Non-neuronal acetylcholine as an endogenous regulator of proliferation and differentiation of Lgr5-positive stem cells in mice

Toshio Takahashi1,*, Hiroe Ohnishi2,*,†, Yuki Sugiura3,4, Kurara Honda3,4, Makoto Suematsu3,5, Takashi Kawasaki2, Tomonori Deguchi2, Takeshi Fujii6, Kaoru Orihashi7, Yoshitaka Hippo7, Takehiro Watanabe1, Tohru Yamagaki1 and Shunsuke Yuba2

1 Suntory Foundation for Life Sciences, Bioorganic Research Institute, Osaka, Japan
2 National Institute of Advanced Industrial Science and Technology, Hyogo, Japan
3 Department of Biochemistry, School of Medicine, Keio University, Tokyo, Japan
4 Precursory Research for Embryonic Science and Technology, Tokyo, Japan
5 Japan Science Technology Agency, Exploratory Research for Advanced Technology, Suematsu Gas Biology Project, Tokyo, Japan
6 Department of Pharmacology, Faculty of Pharmaceutical Sciences, Doshisha Women’s College of Liberal Arts, Kyoto, Japan
7 Division of Cancer Development System, National Cancer Research Institute, Tokyo, Japan

Keywords
cholinergic system; gut; Lgr5; non-neuronal acetylcholine; organoid

Correspondence
T. Takahashi, Suntory Foundation for Life Sciences, Bioorganic Research Institute, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan
Fax: +81 75 962 2115
Tel: +81 75 962 6105
E-mail: takahashi@sunbor.or.jp

Present address:
Department of Otolaryngology, Head and Neck Surgery, Kyoto University Hospital/Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

*These authors contributed equally to this work.

(Received 10 February 2014, revised 22 July 2014, accepted 14 August 2014)

doi:10.1111/febs.12974

Non-neuronal acetylcholine (ACh) is predicted to function as a local cell signaling molecule. However, the physiological significance of the synthesis of non-neuronal ACh in the intestine remains unclear. Here, experiments using crypt–villus organoids that lack nerve and immune cells in culture led us to suggest that endogenous ACh is synthesized in the intestinal epithelium to evoke growth and differentiation of the organoids through activation of muscarinic ACh receptors (mAChRs). The extracts of the cultured organoids showed a noticeable capacity for ACh synthesis that was sensitive to a potent inhibitor of choline acetyltransferase. Imaging MS revealed endogenous ACh localized in the epithelial layer in mouse small intestinal epithelium in vivo, suggesting that there are non-neuronal resources of ACh. Treatment of organoids with carbachol downregulated the growth of organoids and the expression of marker genes for epithelial cells. On the other hand, antagonists for mAChRs enhanced the growth and differentiation of organoids, indicating the involvement of mAChRs in regulating the proliferation and differentiation of Lgr5-positive stem cells. Collectively, our data provide evidence that endogenous ACh released from intestinal epithelium maintains homeostasis of intestinal epithelial cell growth and differentiation via mAChRs in mice.

Abbreviations
ACh, acetylcholine; ChAT, choline acetyltransferase; ChT, choline high-affinity transporter; EGC, enteric glial cell; EGF, epidermal growth factor; ENS, enteric nervous system; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; IMS, imaging MS; iso-OMPA, tetraisopropyl pyrophosphoramide; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; o-organoid, old organoid; P, postnatal day; VACHt, vesicular acetylcholine transporter; y-organoid, young organoid.
Introduction

Choline acetyltransferase (ChAT) catalyzes the synthesis of the classic neurotransmitter acetylcholine (ACh) in the central and peripheral nervous systems of both vertebrates and invertebrates. In higher animals, cholinergic neurons control a variety of physiological and biochemical processes in many effector organs, including the heart, smooth muscle cells, and glands. In Caenorhabditis elegans, the cholinergic nervous system is involved in behavioral responses, including locomotion, egg laying, feeding, and male mating [1]. In 1978, Sastry and Sadavongvivad [2] first observed the expression of components of the cholinergic system outside of the nervous system, demonstrating the presence of a non-neuronal cholinergic system. Moreover, ACh and associated metabolic enzymes have been detected in more primitive life forms, such as plants, lichens, fungi, and even bacteria [3,4], suggesting that ACh is a universal cell molecule in biological systems.

One of the major pathways of excitatory transmission within the enteric nervous system (ENS) is mediated by cholinergic transmission, with the transmitter ACh producing excitatory potentials in postsynaptic effector cells. In addition to ACh-synthesizing and ACh-metabolizing elements in the ENS, the presence of non-neuronal ACh machinery has been reported in epithelial cells of the small and large intestines of rats and humans [5]. Although non-neuronal ACh is predicted to function as a local cell signaling or trophic molecule [5], the physiological significance of the synthesis and release of non-neuronal ACh in the intestine remains unclear. Evidence suggests that ACh is critical for controlling intestinal epithelial ion transport, which strongly influences water movement and hydration [6]. More recently, the non-neuronal release of ACh from colonocytes coupled with propionate stimulation has been shown to play a key role in chloride secretion [7].

Here, we investigated the presence of ACh and the cholinergic system in mouse small intestinal epithelium and crypt–villus organoids lacking nerve and immune cells. Microscopic imaging MS (IMS) was used to examine the localization of ACh in epithelial cell layers of the intestine. In addition, we evaluated the localization of ChAT and specific ACh receptors, including muscarinic acetylcholine receptors (mAChRs), in intestinal epithelial cells. The findings from this study demonstrate that ACh controls the growth and differentiation of crypt–villus organoids, and is involved in both the proliferation and differentiation of Lgr5-positive stem cells in the mouse small intestine via mAChRs.

Results

Immunohistochemical distribution of the cholinergic system in small intestinal epithelium

The apical junctional complex of the intestinal epithelium is composed of tight junctions, adherens junctions, and desmosomes [8]. The major constituent of adherens junctions is E-cadherin, which facilitates homophilic cell–cell interactions. Here, the structural organization of the small intestinal epithelium was visualized with an antibody against E-cadherin. The immunohistochemical analysis revealed that E-cadherin was predominantly localized in the upper region of the intercellular space of the epithelium (Fig. 1A). The cellular distribution of the E-cadherin antibody coincided with the background cellular expression of E-cadherin [9].

ChAT has been extensively used to label cholinergic neurons in the ENS. To examine the immunoreactivity of epithelium for ChAT, indirect immunofluorescence staining was performed with an antibody against ChAT [10]. ChAT was detected in the cytoplasm of epithelial cells throughout the villus (Fig. 1B). We confirmed that there was no background staining with the secondary antibodies [donkey anti-(goat IgG)] (Fig. 1C). The immunohistochemical results suggested that mouse intestinal epithelial cells are able to produce ACh.

Two types of receptor mediate cholinergic transmission in the ENS: nicotinic acetylcholine receptors (nAChRs) and mAChRs. The former are ligand-gated ion channels that are composed of five subunits assembled in homomeric or heteromeric combinations, whereas the latter are G-protein-coupled receptors with five known subtypes (M1–M5) [11,12]. To examine the distribution of mAChRs in mouse epithelial cells, immunohistochemical staining with antibodies against M1, M2, M3, M4 [13,14] and M5 was performed. The analysis showed that all five receptors were located in the cell membranes of epithelial cells and showed similar staining patterns (Fig. 1D–H). No immunological staining was observed in cells in the absence of primary antibody (Fig. 1I). The observed staining patterns showed that ACh has multiple neuronal and non-neuronal sites of synthesis and muscarinic receptors in gut epithelium.

Components of the cholinergic system are involved in crypt and villus development

The mouse small intestine shows a specific developmental pattern. Villus formation begins around embryonic day 15, whereas crypt formation occurs during the first two postnatal weeks [15,16]. However,
with the exception of these morphological rearrange-
mements, the mechanisms underlying the initiation of
lineage differentiation and functional maturation are
less well characterized. Recently, Fordham et al. [17]
reported that the mouse intestine at embryonic day 16
showed high proliferation in the intervillus regions and
scattered immature goblet cells. By postnatal week 2,
mature crypts being formed and mature Paneth cells
can be detected in the proliferative zones [17]. How-
ever, the presence and functional properties of the cho-
linergic system during the course of intestinal
development are largely unknown.

To determine whether components of the cholinergic
system are involved in the regulation of crypt and vil-
lus development in mice, we performed quantitative
RT-PCR in the small intestines at postnatal day (P)2
and postnatal week 8. The small intestine at P2 con-
tains proliferative, immature progenitors, which can be
expanded in vitro as fetal enterspheres [17]. Gene
expression analysis showed that the examined compo-
nents of the cholinergic system in the small intestine,
with the exception of butyrylcholinesterase, had higher
expression at P2 than at postnatal week 8 (Fig. 2).
The data in Fig. 2 suggest that components of the cho-
linergic system affect the proliferation and differentia-
tion of stem cells in the mouse small intestine.

Organoids contain a non-neuronal cholinergic
system
Crypt–villus organoids were cultured from isolated
crypts according to the method of Sato [18]. In

Fig. 1. Immunolocalization of ChAT and M1–M5 in the mouse small intestine.
(A) Visualization of intestinal epithelium (green, E-cadherin). (B) Localization of
ChAT in intestinal epithelium. (C) Control sections labeled with secondary antibody
[Alexa Fluor 568 donkey anti-(goat IgG)] in the absence of primary antibodies. (D–H)
Localization of M1–M5 (red) in intestinal epithelium. (I) Control sections labeled
with secondary antibody [Alexa Fluor 546 donkey anti-(rabbit IgG)] in the absence of
primary antibodies. In all panels, nuclei were stained with Hoechst 33342 (blue).
Bars in (A)–(I) represent 20 μm.
culture, the upper openings of crypts were rapidly sealed (Fig. 3A), and the crypt region underwent continuous budding events, reminiscent of crypt fission (Fig. 3A). Further expansion for ~ 10 days resulted in the formation of organoids that comprised more than 40 crypt domains surrounding a central lumen lined by a villus-like epithelium (Fig. 3A). Both crypts and the central luminal epithelium consisted of a single layer of polarized epithelial cells resting directly on the Matrigel support.

The organoids derived from the crypts were composed of stem cells and epithelial cells. Enteroendocrine cells visualized with antibody against chromogranin A were scattered throughout the organoid structure (Fig. 3B,E). Staining with antibody against mucin-2 demonstrated that goblet cells were also dispersed within organoids (Fig. 3C,F). In addition, Paneth cells visualized with antibody against lysozyme were located at crypt bottoms (Fig. 3D,G), whereas lysozyme protein accumulated in the central lumen of the organoid (Fig. 3D,G).

Following the synthesis of ACh, it is transported via the vesicular ACh transporter (VAChT) to synaptic vesicles in nerve cells. In all tissues, ACh is degraded by acetylcholinesterase. However, a second, nonspecific cholinesterase, designated butyrylcholinesterase, which is abundant in the intestine of mammals, may also contribute to the degradation of ACh [19,20]. After the degradation of ACh, choline is taken up by the sodium-dependent high-affinity choline transporter
(ChT). RT-PCR analysis to confirm the expression of cholinergic components revealed that ChAT, VACHT, ChT, the acetylcholinesterase gene (AChE) and the butyrylcholinesterase gene (BuChE) were expressed in the brain, gut and cultured organoids of mice (Fig. 4A,B). Notably, however, the expression patterns of ACh receptors differed between the examined tissues. Although the expression of all mRNAs encoding muscarinic receptor subtypes was observed in all tissues (Fig. 4A), M5 expression in the gut and organoids was weaker than that in the brain (Fig. 4A). A diverse array of nAChR subtypes was expressed in individual tissues (Fig. 4A). For example, α1 was only expressed in the brain.

Gene expression analysis was also performed to determine whether neuron-specific and glial cell-specific marker genes were expressed in organoids. Enteric glial cells (EGCs), which are characterized by glial fibrillary acidic protein (GFAP) expression, play an important role in maintaining intestinal mucosal integrity [21]. ENS gliogenesis is readily observed under steady-state conditions and after injury, and EGCs have the potential to form neurons and glial cells [22]. RT-PCR revealed that ChAT was expressed in both gut and organoids (Fig. 4B). In contrast, neuron-specific markers (Tubulin βIII and Map2) and the glial cell-specific marker (Gfap) were expressed in the gut, but not in organoids, indicating that the organoids did not contain nerve cells or EGCs.

The global gene expression profiles of gut, crypts, young organoids (y-organoids) (10-day culture) and old organoids (o-organoids) (6-month culture) were next examined with microarray analysis. Heat map analysis showed that the gene expression profile of o-organoids was similar to that of y-organoids (Fig. 5A). Scatter plot analysis of the array results showed that the neuron and glial cell marker genes Neurod2, Map2 and Gfap were highly expressed in the gut (Fig. 5B). The gene expression profiles of crypts were similar to those of y-organoids (Fig. 5C). A comparison of the expression levels of intestinal lineage genes (Notch1, Olfm4, Efd7, Cdca7, Tnf1sf19, Dll4, Tgfa, Egf, Wnt11, Wnt3, Defa1, Vill, Lys1, Muc2, Chga, Lrig1, Lgr5, Sox9, Ngn3, Hnf1, and Klf4) between y-organoids and o-organoids revealed marked similarities (Fig. 5D). In particular, a comparison of the expression levels of intestinal stem cell and epithelial marker genes (Lgr5, Sox9, Ngn3, Hnf1, and Klf4) between y-organoids and o-organoids did not reveal any marked differences (Fig. 5D).

To validate the microarray data, the transcription levels of selected genes were examined with real-time PCR. The analysis confirmed that there were no differences in gene expression patterns between y-organoids and o-organoids (Fig. 5E). Therefore, organoids that were cultured for >6 months appeared to retain
their characteristics. In addition, because no significant induction of neuronal marker genes was observed after 6 months, cultured organoids were considered to constitute a good assay system for studying the capacity of non-neuronal ACh to mediate physiologically important processes in intestinal epithelial cells.

To determine whether ChAT activity and ACh were expressed in the epithelium, the levels of these molecules in organoids lacking neurons was examined biochemically. ACh was detected in organoids at a concentration of 397 fmol/mg tissue with HPLC (Table 1; Fig. 4C). However, the ACh concentrations in gut and brain were 2380 fmol/mg tissue and 1990 fmol/mg tissue, respectively (Table 1). Thus, the difference between ACh levels in organoids and the gut reflects the ACh associated with the ENS.

ACh synthesis was also detected in organoid extracts (Table 1). The capacity of organoids to synthesize ACh (10.0 pmol/mg protein per min) was lower than that of gut and brain (15.5 and 86.5 pmol/mg protein per min, respectively) (Table 1).

To confirm that ACh is produced and released from organoids, the ACh content in culture medium was measured with LC-MS/MS. For the analysis, inhibitors of acetylcholinesterase [10^{-5} M tetraisopropyl pyrophosphoramide (iso-OMPA)] were added to the medium to prevent ACh degradation, and samples were collected after culture of the organoids for 3 days. LC-MS/MS analysis revealed ACh in the organoid culture medium (Fig. 4D), indicating that ACh was endogenously released from organoids.

**Tandem IMS of ACh in mouse gut**

To confirm that ACh is distributed in gut epithelium, tandem IMS analysis for ACh was performed with a MALDI-TOF/TOF-type instrument. Ions with an m/z of 146 were first selected as precursor ions, and the

| Tissue          | ACh synthesis (pmol/mg protein per min) | ACh content (fmol/mg tissue) |
|-----------------|----------------------------------------|-------------------------------|
| Organoid (n = 3)| 10.0 ± 1.1                              | 397                           |
| Gut (n = 3)     | 15.5 ± 1.2                              | 2380                          |
| Brain (n = 3)   | 86.5 ± 1.4                              | 1990                          |
product ion peak was detected at \( m/z \) 87, which is characteristic of the most intense fragment ion of the ACh molecule [23] and is derived from the neutral loss of the trimethylamine group (NL59) from the intact ion (Fig. 6A). The analysis showed that ACh was clearly distributed in epithelial cell layers of the gut sections in blue and/or red colors, but was not detected in the lumen (Fig. 6B,C). Moreover, the observed distribution of the ACh product ion at higher resolution confirmed that ACh was localized in gut epithelial cell layers (Fig. 6D). Immunohistochemical analysis of the cellular distribution of tubulin bIII and E-cadherin demonstrated that ACh was localized in the epithelial cell layer and ENS regions (Fig. 6E).

We also performed immunohistochemical analysis on organoid sections to determine the localization of ChAT and M1–M5. ChAT was localized in epithelial cells throughout the entire organoid (Fig. 7A). In contrast, M1–M5 were localized on the cell membranes of epithelial cells (Fig. 7B–F). These findings confirmed the synthesis and secretion of ACh in organoids.

Non-neuronal ACh is involved in organoid growth and differentiation

The non-neuronal release of ACh from colonocytes stimulated with propionate plays a key role in chloride secretion via the paracrine action of ACh on mAChRs of colonocytes [7]. Although the role of epithelial cell-derived ACh in intestinal epithelium is unclear, Landgraf et al. [24] revealed that ACh may modulate the death and proliferation of embryonic stem cells by binding to ACh receptors. As intestinal stem cells are closely associated with epithelial cells in organoids, we hypothesized that ACh released from the epithelium affects intestinal stem cells. To test this hypothesis, we examined whether the ACh receptor agonist carbachol induced changes in organoid growth. Organoid growth was dramatically inhibited by \( 10^{-4} \) M carbachol as

---

**Fig. 6.** Tandem IMS analysis for localization of ACh in mouse gut sections. (A) Detection of the ACh-derived product ion at \( m/z \) 87, which was derived from the neutral loss of the trimethylamine group (NL59) from the intact ion. (B) Micrographs of gut sections stained with hematoxylin and eosin after IMS measurement. (C) Reconstructed ion intensity map for the ion transition from \( m/z \) 146 to \( m/z \) 87 (tandem MS signature of ACh) in the gut. The obtained ion distribution images were merged with the micrographs in (B). Bars in (B) and (C) represent 500 \( \mu \)m. (D) Reconstructed ion intensity map for the ion transition from \( m/z \) 146 to \( m/z \) 87 in the gut. (E) Visualization of intestinal epithelium (green, E-cadherin) and ENS (red, tubulin bIII) in a serial section of gut tissue. Nuclei were stained with Hoechst 33342 (blue). The obtained ion distribution images were merged with the micrographs of the same section stained with haematoxylin and eosin after IMS measurement. Bars in (D) and (E) represent 100 \( \mu \)m.
compared with the untreated control (Fig. 8A). A
time-course analysis revealed that the inhibition of
organoid growth first reached the level of significance
at day 3 of carbachol treatment, and that growth was
inhibited in a dose-dependent manner (Fig. 8B).

We next examined the expression of intestinal stem
cell (Lgr5) and epithelial marker (Sox9, Ngn3, Hnf1,
and Klf4) genes in the presence and absence of 10^{-4} M
carbachol. RT-PCR analysis of organoids treated with
carbachol for 3 days revealed that the levels of Sox9,
Ngn3, Hnf1 and Klf4 transcripts were markedly
reduced as compared with control organoids (Fig. 8C).
In contrast, carbachol only slightly reduced Lgr5

transcript levels (Fig. 8C). Quantitative RT-PCR
analysis confirmed that the expression of Sox9, Hnf1
and Klf4 was significantly decreased in carbachol-trea
ted organoids, whereas the expression of Lgr5 and
Ngn3 was not decreased as compared with control
organoids (Fig. 8D). Thus, we concluded that carba-
chol had no effect on the expression levels of Lgr5

mAChRs have recently been suggested to be impli-
cated in the control of cell growth and proliferation
[25–27]. To determine whether mAChRs are involved
in the growth and differentiation of organoids, we
examined the effects of atropine, a muscarinic receptor

Fig. 7. Immunolocalization of ChAT and
M1–M5 in organoids. (A) ChAT (red).
Nuclei were stained with Hoechst 33342
(blue). (B–F) Localization of M1–M5,
respectively (red). Bars in (A)–(F) represent
20 μm.
Pharmacological effects of carbachol and atropine on organoid growth and differentiation. (A) Micrographs of organoids after treatment with $10^{-4}$ M carbachol for 1 day and 3 days of culture. (B) Effect of carbachol on the size of cultured organoids. Each sample represents an average of three independent experiments. Error bars represent the standard deviation of the mean. An asterisk indicates a statistically significant difference from untreated control organoids (Mann–Whitney test, $P < 0.05$). (C) Effect of carbachol ($10^{-4}$ M) on the expression of markers for intestinal stem cells ($Lgr5$) and epithelium ($Sox9$, $Ngn3$, $Hnf1$, and $Klf4$) in a 3-day culture of organoids. The GAPDH level was used as an internal control. (D) Relative quantification of the marker genes after treatment of cultured organoids with $10^{-4}$ M carbachol for 3 days at $37^\circ\text{C}$. The results are based on three independent experiments and expressed as mean values ± standard errors. The statistical significance was calculated with Student’s $t$-test ($^{*}P < 0.05$ as compared with control). (E) Enhancement of growth of organoids by treatment with $10^{-5}$ M atropine for 1 day and 3 days of culture. (F) Effect of atropine on the size of cultured organoids. Each sample represents an average of three independent experiments. Error bars represent the standard deviation of the mean. An asterisk indicates a statistically significant difference from the control (Mann–Whitney test, $P < 0.05$). (G) Effect of atropine ($10^{-5}$ M) on the expression of markers for intestinal stem cells and epithelium in a 3-day culture of organoids. (H) Relative quantification of the marker genes after treatment with $10^{-5}$ M atropine for 3 days at $37^\circ\text{C}$. The results are based on three independent experiments and are expressed as mean values ± standard errors. The statistical significance was calculated with Student’s $t$-test ($^{*}P < 0.05$ as compared with control).
antagonist, on cultured organoids. Organoid growth was clearly enhanced by treatment with $10^{-5} \text{ M}$ atropine as compared with controls at day 3 (Fig. 8E). The enhancement of organoid growth occurred in dose-dependent manner until day 7, at which point no statistically significant effect was detected (Fig. 8F). We also examined the expression levels of selected marker genes in the presence and absence of $10^{-5} \text{ M}$ atropine. RT-PCR analysis of organoids treated with atropine for 3 days revealed that the levels of all examined marker gene transcripts were slightly enhanced as compared with the untreated control (Fig. 8G). Quantitative RT-PCR analysis confirmed that the expression of all marker genes was significantly enhanced by treatment with $10^{-5} \text{ M}$ atropine (Fig. 8H).

To determine the types of mAChR that are involved in the growth and differentiation of organoids, we further treated organoids with selective mAChR antagonists. The effects of four mAChR antagonists on the growth of organoids at concentrations of $10^{-6} \text{ M}$, $10^{-5} \text{ M}$ and $10^{-4} \text{ M}$ was examined over a 7-day period (Fig. 9A–D). Treatment with the M1 antagonist pirenzepine, the M2 antagonist AF-DX-116 and the M3 antagonist 4-DAMP at $10^{-5} \text{ M}$ significantly enhanced the growth of organoids by day 3 (Fig. 9A–C). In contrast, the M4 antagonist tropicamide did not show an antagonistic effect on the growth of organoids at any concentration tested (Fig. 9D). We also found that treatment with pirenzepine at $10^{-4} \text{ M}$ weakly reduced organoid growth as compared with treatment at $10^{-5} \text{ M}$ (Fig. 9A). It is possible that pirenzepine was toxic to cells at the higher concentration.

The positive effects of the mAChR antagonists on organoid growth suggested that they may increase the expression levels of marker genes. To test this
hypothesis, we examined the expression of selected marker genes by organoids in the presence and absence of the four antagonists at $10^{-5}$ M. Real-time PCR analysis revealed that the expression of Lgr5, Sox9 and Ngn3 was significantly increased by treatment with pirenzepine, whereas the expression of Hnf1 and Klf4 was not changed (Fig. 9E). Both AF-DX-116 and 4-DAMP resulted in a 1.2-fold to 1.6-fold increase in the expression of Lgr5, Sox9, Hnf1, and Klf4, but Ngn3 expression was not affected (Fig. 9F,G). In contrast, the expression of Lgr5 and Klf4 was significantly decreased following tropicamide treatment, whereas the expression of Sox9, Ngn3 and Hnf1 was not markedly changed as compared with untreated organoids (Fig. 9H).

Atropine increases the number of Lgr5-positive cells

To investigate the function of non-neuronal ACh at the cellular level, we conducted a biological assay using mouse crypts. Crypts were isolated from lgr5-EGFP-ires-CreERT2 mice and cultured in the presence and absence of $10^{-4}$ M carbachol and $10^{-5}$ M atropine (Fig. 10). With increasing duration of treatment with atropine, the green fluorescent protein (GFP)-positive area of organoids appeared to increase as compared with that of controls, whereas carbachol appeared to have no effect on the GFP-positive area of organoids (Fig. 10A). After treatment with carbachol and atropine, the organoids were dissociated into suspensions of single cells (Fig. 10B), and Lgr5-GFP-positive cells were counted. The analysis showed that treatment with atropine significantly increased the number of Lgr5-GFP-positive cells by ~1.5-fold at day 3 as compared with the untreated organoids ($P < 0.05$) (Fig. 10C). In addition, the percentage of Lgr5-GFP-positive cells among GFP-positive cells after 3 days of carbachol and atropine treatment was quantified by flow cytometry analysis (Fig. 10D). After treatment with carbachol, the percentage of Lgr5-GFP-positive cells (19.5%) was slightly decreased as compared with that of the control (24.3%). In contrast, treatment with atropine clearly increased the percentage of Lgr5-GFP-positive cells (36.5%).

Discussion

ACh is regarded as a classic neurotransmitter that binds to nicotinic and muscarinic receptors, and...
mediates signal transmission. The traditional view that ACh acts solely as a neurotransmitter must be revised, because of the identification of a non-neuronal cholinergic system in several primitive unicellular and multicellular organisms. This system is also common in non-neuronal cells of animals and humans [3,4,28,29].

The structure and function of the gut vary along the crypt–villus and duodenum–colon axes. The gut epithelium is a dynamic structure that is constantly undergoing self-renewal as a result of continuous growth and differentiation. The interstitial epithelium consists of four lineages of differentiated cells: goblet cells, enteroendocrine cells, Paneth cells, and enteroendocytes [30]. In mice, cells are newly generated in crypts and are lost following apoptosis at the tips of the villi within 5 days. Self-renewing stem cells have long been known to reside near the crypt bottom, where four to six stem cells are typically found.

Recently, Barker et al. [31] showed that Lgr5 is specifically expressed in cycling crypt base columnar cells, which are interspersed between the Paneth cells. Lgr5 is an orphan leucine-rich repeat-containing G-protein-coupled receptor that belongs to the glycoprotein hormone receptor family [32]. Recently, the cognate ligand for these receptors was identified as R-spondin [33]. The same group established long-term culture conditions under which single crypts undergo multiple crypt fission events while simultaneously generating villus-like epithelial domains containing all types of differentiated cells [18]. Furthermore, single-sorted Lgr5-positive stem cells can also initiate the formation of these crypt–villus organoids [18]. However, Lgr5-positive stem cells cannot generate nerve or immune cells [18].

Here, we demonstrated that ACh synthesis occurs in non-neuronal cells in the mouse gut, based on the following findings. First, biochemical, enzymological and LC-MS/MS analyses revealed that organoids lacking nerve and immune cells express and release ACh (Table 1; Fig. 4). Second, immunohistochemical, tandem IMS and gene expression analyses showed that the components of the cholinergic system, including ACh receptors, are expressed in intestinal epithelium (Figs 1, 4, 6, and 7). In tissue sections of organoids, previous immunohistochemical studies showed that ChAT-positive epithelial cells were distributed throughout organoids, thus confirming that the in vitro reactions cannot be ascribed to tissue culture artefacts. Here, polyclonal antibodies against mAChRs were used in the immunohistochemical analyses, and some nonspecific background staining was observed. Although these false-positive findings should be interpreted with caution, it seems likely that muscarinic M1, M2 and M3 receptors are found within the intestinal epithelium, according to the functional results.

The main finding of the present study is that ACh is a regulator of stem cells in the mouse intestine. We demonstrated that key components of the cholinergic system are present during the course of crypt and villus development in the small intestine (Fig. 2). In the mouse, the development of nerve terminals within various target tissues innervated by enteric neurons has been examined. Cholinergic (VACHT-immunoreactive) nerve terminals are present in the colon at birth, and the density of VACHT terminals in colonic circular muscle increases dramatically between P0 and P10 [34]. Nerve terminals are also present in the mucosa of the mouse small intestine just prior to birth [35]. Although the mouse small intestine at P2 contains components of both the neuronal and non-neuronal cholinergic systems, the findings of the present study provide the first direct evidence for functional expression of the cholinergic system involved in the regulation of crypt and villus development. To further investigate whether the non-neuronal cholinergic system is expressed and functional in immature intestinal epithelium, fetal enterospheres, which can be established from immature progenitor cells in the mouse small intestine up to the P2 stage [17], would constitute a good assay system.

Although ACh was one of the first neurotransmitters to be characterized, it has yet to be specifically localized. However, Geffard et al. [36] established methods for producing specific antibodies against ACh and directly visualizing the ACh pathway in the locust brain. This group also successfully produced a conjugated mAb against ACh for immunostaining of the rat nervous system [37]. Although antibodies against ACh have been produced, staining ACh in fixed tissue sections remains difficult. To overcome this limitation, Sugiuira et al. [38] recently used tandem IMS, which is a powerful tool for investigating the distribution of small molecules in tissue and cells [39–41], to visualize the ACh distribution in mouse brain and spinal cord sections for the first time. IMS is therefore expected to allow the imaging of ACh not only in the nervous system, but also in other tissues, such as epithelium. Using IMS, we revealed that ACh is localized in mouse intestinal epithelium (Fig. 6C,D). Our findings suggest that tandem IMS may be a valuable method for studying non-neuronal cholinergic pathways and determining their role in the maintenance and differentiation of intestinal stem cells.

Analysis of the pharmacological effects of the cholinergic agonist carbachol revealed that ACh inhibits the growth and differentiation of organoids...
Acetylcholine controls Lgr5-positive stem cells

T. Takahashi et al.

(Fig. 8A–D). However, because carbachol did not affect the expression of Lgr5 (Fig. 8C,D), ACh may impact on organoid growth through two distinct mechanisms: maintenance of Lgr5-positive stem cells, and inhibition of the differentiation of Lgr5-positive stem cells into enterocytes, goblet cells, Paneth cells, and enteroendocrine cells, which together constitute the intestinal epithelium. Carbachol is a general agonist that binds to multiple ACh receptors on different cells. We also examined the effects of atropine on the growth and differentiation of organoids, and found that this antimuscarinic agent had an antagonistic effect as compared with carbachol (Fig. 8E–H). These findings support the hypothesis that activation of muscarinic receptors by ACh stimulates the growth and differentiation of organoids.

To obtain further insights into the role of mAChRs in organoids, we examined the growth and differentiation of organoids after treatment with selective mAChR antagonists. Organoids exposed to the M1, M2 and M3 antagonists pirenzepine, AF-DX-116, and 4-DAMP, respectively, showed enhanced growth (Fig. 9A–C), whereas organoids treated with the M4 antagonist tropicamide did not show any effects, even at the highest examined concentration (Fig. 9D). Additionally, treatment with M1, M2 and M3 antagonists resulted in increased Lgr5 transcription (Fig. 9E–G), whereas decreased gene expression was observed in tropicamide-treated organoids (Fig. 9H). The M1 antagonist pirenzepine increased the expression of the intestinal epithelial cell marker genes Sox9 and Ngn3, and the M2 and M3 antagonists AF-DX-116 and 4-DAMP, respectively, increased the expression of Sox9, Hnf1, and Klf4 (Fig. 9E–G). In contrast, the M4 antagonist decreased Klf4 expression (Fig. 9H). Although the changes in the transcript levels of the marker genes were relatively weak, the present pharmacological results suggest that the activation of Lgr5-positive stem cells is most likely to involve the M1, M2 and M3 muscarinic receptor subtypes. The muscarinic receptors expressed on epithelial and/or Lgr5-positive stem cells may indirectly modulate the renewal rate of the intestinal epithelium. However, because muscarinic receptor stimulation itself influences the proliferation and differentiation of Lgr5-positive stem cells, such stimulation may induce cell growth and proliferation in conjunction with other stimuli. For example, in combination with epidermal growth factor (EGF), which acts as a proliferative stimulus, muscarinic stimulation enhances the proliferative effect of airway smooth muscle [42,43]. ACh has been suggested to transactivate EGF regulatory pathways [44] through binding to muscarinic M1, M2 and M3 receptors [45–47]. We speculate that the signaling mechanisms underlying the proliferation and differentiation of Lgr5-positive stem cells may have induced the weak changes observed in the transcription levels of the marker genes.

We also revealed that the number of Lgr5-positive stem cells markedly increased in organoids upon treatment with atropine (Fig. 10). Thus, endogenously released ACh contributes to the maintenance of Lgr5-positive stem cells and inhibition of their differentiation into intestinal epithelial cells through activation of M1, M2 and M3. As mucosal afferent nerves control intestinal epithelial stem/progenitor cells [48], in the absence of any influence from the ENS, non-neuronal ACh would control the maintenance and differentiation of Lgr5-positive stem cells. These findings indicate that the function of Lgr5-positive stem cells is, at least in part, under the control of an independent epithelial cholinergic system.

Genetic inducible fate mapping studies have identified two principal epithelial stem cell pools in the small intestine [49,50]. One pool consists of Lgr5-expressing cells. The other pool consists of Bmi1-expressing cells that largely reside above the crypt base [49]. Lineage tracing showed that Bmi1-expressing cells gave rise to Lgr5-expressing cells, pointing to a hierarchy of stem cells in the intestinal epithelium [50]. Indeed, clonogenic culture of isolated single Bmi1-expressing cells yields long-lived self-renewing organoids of intestinal epithelium that produce Lgr5-expressing cells, thereby establishing a lineage relationship between these two populations in vitro [51]. Isolated Bmi1-expressing cells can give rise to Lgr5-expressing cells under homeostasis, although the frequency of this occurrence is unknown. Our preliminary result showed that carbachol decreased Bmi1 expression (T. Takahashi, T. Kawasaki & S. Yuba unpublished results). If it is the case that non-neuronal ACh regulates Bmi1-expressing stem cells, this lineage relationship could possibly occur with directional regulatory mechanisms to control the total number of intestinal stem cells, to regulate the balance of active and quiescent intestinal stem cells within the total stem cell pool, and to restrain homeostatic Bmi1-expressing cell proliferation.

M1–M5 are abundantly expressed in most cancer cells, and are involved in various aspects of cell function, particularly proliferation [27,52]. The muscarinic influence on proliferation is at least partly mediated by the ‘classic’ signaling pathway for muscarinic receptors, namely the inositol–phospholipid signaling cascade [52] involving two secondary messengers, diacylglycerol and inositol 1,4,5-triphosphate. Notably, however, this pathway is not the only one involved in mediating the
muscarnic effect. Several studies have reported that the muscarinic receptor is also coupled to the mitogen-activated protein kinase pathway, probably via transactivation of the EGF receptor [25,27,47,53,54] located on the basolateral membranes of crypt cell epithelium [55]. We previously demonstrated that organoids are formed from isolated crypts when grown in a Matrigel-based culture system in the presence of EGF. It has also been shown that Paneth cells from mice express EGF as one of several essential signals required for maintenance of cultured stem cells [56]. In the present study, we have demonstrated that non-neuronal ACh contributes to the maintenance and differentiation of Lgr5-positive stem cells. On the basis of the experimental evidence, we speculate that EGF produced by Paneth cells stimulates the proliferation of stem cells by transactivation of the EGF receptor via muscarinic receptors. To control this effect, epithelial cells produce ACh, which blocks both stem cell commitment and movement along the epithelial cell differentiation pathway without transactivation of the EGF receptor. Thus, EGF and ACh may modulate each other’s effects or act on different parts of the differentiation pathway.

In conclusion, our data suggest that non-neuronal ACh is involved in the maintenance and differentiation of Lgr5-positive stem cells to regulate the homeostasis of intestinal epithelial cell density. The non-neuronal cholinergic system described in this study is consistent with the speculation that a novel combinatorial pathway is controlled by cholinergic and antagonistic signals, such as Wnt, which causes the typical crypt–villus architecture to change, resulting in round cysts devoid of differentiated cell types [56]. The identification of this pathway may help to determine the mechanisms underlying developmental processes in the crypt–villus axis.

**Experimental procedures**

**Animals**

This study was approved by the Animal Committee of the National Institute of Advanced Industrial Science and Technology, and all animals were maintained in accordance with the guidelines of this committee for the care and use of laboratory animals. C57/BL6 mice (aged 2 days and 8 weeks) were used in this study. lgr5-EGFP-ires-CreERT2 mice (aged 8 weeks) were purchased from Jackson Laboratory (Sacramento, CA, USA). Mice were killed by cervical dislocation.

**Crypt isolation and crypt–villus organoid culture**

Crypt isolation and crypt–villus organoid culture were performed according to the method of Sato [18]. Isolated small intestines were opened longitudinally and washed with cold NaCl/Pi. The tissue was cut into ~ 5 × 5-mm pieces, which were further washed with cold NaCl/Pi. The washed tissue fragments were incubated in NaCl/Pi, containing 2 mM EDTA for 30 min on ice. After removal of the EDTA solution, the tissue fragments were vigorously suspended in cold NaCl/Pi, with a 10-mL pipette. The resulting suspension was passed through a 70-μm cell strainer (BD Biosciences, MA, USA) to remove residual villous material, and the isolated crypts were then centrifuged (390 g, 3 min, 4 °C). The final fraction consisted of essentially pure crypts, and was used for culture. Isolated crypts were counted and pelleted. A total of 100 crypts were mixed with 40 μL of Matrigel (BD Biosciences) and plated in 24-well plates. After polymerization of Matrigel, 500 μL of crypt culture medium (Advanced DMEM/F12; Invitrogen, CA, USA) containing growth factors (20 ng/mL EGF; R&D Systems, MN, USA), 500 ng/mL R-spondin 1 (R&D Systems) and 100 ng/mL Noggin (R&D Systems) was added. Culture medium was changed every other day. For passaging, organoids were removed from the Matrigel, mechanically dissociated into single-crypt domains, and then transferred to fresh Matrigel. Passaging was performed every 1–2 weeks with a 1 : 3 split ratio.

**RT-PCR**

Total RNA from brain, gut and organoid tissues was extracted with TRIzol reagent (Gibco BRL, OK, USA) according to the manufacturer’s instructions. Each total RNA (2 μg) was used as a template for cDNA synthesis. Reverse transcription was performed with SuperScript II and an oligo-dT primer according to the protocol recommended by the manufacturer (Invitrogen). With the first-strand cDNA as a template, cDNAs encoding neuron-specific marker genes, intestinal stem cell marker genes, epithelial cell marker genes and genes encoding cholinergic components were amplified by PCR with designed PCR primers (Table S1). The PCR conditions were as follows: 94 °C for 3 min followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was amplified as an internal control with a specific primer set (Table S1).

**Preparation of extracts and determination of ACh content**

Extracts of brain, gut and organoids were homogenized in 0.1 M perchloric acid (1 g 5 mL⁻¹ of sample) containing 0.1 M EDTA. After centrifugation at 20 400 g for 15 min at 4 °C, samples were adjusted to ~ pH 7.4 with 0.2 M KHCO₃. Each supernatant was filtered through an Ultrafree-MC Centrifugal Filter (0.22-μm filter; Millipore, Tokyo, Japan), 10 μL of the filtrate was injected onto an
AC-GEL column (2.0 × 150 mm²; Eicom, Kyoto, Japan), and ACh was detected with an electrochemical detector (HTEC-500; Eicom). The working electrode potential was maintained at +300 mV against Ag/AgCl. A PC-03 column (3.0 × 4.0 mm²; Eicom) was placed between the injector and the analytical column. An AC-ENZYMpak column (1.0 × 4.0 mm²; Eicom) was placed between the analytical column and the electrochemical detector. The temperature was kept constant at 33 °C. The mobile phase (300 mg L⁻¹ SDS, 50 mM KHCO₃, 50 mg L⁻¹ EDTA) was delivered at 150 µL-min⁻¹. The retention time of ACh was ~14.6 min.

Preparation of extracts and measurement of ACh-synthesizing activity

Extracts for determination of ACh-synthesizing activity were prepared from brain, gut and organoid tissues as previously described [3]. ACh-synthesizing activity in each enzyme extract was assayed with a modified procedure of Fonnum [57]. Briefly, a 50-µL sample of enzyme extract was incubated for 60 min at 37 °C in 100 µL of assay mixture (0.15 mM [³H]acetyl-coenzyme A, 24 mM sodium phosphate buffer, pH 7.4, 15 mM choline chloride, 0.15 mM physostigmine). The reaction was then terminated by addition of 500 µL of ice-cold NaCl/Pi to the mixture in an ice/water bath. ACh-synthesizing activity was calculated by subtracting the ACh synthesized in the presence of 10 µM bromoacetylcholine (Nacalai Tesque, Kyoto, Japan), a specific ChAT inhibitor, from that synthesized in the absence of bromoacetylcholine.

Detection of ACh in the culture medium of organoids

Organoids were cultured in a medium supplemented with inhibitors of acetylcholinesterase (10⁻⁵ M eserine) (Sigma, CA, USA) and butryrylcholinesterase (10⁻⁵ M iso-OMPA) (Alfa Aesar, MA, USA) for 3 days. The medium (1 mL) was applied to an LC-MS/MS system (LCMS-8030; Shimadzu, Kyoto, Japan) equipped with an amino acid column (50 × 3.0 mm internal diameter, 3 µm; Imtakt, Kyoto, Japan) operated at 40 °C for chromatographic separation. The mobile phase consisted of solvent A [0.2% (v/v) formic acid, 100 mmol L⁻¹ ammonium formate, pH 4.0] and solvent B (acetonitrile), and was delivered at a flow rate of 0.15 mL min⁻¹. The linear gradient used was as follows: 0–5 min, 90–60% solvent B; 5–12 min, 60% solvent B; 12–15 min, 60–30% solvent B; and 15–16 min, 30–90% solvent B. The ESI (positive ionization mode) mass spectrometer was operated in multiple reaction monitoring mode to observe the transition of m/z 146.10 to m/z 87.05 for ACh quantitation at a collision energy of 20 eV. The retention time of ACh was ~6.3 min.

Immunohistochemistry

The collected gut and organoid tissue samples were embedded in Tissue-Tek OCT compound (Qiagen, Tokyo, Japan), frozen at −20 °C, and then cut serially into 10-µm-thick sections with a cryostat (Leica, Heidelberg, Germany). For immunohistochemistry analysis, the sections were fixed with NaCl/Pi, containing 4% paraformaldehyde for 10 min at room temperature. After being washed three times with NaCl/Pi, the sections were treated with 1% sodium borohydride (Nacalai Tesque) for 10 min at room temperature to reduce autofluorescence, and were again washed three times with NaCl/Pi. The sections were then treated with NaCl/Pi containing 0.1% Triton X-100 for 10 min at room temperature, and washed three times with NaCl/Pi. The sections were then treated with 1% BSA (Sigma) for 2 h at room temperature. The treated sections were incubated for 60 min at room temperature in primary antibodies diluted appropriately in 1% BSA. The primary antibodies used for immunohistochemistry are shown in Table S2. After incubation, the sections were washed three times with NaCl/Pi, and further incubated for 30 min at room temperature in secondary antibodies diluted in 1% BSA. The secondary antibodies used were Alexa Fluor 568 goat anti-(mouse IgG) (1 : 1000), Alexa Fluor 546 goat anti-(rabbit IgG) (1 : 1000), Alexa Fluor 488 goat anti-(rabbit IgG) (1 : 1000), Alexa Fluor 488 and 488 donkey anti-(mouse IgG) (1 : 1000), and Alexa Fluor 568 donkey anti-(goat IgG) (1 : 1000) (all from Molecular Probes, CA, USA). Nuclei were stained with Hoechst 33342 (1 : 1000; AnaSpec, CA, USA). Control sections were labeled in the absence of primary antibodies. Sections were mounted in Mowiol mounting medium (Mowiol 4-88; Sigma) under a cover glass, and observed by the use of confocal immunofluorescence microscopy (LV1000; Olympus, Tokyo, Japan). Whole-mount immunohistochemical staining was also conducted as described previously [18]. Three-dimensional images were acquired by confocal microscopy (LV1000; Olympus).

IMS

We adopted a previously described procedure for MALDI-TOF IMS [58–60]. For tissue sections and matrix coating for IMS analyses, 8-µm cryosections of frozen gut tissue samples were prepared on ITO-coated glass slides (Bruker Daltonics, Leipzig, Germany) at −20 °C. The sections were then uniformly sprayed with 50 mg mL⁻¹ 2,5-dihydroxybenzoic acid (Bruker Daltonics) in 70% methanol and 0.1% trifluoroacetic acid by use of a Procon Boy FWA Platinum 0.2-mm caliber airbrush (Mr Hobby, Tokyo, Japan). The sections were analyzed with a MALDI quadrupole ion trap-type instrument, which is a combination of a mass microscope (Shimadzu) and a MALDI-TOF/TOF-type instrument (Autoflex3; Bruker Daltonics), with data
point intervals of 100 μm (Fig. 6C) and 10 μm (Fig. 6D), respectively. To examine the distribution of ACh in the gut, MS/MS imaging was performed with the mass microscope in MS/MS mode, in which the data acquisition conditions (laser power, collision energy, and number of laser irradiations) were optimized to obtain product ion mass spectra with high signal-to-noise ratios for the fragment peaks.

Microarray analysis

Microarray analysis was conducted at Miltenyi Biotec K.K. (Tokyo, Japan). Total RNAs were extracted from the intestine, crypt, γ-organoids (short-term cultured organoids), and δ-organoids (long-term cultured organoids). The RNA samples were checked for quality with the Agilent 2100 Bioanalyzer platform (Agilent Technologies, CO, USA). For the linear T7-based amplification step, 20 ng of each total RNA sample was used. To produce Cy3-labeled cRNA, the RNA samples were amplified and labeled with the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies), according to the manufacturer’s protocol. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol with the Agilent Gene Expression Hybridization kit (Agilent Technologies). Briefly, 1.65 μg of Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight at 65 °C to a Whole Mouse Genome Oligo Microarray (4000 × 44 000 slide format, V2; Agilent Technologies). Fluorescence signals were detected with the Microarray Scanner System (Agilent Technologies), and Agilent Feature Extraction Software (fes) was used to read and process the microarray image files. The cluster analysis was performed with the L2L Microarray Analysis Tool (http://depts.washington.edu/∼l2l/about.html). The ratio experiments were designated as control versus sample experiments (automated data output of the Resolver system). The results of the ratio experiments were visualized in double-log scatter plots. Differentially expressed genes were selected if they showed a greater than two-fold change between the two groups with a P-value of < 0.01. The data were deposited in the Gene Expression Omnibus database (accession number GSE49864).

Pharmacological assay

A pharmacological assay was performed with mechanically dissociated single-crypt domains, which were allowed to grow into organoids in the presence or absence of carbachol, atropine, or selective mACHR antagonists (pirenzepine, AF-DX-116, 4-DAMP, and tropicamide; Nacalai Tesque). The culture medium with or without drugs was replaced every other day. To assay growth, organoid size was analyzed with IMAGE J 1.41 (NIH, MD, USA). The effects of the drugs on marker gene expression in intestinal stem cells (Lgr5) and epithelium (Sox9, Ngn3, Hnf1, and Klf4; differentiation markers for Paneth cells, enteroendocrine cells, enterocytes, and goblet cells, respectively) were analyzed by RT-PCR with treated and untreated (control) organoids. The primer sequences used for PCR amplification are shown in Table S1.

Quantitative RT-PCR analysis

Organoids were treated with carbachol, atropine and selective mACHR antagonists in culture medium for 3 days at 37 °C. Quantitative RT-PCR for intestinal stem cell and epithelial cell marker genes with the SYBR Green master mixture (Applied Biosystems, CA, USA) was performed three times, according to the manufacturer’s procedure. GAPDH was amplified as an internal control. All primers for real-time PCR are shown in Table S3. For relative quantification of gene expression, the comparative C_{T} method was used.

Biological assay

Cell dissociation has been described previously [56]. Cell proliferation analysis was performed as follows. Crypts were isolated from lgr5-EGFP-ires-CreERT2 mice, and cultured as described above. Lgr5–GFP-positive cells retain stem cell properties. For the assay, 100 organoids (passage 5) were incubated with carbachol (10^{-4} m) and atropine (10^{-5} m) for 3 days. The solutions were renewed every other day. After treatment, the organoids were dissociated into a suspension of single cells with TrypLE express (Invitrogen) for 30 min at 37 °C to score for GFP-positive cells. The cell proliferation assay was performed at least three times independently.

Flow cytometry analysis

The carbachol-treated and atropine-treated organoids and untreated control organoids were dissociated by treatment with TrypLE express for 30 min at 37 °C. Dissociated cells were passed through a 20-μm cell strainer (BD Biosciences, MA, USA), washed with NaCl/Pi, and then sorted with a BD FACSVersus (BD Biosciences). Viable cells were gated by staining for 7-amino-actinomycin D (BD Biosciences). Mean fluorescence values of 2500 events per sample were read via flow cytometry and analyzed with BD FACSuite (BD Biosciences).

Statistical analysis

Comparisons between two groups of data were made with the Mann–Whitney test or Student’s t-test, wherever applicable. Data and statistical analyses were performed with Microsoft Excel. In all cases, differences were considered to
be significant at \( P < 0.05 \). All experiments were repeated at least three times.

**Acknowledgements**

We thank H. Satake and J. Murata (Suntory Foundation for Life Sciences, Bioorganic Research Institute, Japan) for fruitful discussions. This work was supported by Research Grants for PRESTO (870) (Y. Sugiuira) and ERATO (1865) (M. Suematsu) from JST.

**Author contributions**

M. Suematsu, Y. Hippo, T. Yamagaki, S. Yuba and T. Takahashi planned the experiments. H. Ohnishi, T. Fujii, Y. Sugiuira, K. Honda, K. Orihashi, T. Watanabe and T. Takahashi performed the experiments. T. Kawasaki and T. Deguchi analyzed the data. T. Takahashi wrote the paper.

**References**

1. Rand JB (2007) Acetylcholine. In WormBook (Erik MJ & Joshua MK, eds), pp. 1–21. The C. elegans Research Community, WormBook, doi: 10.1895/wormbook.1.131.1.
2. Sastry BV & Sadavongvivad C (1978) Cholinergic systems in non-nervous tissues. *Pharmacol Rev* 30, 65–132.
3. Horiiuchi Y, Kimura R, Kato N, Fujii T, Seki M, Endo T, Tako T & Kawashima K (2003) Evolutional study on acetylcholine expression. *Life Sci* 72, 1745–1756.
4. Wessler I, Kilbinger H, Bittinger F & Kirkpatrick CJ (2001) The biological role of non-neuronal acetylcholine in plants and humans. *Ipn J Pharmacol* 85, 2–10.
5. Klapproth H, Reinheimer T, Metzen J, Munch M, Bittinger F, Kirkpatrick CJ, Hohle KD, Schemann M, Racke K & Wessler I (1997) Non-neuronal acetylcholine, a signaling molecule synthesized by surface cells of rat and man. *Naunyn Schmiedebergs Arch Pharmacol* 355, 515–523.
6. Hirota CL & McKay DM (2006) Cholinergic regulation of epithelial ion transport in the mammalian intestine. *Br J Pharmacol* 149, 463–479.
7. Yajima T, Inoue R, Matsumoto M & Yajima M (2011) Non-neuronal release of ACh plays a key role in secretory response to luminal propionate in rat colon. *J Physiol* 589, 953–962.
8. Laukoetter MG, Nava P & Nusrat A (2008) Role of the intestinal barrier in inflammatory bowel disease. *World J Gastroenterol* 14, 401–407.
9. Schneider MR, Dahlhoff M, Horst D, Hirschi B, Trulzsch K, Muller-Hocker J, Vogelmann R, Allgauer M, Gerhard M, Steininger S et al. (2010) A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. *PLoS One* 5, e14325.
10. Tatetsu M, Kim J, Kina S, Sunakawa H & Takayama C (2012) GABA/glycine signaling during degeneration and regeneration of mouse hypoglossal nerves. *Brain Res* 1446, 22–33.
11. Gotti C & Clementi F (2004) Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol* 74, 363–396.
12. Caulfield MP & Birdsall NJ (1998) International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 50, 279–290.
13. Shiozaki K, Iseki E, Uchiyama H, Watanabe Y, Haga T, Kameyama K, Ikeda T, Yamamoto T & Kosaka K (1999) Alternations of muscarinic acetylcholine receptor subtypes in diffuse Lewy body disease: relation to Alzheimer’s disease. *J Neurol Neurosurg Psychiatry* 67, 209–213.
14. Shiozaki K, Iseki E, Hino H & Kosaka K (2001) Distribution of m1 muscarinic acetylcholine receptors in the hippocampus of patients with Alzheimer’s disease and dementia with Lewy bodies – an immunohistochemical study. *J Neurol Sci* 193, 23–28.
15. Montgomery RK, Mulberg AE & Grand RJ (1999) Development of the human gastrointestinal tract: twenty years of progress. *Gastroenterology* 116, 702–731.
16. Spence JR, Lauf R & Shroyer NF (2011) Vertebrate intestinal endoderm development. *Dev Dyn* 240, 501–520.
17. Fordham RP, Yui S, Hannan NRF, Soendergaard C, Madgwick A, Schweiger PJ, Nielsen OH, Vallier L, Pedersen RA, Nakamura T et al. (2013) Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* 13, 734–744.
18. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ et al. (2009) Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. *Nature* 459, 262–265.
19. Massoulie J, Sussman J, Bon S & Silman I (1993) Structure and functions of acetylcholinesterase and butyrylcholinesterase. *Prog Brain Res* 98, 139–146.
20. Li B, Stribley JA, Ticu A, Xie W, Schopfer LM, Hammond P, Brimijoin S, Hinrichs SH & Lockridge O (2000) Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. *J Neurochem* 75, 1320–1331.
21. Cabbarocas J, Savidge T & Liblau R (2003) Role of enteric glial cells in inflammatory bowel disease. *Glia* 41, 81–93.
22. Joseph NM, He S, Quintana E, Kim YG, Nunez G & Morrison SJ (2011) Enteric glia are multipotent in...
culture but primarily form glia in the adult rodent gut. J Clin Invest 121, 3398–3411.

23 Carrozzo MM, Cannazza G, Pinetti D, Di Viesti V, Battisti U, Braghiroli D, Parenti C & Baraldi M (2010) Quantitative analysis of acetylcholine in rat brain microdialysates by liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Neurosci Methods 194, 87–93.

24 Landgraf D, Barth M, Layer PG & Sperling LE (2010) Acetylcholine as a possible signaling molecule in embryonic stem cells: studies on survival, proliferation and death. Chem Biol Interact 187, 115–119.

25 Ukegawa JI, Takeuchi Y, Kusayanagi S & Mitamura K (2004) Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front Biosci 9, 2063–2085.

26 Kawashima K & Fujii T (2004) Expression of non-neuronal acetylcholinesterase in neurons of the nervous system tissue sections by tandem imaging mass spectrometry. J Neurochem 93, 1–6.

27 Shah N, Khurara S, Cheng K & Paufman JP (2009) Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296, C221–C232.

28 Takahashi T & Hamaue N (2010) Molecular characterization of Hydra acetylcholinesterase and its catalytic activity. FEBS Lett 584, 511–516.

29 Wessler I & Kirkpatrick CJ (2008) Acetylcholine beyond neurons: the non-neuronal cholinergic system in human. Br J Pharmacol 154, 1558–1571.

30 Cheng H & Leblond CP (1974) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am J Anat 141, 537–561.

31 Barker N, van Es JH, Kuijpers J, Kujala P, van den Born M, Cozijnse M, Haegebarth A, Korving J, Begthel H, Peters PJ et al. (2007) Identification of stem cells in small intestine and colon by maker gene Lgr5. Nature 449, 1003–1007.

32 Hsu SY, Kado M, Chen T, Nakabayashi K, Bhalla A, van der Spek PJ, van Duin M & Hsueh AJ (2000) The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors (LGR): identification of LGR6 and LGR7 and the signaling mechanism for LGR7. Mol Endocrinol 14, 1257–1271.

33 de Lau W, Barker N, Low TY, Koo B-K, Li VSW, Teunissen H, Kujala P, Haegebarth A, Peters PJ, van de Wetering M et al. (2011) Lgr5 homologues associate with Wnt receptors and mediate R-spondin signaling. Nature 476, 293–297.

34 Roberts RR, Murphy JF, Young HM & Bornstein JC (2007) Development of colonic motility in the neonatal mouse – studies using spatiotemporal maps. Am J Physiol Gastrointest Liver Physiol 292, G930–G938.

35 Young HM & Ciampoli D (1998) Transient expression of neuronal nitric oxide synthase by neurons of the submucous plexus of the mouse small intestine. Cell Tissue Res 291, 395–401.

36 Geffard M, Vieillemaringe J, Heinrich-Rock A-M & Duris P (1985) Anti-acetylcholine antibodies and first immunocytochemical application in insect brain. Neurosci Lett 57, 1–6.

37 Chagnaud JL, Souan ML, Charrier MC & Geffard M (1989) Monoclonal anti-conjugated acetylcholine antibody and immunohistochemical applications in rat nervous system. J Neurochem 53, 383–391.

38 Sugiuara Y, Zaima N, Setou M, Ito S & Yao I (2012) Visualization of acetylcholine distribution in central nervous system tissue sections by tandem imaging mass spectrometry. Anal Bioanal Chem 403, 1851–1861.

39 Hattori K, Kajimura M, Hishiki T, Nakanishi T, Kubo A, Nagahara Y, Ohmura M, Yachie-Kinoshita A, Matsuura T, Morikawa T et al. (2010) Paradoxical ATP elevation in ischemic penumbra revealed by quantitative imaging mass spectrometry. Antioxid Redox Signal 13, 1157–1167.

40 Kubo A, Ohmura M, Wakui M, Harada T, Kajihara S, Ogawa K, Suemizu H, Nakamura M, Setou M & Suematsu M (2011) Semi-quantitative analyses of metabolic systems of human colon cancer xenografts in livers of superimmunodeficient NOG mice. Anal Bioanal Chem 400, 1895–1904.

41 Morikawa T, Kajimura M, Nakamura T, Hishiki T, Nakanishi T, Yukutake Y, Nagahata Y, Ishikawa M, Hattori K, Takenouchi T et al. (2012) Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway. Proc Natl Acad Sci USA 109, 1293–1298.

42 Krymskaya VP, Orsini MJ, Eszterhas AJ, Brodbeck KC, Benovic JL, Panetieri RA Jr & Penn RB (2000) Mechanisms of proliferation synergy by receptor tyrosine kinase and G protein-coupled receptor activation in human airway smooth muscle. Am J Respir Cell Mol Biol 23, 546–554.

43 Goksen R, Nelemans SA, Groote Bromhaar MM, McKay S, Zaagsma J & Meurs H (2003) Muscarinic M3 receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. Am J Respir Cell Mol Biol 28, 257–262.

44 Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C & Ullrich A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402, 884–888.

45 Tsai W, Morielli AD & Peralta EG (1997) The m1 muscarinic acetylcholine receptor transactivations the EGF receptor to modulate ion channel activity. EMBO J 16, 4597–4605.

46 Stirnweiss J, Valkova C, Ziesche E, Drube S & Liebmann C (2006) Muscarinic M2 receptors mediate transactivation of EGF receptor through Fyn kinase
and without matrix metalloproteases. *Cell Signal* **18**, 1338–1349.

47 Cheng K, Zimniak P & Raufman J-P (2003) Transactivation of the epidermal growth factor receptor mediates cholinergic agonist-induced proliferation of H508 human colon cancer cells. *Cancer Res* **63**, 6744–6750.

48 Lundgren O, Jodal M, Jansson M, Ryberg AT & Svensson L (2011) Intestinal epithelial stem/progenitor cells are controlled by mucosal afferent nerves. *PLoS One* **6**, e16295.

49 Sangiorgi E & Capecchi MR (2008) *Bmi1* is expressed in vivo in intestinal stem cells. *Nat Genet* **40**, 915–920.

50 Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD & de Sauvage FJ (2011) A reverse stem cell population in small intestine renders *Lgr5*-positive cell dispensable. *Nature* **478**, 255–259.

51 Yan KS, Chia LA, Li X, Ootani A, Su J, Lee JY, Nan Su, Luo Y, Heilshorn SC, Amieva MR et al. (2012) The intestinal stem cell markers *Bmi1* and *Lgr5* identify two functionally distinct populations. *Proc Natl Acad Sci USA* **109**, 466–471.

52 Costa LG, Guizzetti M, Oberdoerster J, Yagle K, Costa-Mallenn P, Titu B, Bordi F, Vitalic A, Palmery M & Valeri P (2001) Modulation of DNA synthesis by muscarinic cholineric receptors. *Growth Factors* **18**, 227–236.

53 Slack BE (2000) The m3 muscarinic acetylcholine receptor is coupled to mitogen-activated protein kinase via protein kinase C and epidermal growth factor receptor kinase. *Biochem J* **348**, 381–387.

54 Cheng K, Xie G & Raufman JP (2007) Matrix metalloproteinase-7-catalyzed release of HM-EGF mediates deoxycholyltarurine-induced proliferation of a human cancer cell line. *Biochem Pharmacol* **73**, 1001–1012.

55 Scheving LA, Shiurba RA, Nguyen TD & Gray GM (1989) Epidermal growth factor receptor in the intestinal enterocyte. Localization to laterobasal but not brush border membrane. *J Biol Chem* **264**, 1735–1741.

56 Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M & Clevers H (2011) Paneth cells constitute the niche for *Lgr5* stem cells in intestinal crypts. *Nature* **469**, 415–418.

57 Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J Neurochem* **24**, 407–409.

58 Shimma S, Sugiura Y, Hayasaki T, Hoshikawa Y, Noda T & Setou M (2007) MALDI-based imaging mass spectrometry revealed abnormal distribution of phospholipids in colon cancer liver metastasis. *J Chromatogr B Anal Technol Biomed Life Sci* **855**, 98–103.

59 Sugiura Y, Shimma S & Setou M (2006) Two-step matrix application technique to improve ionization efficiency for matrix-assisted laser desorption/ionization in imaging mass spectrometry. *Anal Chem* **78**, 8227–8235.

60 Yamamoto T, Takano N, Ishiwata K, Ohmurka M, Nagahata Y, Matsuura T, Kamata A, Sakamoto K, Nakanishi T, Kubo A et al. (2014) Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. *Nat Commun* **5**, 3480.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s website:

Table S1. Primers used for RT-PCR of brain, gut, and organoids.

Table S2. List of antibodies used in this study.

Table S3. Primers used for quantitative RT-PCR.