3'; PDGB (NM_013551), 5'-TGCACGATCCTGAAACTCTG-3', 5'-TGCATGCTATCTGAGCCATC-3'.

Immunofluorescence

Immunofluorescence staining was performed as previously described[21]. The following antibodies were used at the indicated dilutions: FITC-conjugated anti-mouse IgG (1:200, Vector, Burlingame, CA, United states), FITC-conjugated anti-rabbit IgG (1:200, Vector), AlexaFluor 568 donkey anti-goat (1:400, Invitrogen, Grand Island, NY, United states), AlexaFluor 488 donkey anti-goat (1:400, Invitrogen), AlexaFluor 488 donkey anti-rabbit (1:400, Invitrogen), rabbit anti-SP-1 CgA (1:1000, ImmunoStar, Hudson, WI, United states), goat anti-CgA (1:50, SantaCruz, Santa Cruz, CA, United states), rabbit anti-gastrin (1:200, Chemicon, Billerica, MA, United states), goat anti-GIP (1:100, SantaCruz), mouse anti-serotonin (1:200, LabVision, Kalamazoo, MI, United states), rabbit anti-secretin (1:1000, Phoenix pharmaceuticals, Burlingame, CA, United states), goat anti-SST (1:100, SantaCruz), goat anti-ghrelin (1:100, SantaCruz), rabbit anti-CCK (1:100, ab92128 gift from Rehfeld JF)[22].

Measurement of circulating hormone levels

Blood was collected on 16 h fasted mice by cardiac puncture. Serum levels of total ghrelin and GIP were measured using Millipore ELISA kits (EZRGRT-91K, EZRMGIP-55K) (Millipore, Billerica, MA, United states) according to manufacturer's instructions. Serum levels of SST were measured using the Phoenix Pharmaceuticals ELISA kit EK-060-03, according to the manufacturer's instructions.

Statistical analysis

All cell count analyses were performed using continuous serial sections from low-powered fields of well-oriented intestinal cross-sections in a blind manner on an average of 10 independent fields per animal. Three different intestinal sections were evaluated: duodenum, jejunum and ileum. The total number of enteroendocrine cells was counted per crypt-villus axis. Image magnification was calibrated by comparison with a stage micrometer (graticulesTM Ltd., Tonbridge, Kent, England). Statistical analyses were performed using two-way ANOVA. For qRT-PCR, data were analyzed using the Mann Whitney-test for abnormal distribution. Differences were considered significant with a P value of < 0.05. All statistical analyses were carried out using Graph Pad Prism 5 (Graph Pad Inc., San Diego, CA).

RESULTS

CgA is not expressed in all enteroendocrine cell subtypes of the mouse small intestinal epithelium

Mice homozygous for the floxed exon 5 of the *Pten* gene[23] were bred to the *villin*-Cre transgenic line, which directs expression of the transgene in all epithelial cells of the small intestine and colon, including stem cells, but not in the mesenchymal compartment[20]. Conditional knockout mice for *Pten* ($Pten^{\Delta IEC}$) were born at the expected Mendelian ratios, survived for more than 1 year, and grew normally without obvious gross physical abnormalities[10]. In a previous study with these mice, we reported an overall decrease in the number of enteroendocrine cells using a CgA antibody[10]. Over the years, there has been a lingering controversy where a number of studies showed that all endocrine cell subpopulations express CgA[18,24,25] while others reported that some cell subpopulations do not express CgA[17,26]. Therefore, individual analysis of various intestinal endocrine subpopulations was first performed for their co-expression with CgA in the mouse small intestine. Double-labelling with CgA (Figure 1B, E, H and K) and specific antibodies directed against ghrelin (Figure 1A), CCK (Figure 1D), gastrin (Figure 1G) and secretin (Figure 1J) confirmed co-expression of CgA with ghrelin (Figure 1C) as well as with CCK- (Figure 1F), gastrin- (Figure 1I) and secretin- (Figure 1L) producing enteroendocrine cells in the mouse small intestine. On the other hand, double-labelling with CgA antibody (Figure 1N and Q) and specific antibodies directed against GIP (Figure 1M) and SST (Figure 1P) supported the exclusion of co-expression between GIP (Figure 1O), SST (Figure 1R) and CgA in the mouse small intestine. The specificity of our CgA antibodies was confirmed with the use of two different CgA antibodies from two different commercial sources, in which the exact same cells were labeled in consecutive sections from a same specimen with both antibodies.

Loss of intestinal epithelial Pten impairs the specification of CgA expressing enteroendocrine cells

Enteroendocrine subtype specification appears to be regulated by distinct mechanisms[17,26]. Since our previous study only investigated CgA-expressing cells, the impact of *Pten* loss of expression on the specification of the various enteroendocrine cell subpopulations in the small intestine was further analyzed. We first analyzed how the loss of epithelial *Pten* alters specification of CgA-expressing en-

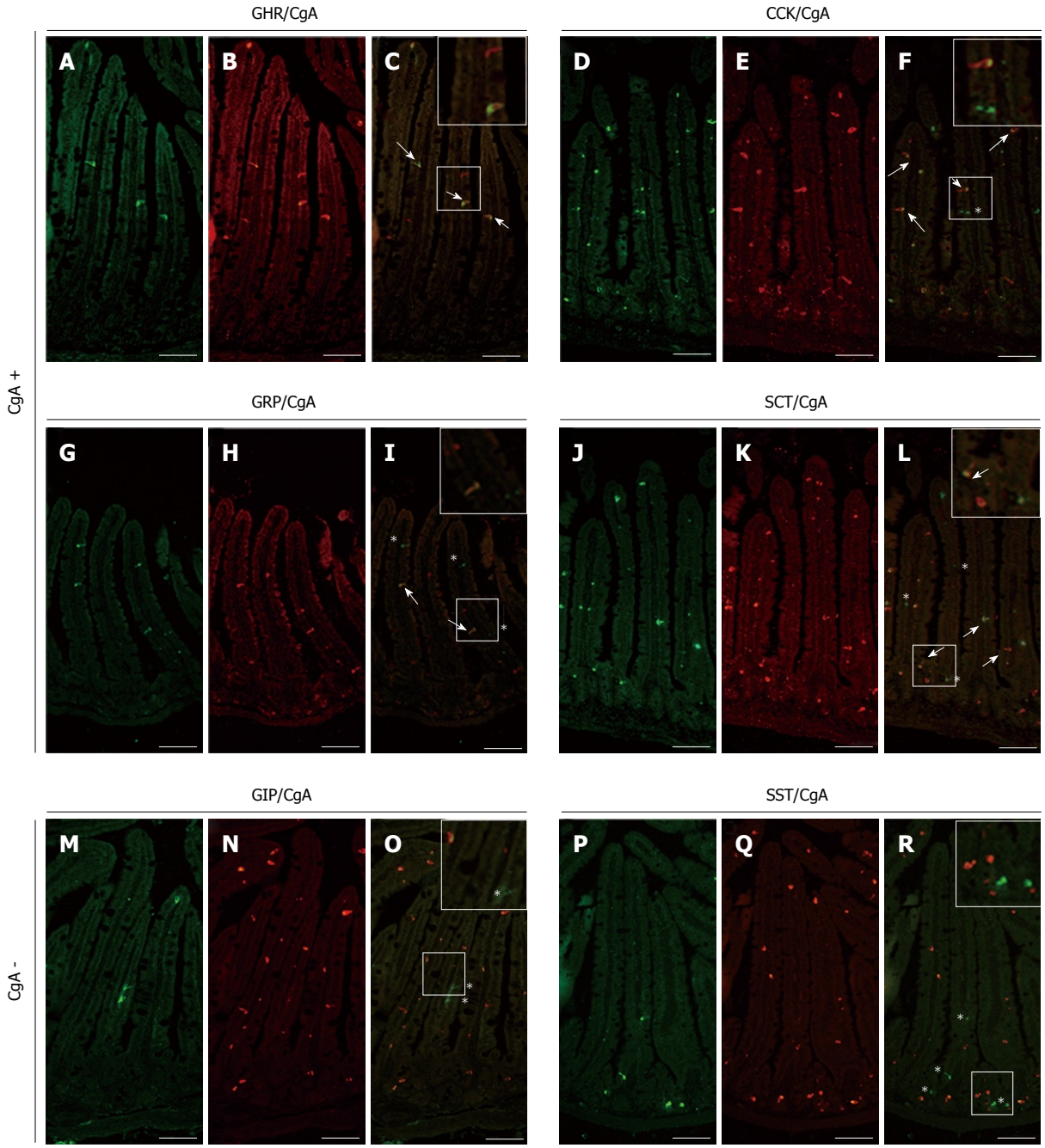

Figure 1 Analysis of chromogranin A co-expression in mouse small intestinal endocrine subpopulations. Small intestine sections of adult control mice were co-immunostained with antibodies directed against ghrelin (GHR) (A), cholecystokinin (CCK) (D), gastrin-releasing peptide (GRP) (G), secretin (SCT) (J) glucose-dependent insulinotropic peptide (GIP) (M) or somatostatin (SST) (P) and against chromogranin A (CgA) (respectively B, E, H, K, N and Q). The arrows in images C, F, I and L show co-expression of CgA respectively with GHR, CCK, GRP and SCT while asterisks in images F, I, L, O and R point to CgA-negative enteroendocrine cells. The number of arrows and asterisks within the crypt-villus axis represents the average proportion of labelled cells per units. Scale bar: 50 μm.

teroendocrine cells along the various sections of the small intestine (duodenum, jejunum and ileum). Although some enteroendocrine cells are restricted to specific regions of the small intestine, each region was analyzed in order to verify the possible delocalization of subpopulations along the rostro-caudal axis of the gut. The intestinal mucosa of $Pten^{\Delta IEC}$ and control mice was stained with specific markers for each enteroendocrine cell subtype and positive cells were counted (Figure 2). A significant decrease of 29% in the jejunum (1.2 positive cells per crypt-villus axis *vs* 1.7) and 51% in the ileum (0.25 cell *vs* 0.52 cell) was observed (Figure 2C) in the ratio of positive ghrelin cells in *Pten* mutant mice (Figure 2B) compared to control littermates (Figure 2A). A modest but significant decrease of 10% (Figure 2F) was also observed in the ratio of positive CCK cells in the duodenum of the mutant mice (4.4 cell *vs* 4.7 cell) (Figure 2E). There was also a significant 23% decrease in gastrin-positive cells in the jejunum (1 cell *vs* 1.3 cell) and a decrease of 29% in the ileum (0.34 cell *vs* 0.44 cell) in $Pten^{\Delta IEC}$ (Figure 2H and I) when compared to

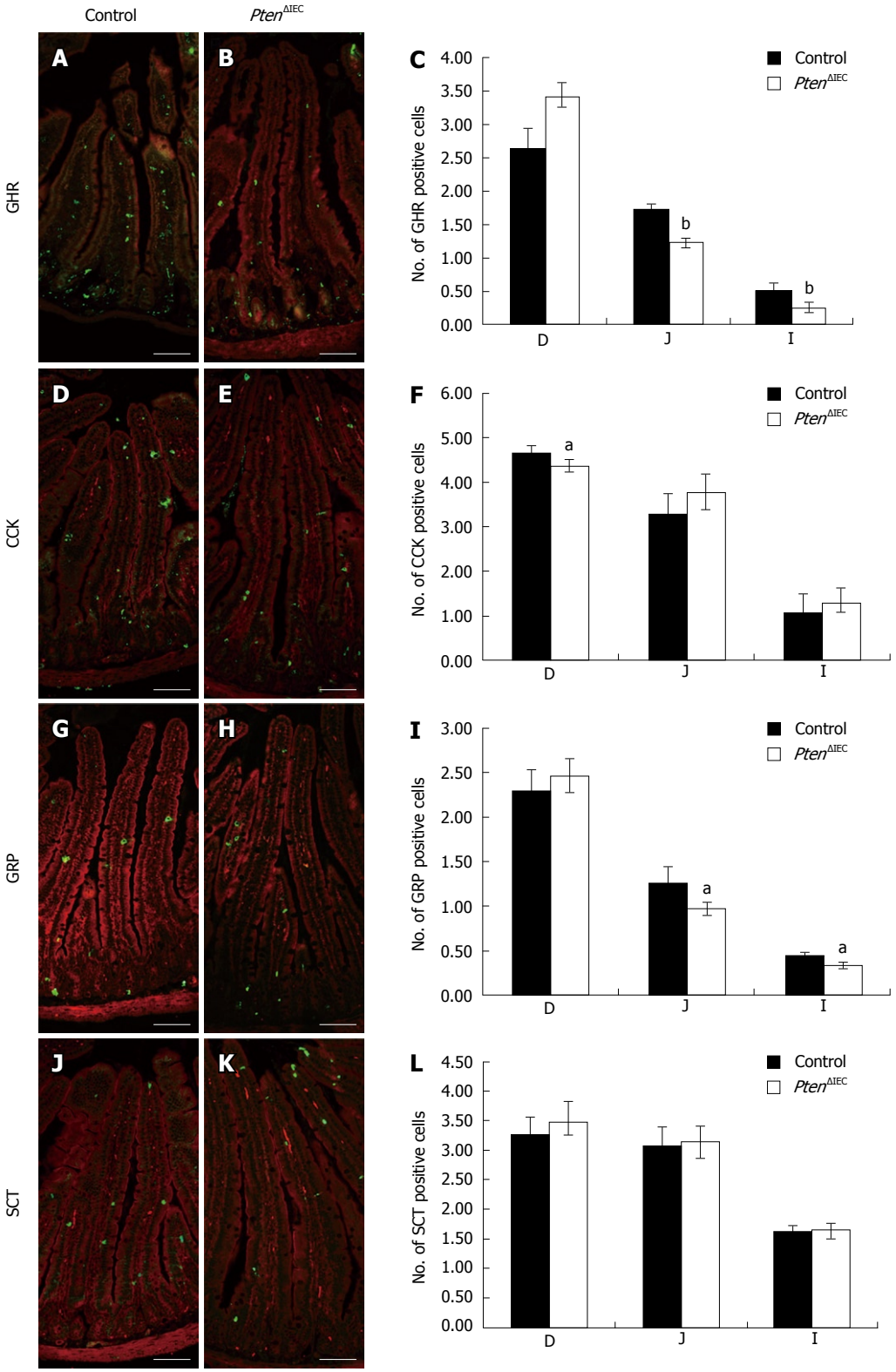

Figure 2 Epithelial *Pten* positively regulates commitment of chromogranin A-positive enteroendocrine subpopulations in the small intestine. Duodenum, jejunum and ileum of adult control and *Pten*$^{\Delta IEC}$ mice were immunostained with antibodies against ghrelin (GHR) (A and B), cholecystokinin (CCK) (D and E), gastrin-releasing peptide (GRP) (G and H) and secretin (SCT) (J and K). Positive cells were counted from intestinal sections of controls (n = 6) and mutants (n = 5). Statistical analysis (C, F, I, L) represents the average number of positive cells per crypt-villus axis in each section of the intestine. Error bars represent SE. Scale bar: 50 μm. D: Duodenum; J: Jejunum; I: Ileum. $^{a}P < 0.05$, $^{b}P < 0.001$.

control mice (Figure 2G and I). Finally, secretin immunostaining showed no modulation in the number of secretin-positive cells in *Pten*$^{\Delta IEC}$ (Figure 2K and L) *vs* control mice (Figure 2J and L). Taken together, these results suggest that *Pten* positively influences production of CgA-expressing enteroendocrine cell subpopulations.

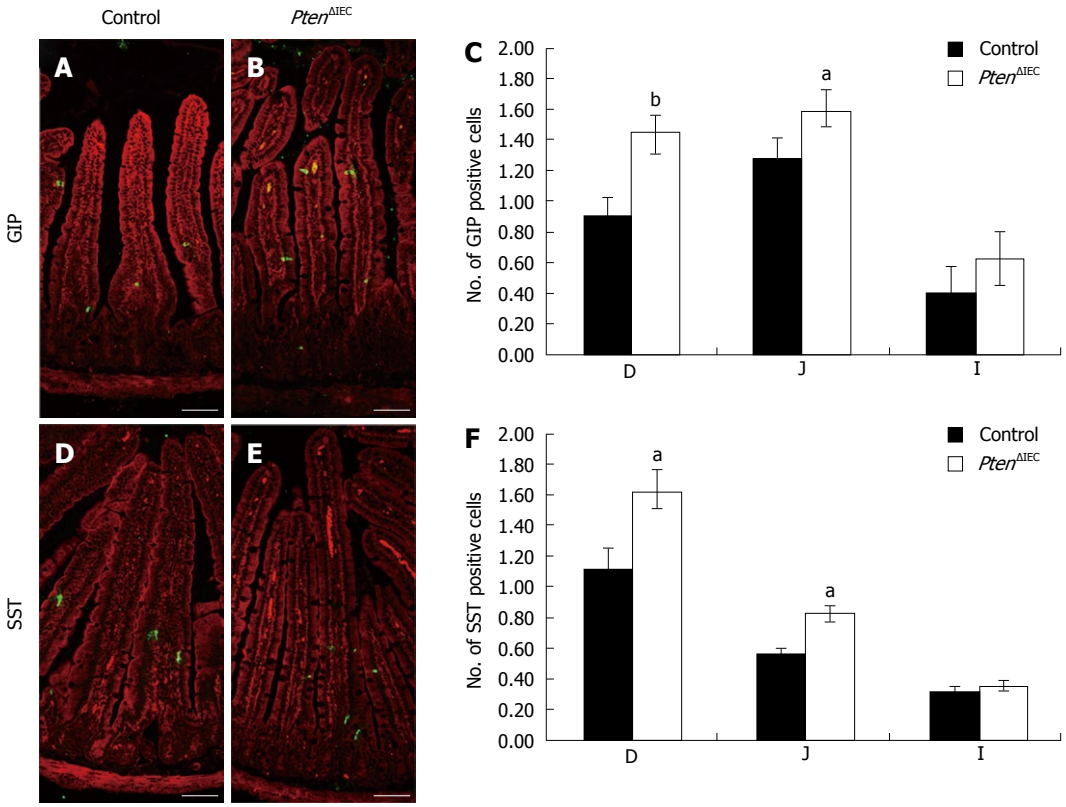

Figure 3 Epithelial *Pten* negatively regulates commitment of chromogranin A-negative enteroendocrine subpopulations in the small intestine. Duodenum, jejunum and ileum of adult control and *Pten*$^{\Delta IEC}$ mice were immunostained with antibodies against glucose-dependent insulinotropic peptide (GIP) (A and B) and somatostatin (SST) (D and E). Positive cells were counted from intestinal sections of controls (n = 6) and mutants (n = 5). Statistical analysis (C and F) represents the average number of positive cells per crypt-villus axis in each section of the intestine. Error bars represent SE. Scale bar: 50 μm. D: Duodenum; J: Jejunum; I: Ileum. [a]$P < 0.05$, [b]$P < 0.01$.

Loss of epithelial intestinal Pten positively influences the specification of CgA negative enteroendocrine cells

We next examined if the specification of CgA-negative cells was affected following the loss of epithelial *Pten*. As illustrated in Figure 3, there was a marked 61% (duodenum) and 25% (jejunum) increase in GIP-positive cells in *Pten*$^{\Delta IEC}$ (Figure 3B and C) when compared to control mice (Figure 3A and C) (respectively 1.45 positive cells *vs* 0.9 in the duodenum and 1.6 cells *vs* 1.28 in the jejunum). SST immunostaining in both duodenum and jejunum revealed an increase of 45% in the number of SST-positive cells in *Pten*$^{\Delta IEC}$ (Figure 3E and F) *vs* control mice (Figure 3D and F) (respectively 1.65 cells *vs* 1.15 cell and 0.85 cell *vs* 0.6 cell). Hence, these data suggest that Pten signalling negatively controls specification of CgA-negative cells in the intestinal epithelium.

Loss of epithelial Pten signalling leads to deregulation of circulating GIP and SST levels

In light of these observations, we next investigated whether deregulation in the number of enteroendocrine cells in the intestinal epithelium of the *Pten*$^{\Delta IEC}$ mice has an impact on their circulating levels. We chose to focus on the enteroendocrine subpopulations where the deregulation was more considerable. Circulating ghrelin, GIP and SST levels were analysed by ELISA assay. A 1.5-fold and 1.3-fold increase in GIP (Figure 4B) and SST (Figure 4C) levels, respectively, were observed in *Pten*$^{\Delta IEC}$ mice when compared to control littermates. No significant difference in ghrelin levels was observed between *Pten*$^{\Delta IEC}$ mice and control mice (Figure 4A).

Pten expression impacts differently on various pro-enteroendocrine specification factors

Comparative analysis of secretory lineage and specific pro-enteroendocrine determination factors was next investigated by quantitative PCR to clarify the role of *Pten* during enteroendocrine subtype specification. The Notch pathway, and more specifically the transcription factors Hairy enhancer of Split (Hes-1) and Math1, is crucial in the determination of the intestinal progenitor cell to absorptive or secretory cell fate (Figure 5)[27,28]. We also investigated if loss of epithelial *Pten* could deregulate the production of secretory precursors. Quantitative PCR analysis of mutant *vs* wild-type littermates revealed no modulation of Math1 or Hes-1 mRNA levels in the mutant animals (Table 1). Modifications downstream of the Notch pathway during enteroendocrine cell determination were also subsequently assessed. The proendocrine bHLH transcription factor Ngn3 has been shown to contribute to the maintenance and specification of enteroendocrine precursors (Figure 5)[29]. Our analysis revealed that the Ngn3 mRNA expression was significantly reduced by 2.07-fold in the mutant animals (Table 1). BETA2/

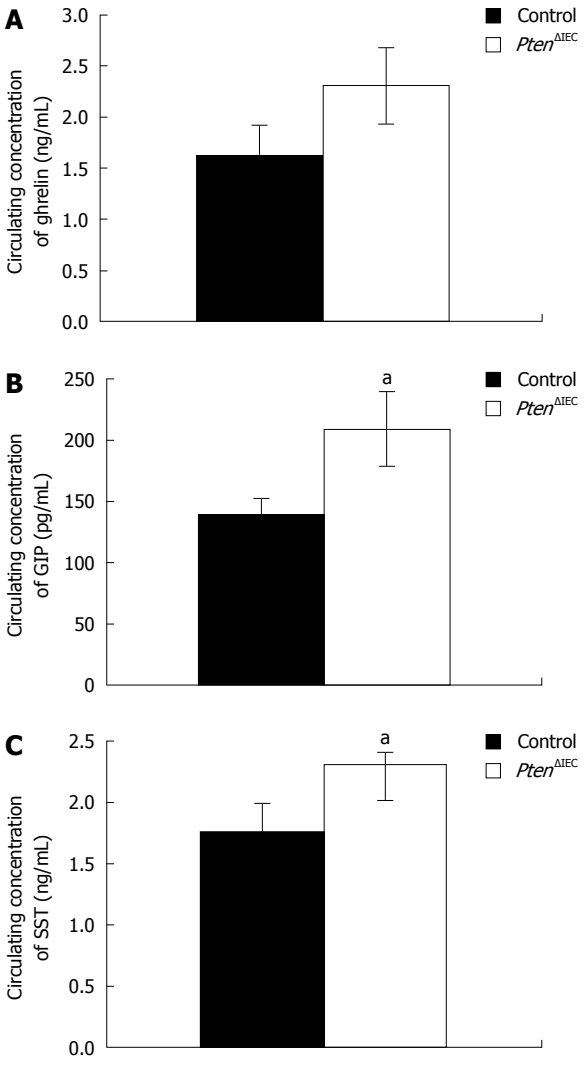

Figure 4 Loss of epithelial *Pten* signalling modulates circulating levels of glucose-dependent insulinotropic peptide and somatostatin. A: Analysis of circulating ghrelin level revealed no significant modulation between adult *Pten*$^{\Delta IEC}$ mice (n = 10) and control littermates (n = 10); B: Analysis of circulating glucose-dependent insulinotropic peptide (GIP) level revealed a 1.5-fold increase in adult *Pten*$^{\Delta IEC}$ mice (n = 10) when compared to control littermates (n = 10); C: Analysis of circulating somatostatin (SST) level revealed a 1.3-fold increase in adult *Pten*$^{\Delta IEC}$ mice (n = 10) when compared to control littermates (n = 10). Error bars represent SE. $^{a}P < 0.05$.

NeuroD1, Pancreatic and duodenal homeobox 1 gene (*Pdx1*), the winged helix Foxa1 and the forkhead box-containing (FoxO1) transcription factors have also been shown to control the determination of specific enteroendocrine subpopulations[16,30-33]. The gene transcript level of BETA2/NeuroD1, linked to specification of secretin and CCK producing cells[34], was reduced by 1.40-fold in mutant animals (Table 1). Pdx1, which regulates serotonin and GIP producing cells (Figure 5)[31,32], was found to be significantly increased by 2.15-fold at the gene transcript level in mutant mice (Table 1). FoxO1 factors are downstream targets of the PI3K/AKT pathway[35] and affect the subcellular localization of Pdx1 in the pancreas and, hence, its transcriptional activity[36]. *FoxO1* gene transcript level was found to be reduced by 1.95-fold in the *Pten*$^{\Delta IEC}$ mice (Table 1). Finally, the winged helix transcription factors Foxa1 is essential for the differentiation of SST-, GLP-1- and PYY-expressing endocrine cells (Figure 5)[33]. Accordingly, we found an increase of 2.64-fold in *Foxa1* gene transcript expression in *Pten*$^{\Delta IEC}$ mice (Table 1).

Table 1 Gene expression changes in the small intestine of *Pten*$^{\Delta IEC}$ mice

Gene description	Gene symbol	Fold	*P* value
Hairy and enhancer of split 1	*Hes1*	-1.08	NS
Atonal homolog 1	*Math 1*	-2.05	NS
Neurogenin 3	*Ngn3*	-2.07	0.033
Growth factor independent 1	*Gfi1*	1.07	NS
Neurogenic differentiation 1	*NeuroD1*	-1.40	0.002
Pancreatic and duodenal homeobox 1	*Pdx1*	2.15	0.048
Forkhead box O1	*FoxO1*	-1.95	0.017
Forkhead box a1	*Foxa1*	2.64	0.017
Forkhead box a2	*Foxa2*	-1.25	NS

Target expression was quantified relatively to PDGB expression. Fold changes represent the ratio of mean expression values (control/mutant). Negative values indicate reduction in *Pten*$^{\Delta IEC}$ intestines. NS: Non significant fold change (Mann-Whitney test).

DISCUSSION

Endocrine cells found scattered in the gastrointestinal epithelium represent the major endocrine organ of the body[15,16]. The various hormones produced by these endocrine cells control numerous physiological functions[16,18,19]. Recently, by using conditional tissue-specific disruption of *Pten* in the epithelium of the gut, we revealed a key role for epithelial Pten in intestinal morphogenesis, in the maintenance of crypt-villus axis architecture, in cell proliferation and in secretory cell commitment[10]. We had also reported an overall decrease in the number of enteroendocrine cells using a CgA antibody. However, the choice of CgA as a pan marker for all enteroendocrine cells has been challenged. Commonly used as a biomarker for endocrine granules, CgA plays a role in the biogenesis of mobile secretory granules and the release of hormones through the regulated secretory pathway[37]. Over the years, there has been a lingering controversy in which some studies showed that all endocrine cell subpopulations express CgA[18,24,25] while others reported that enteroendocrine cell subpopulations producing GIP, GLP-1 or SST do not express CgA[17,26]. Fixation artefacts and different CgA antibodies may account for this controversy. Also, it has been demonstrated that CgA expression in enteroendocrine subpopulations varies from one species to another as well as in pathologies such as colorectal cancer and inflammatory bowel diseases[38-42]. Herein, our analysis of the various intestinal endocrine subpopulations with CgA antibodies confirmed the absence of co-expression between GIP and SST with CgA. Therefore, the important role of enteroendocrine cells in whole body homeostasis prompted us to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various en-

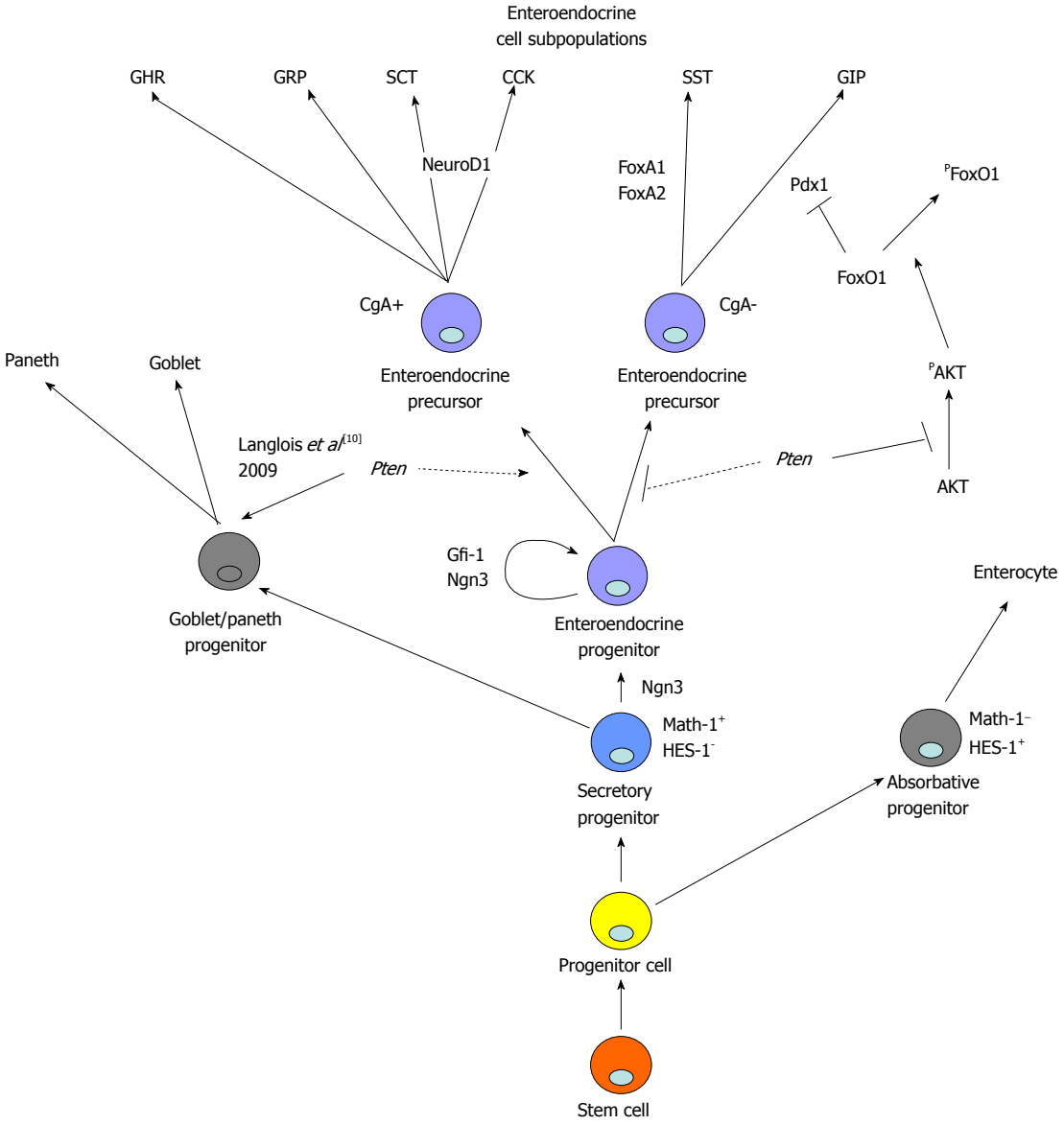

Figure 5 Proposed model for mode of action of epithelial Pten signalling in intestinal epithelial determination and specification of enteroendocrine progenitor cell fate. Epithelial Pten signalling is not essential for maintenance or determination of the secretory precursor. Pten represses specification of glucose-dependent insulinotropic peptide (GIP)-expressing cells by maintaining FoxO1 in the nucleus. GHR: Ghrelin; GRP: Gastrin-releasing peptide; SCT: Secretin; CCK: Cholecystokinin; SST: Somatostatin; GIP: Glucose-dependent insulinotropic peptide; CgA: Chromogranin A.

teroendocrine subpopulations. Since all enteroendocrine subtype cells are still detectable in the mutant mice, our results suggest that Pten is not a direct and indispensable regulator of enteroendocrine cell determination. Nevertheless, our data revealed a dual role for Pten signalling in enteroendocrine cell specification. Indeed, our results indicate that Pten signalling facilitates the specification of CgA-expressing enteroendocrine cell subpopulations while it negatively controls specification of CgA-negative cells in the intestinal epithelium. Furthermore, our results showed that the number of GIP and SST cells as well as their associated circulating hormone levels was increased in mutant mice. Although the number of ghrelin cells was decreased, no significant modulation in ghrelin serum level was observed in the $Pten^{\Delta IEC}$ mice. This may be explained by the fact that ghrelin endocrine cells found in the stomach epithelium are strong contributors for total circulating ghrelin levels[43,44], and are not likely affected by the loss of epithelial *Pten* in the intestine. Nevertheless, the lack of modulation in circulating levels of ghrelin does not imply that the reduction observed in the cell number in the intestine has no local consequences in this tissue. Indeed, such a reduction could influence specific physiological intestinal functions, such as motility, digestion and epithelial renewal[16,18,19]. Finally, analyses of each enteroendocrine cell subtypes along the rostro-caudal axis of the small intestine confirmed that the loss of *Pten* does not influence normal distribution of these endocrine cell subpopulations.

Our data also indicate that Pten affects the expression of key regulators for cell lineages and/or proenteroendocrine determination. Since the Notch/Hes-1

path is required for the specification of progenitor cells into the absorptive lineage and since Math1 is required for specification into the secretory lineage[27,28], we therefore analyzed whether the loss of epithelial *Pten* could alter their expression. Lack of modulation in Math1 and Hes-1 gene transcripts suggest that Pten is not involved in the initial decision steps for lineage determination. Once the initial decision is made between secretory and absorptive cell lineages, the fate of enteroendocrine progenitor cells is defined by proendocrine bHLH transcription factors such as Ngn3 and BETA2/NeuroD1. Ngn3 acts downstream of Math1[27,29] and has been shown to contribute to the maintenance of the enteroendocrine precursors and to the differentiation of all enteroendocrine subpopulations in mice[29,45,46]. Unlike Ngn3, expression of BETA2/NeuroD1 is restricted to a subset of enteroendocrine cells[34]. BETA2/NeuroD1 controls terminal differentiation of secretin and CCK producing cells in the intestine as revealed by the absence of these subpopulations in BETA2/NeuroD1 null mice[34]. In addition, BETA2/NeuroD1 acts downstream of Ngn3[45]. Our analysis revealed that the expression of both bHLH transcription factors was reduced in absence of epithelial Pten, thereby impacting on the production of specific enteroendocrine subpopulations (Figure 5). Over the years, other factors have been shown to be important in the differentiation/specification of several enteroendocrine cell subpopulations[16,30-33]. Such is the case for the winged helix transcription factor Foxa1, previously shown to be essential for the differentiation of SST, GLP-1 and PYY expressing cells[33]. Foxa1 expression was found to be increased in $Pten^{\Delta IEC}$ mice, hence correlating with the increased production of SST-expressing cells in these mice (Figure 5). The same logic can be applied to Pdx-1. Indeed, studies from Pdx1-null mice revealed an increase in the number of serotonin cells and a decrease in the GIP-expressing cell population[31,32]. Herein, *Pdx-1* gene transcript was found to be significantly increased in absence of epithelial *Pten*, thereby matching the deregulation seen in GIP cell specification (Figure 5). In addition, *FoxO1* gene transcript was found to be significantly reduced in the absence of epithelial Pten. FoxO1 competes with FoxA2 for binding to the Pdx1 promoter, resulting in inhibition of Pdx1 transcription[36] (Figure 5). Aside from these observations, one could speculate that phosphorylation of FoxO1 affects its subcellular localisation leading to its exclusion from the nucleus. This nuclear/cytoplasm shuttling phosphorylation of FoxO1 ultimately decreases its transactivation potential[36,47]. Furthermore, PI3K/Akt is a major upstream signalling pathway leading to the phosphorylation of FoxO1 and its exclusion from the nucleus[35]. In a previous study with $Pten^{\Delta IEC}$ mice, we reported that loss of *Pten* resulted in increased phosphorylation levels of Akt[10]. Thus, it is tempting to extrapolate that following the loss of intestinal epithelial Pten and activation of Akt, targeted FoxO1 protein would become more phosphorylated and exported to the cytoplasm allowing expression of Pdx1 and specification of GIP-expressing cells.

In summary, our results reveal a distinctive role for Pten in specification/differentiation of enteroendocrine cell subpopulations. Pten signalling negatively regulates the enteroendocrine subtype specification of non-expressing CgA cells such as GIP and SST expressing cells. In contrast, Pten signalling positively affects CgA-expressing cells such as ghrelin, gastrin and CCK cells. Many of these enteroendocrine cell subtypes are known to play critical roles in whole body physiological functions. Incretin hormones such as GLP-1 and GIP have been shown to potentiate glucose-stimulated insulin secretion[48], while double-mutant mice for GIP and GLP-1 exhibit glucose intolerance[49]. Likewise, the importance of enteroendocrine cells in lipid absorption has recently been shown with the generation of intestinal-conditional Ngn3 null mice[46]. A study with Gip-receptor null mice revealed a crucial role for GIP in promoting the efficient storage of ingested fat suggesting that inhibition of the GIP signal could represent a therapeutic approach against obesity[50]. Further analysis will be needed to better evaluate the impact and possible networking of small intestinal endocrine cell deregulation following the loss of Pten signalling on overall metabolism in the mouse.

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COMMENTS

Background

The phosphatase and tensin homolog (*PTEN*) tumour suppressor gene is a lipid and protein phosphatase frequently mutated/deleted in various human cancers. Its best-known substrate, the phosphatidylinositol 3,4,5-trisphosphate, is a lipid second messenger mainly produced by class IA phosphatidylinositol 3-kinases (PI3Ks). PI3Ks have been implicated in many signalling pathways that regulate cell survival, growth, proliferation, migration, phagocytosis, and metabolism. In previous study, authors reported that *Pten* is important for intestinal homeostasis as well as in the commitment of enteroendocrine cells. The important role of enteroendocrine cells in whole body homeostasis prompted people to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various enteroendocrine subpopulations.

Research frontiers

Enteroendocrine cells located in the gut epithelium are the largest and least understood population of hormone-producing cells in the body. The various hormones and peptides produced by these endocrine cells control important physiological functions, such as gastrointestinal motility, glycaemia, exocrine pancreatic secretion, biliary secretion, digestion, gut epithelial renewal and appetite. In recent years, studies have placed the regulation of these gut hormones as potential targets for novel treatments of metabolic diseases such as type 2 diabetes and obesity.

Innovations and breakthroughs

In the current study, the authors report a distinctive role for Pten in specification/differentiation of enteroendocrine cell subpopulations. Pten signalling negatively regulates the enteroendocrine subtype specification of non-expressing chromogranin A (CgA) cells such as glucose-dependent insulinotropic peptide and somatostatin expressing cells. In contrast, Pten signalling affects positively CgA-expressing cells such as ghrelin, gastrin and cholecystokinin cells.

Applications

Many of these enteroendocrine cell subtypes are known to play critical roles in whole body homeostasis. These experimental data can be used in further studies to better evaluate the impact on general metabolism and possible networking of small intestinal endocrine cell deregulation following the loss of Pten signalling.

Peer review

This is a high quality descriptive study in which authors analyze the impact of the *PTEN* intestinal knockdown in the specification of intestinal enteroendocrine subpopulations.

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