Paneth cell maturation is related to epigenetic modification during neonatal–weaning transition

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Abstract
Paneth cells are antimicrobial peptide-secreting epithelial cells located at the bottom of the intestinal crypts of Lieberkühn. The crypts begin to form around postnatal day 7 (P7) mice, and Paneth cells usually appear within the first 2 weeks. Paneth cell dysfunction has been reported to correlate with Crohn’s disease-like inflammation, showing narrow crypts or loss of crypt architecture in mice. The morphology of dysfunctional Paneth cells is similar to that of Paneth/goblet intermediate cells. However, it remains unclear whether the formation of the crypt is related to the maturation of Paneth cells. In this study, we investigated the histological changes including epigenetic modification in the mouse ileum postnatally and assessed the effect of the methyltransferase inhibitor on epithelium development using an organoid culture. The morphological and functional maturation of Paneth cells occurred in the first 2 weeks and was accompanied by histone H3 lysine 27 (H3K27) trimethylation, although significant differences in DNA methylation or other histone H3 trimethylation were not observed. Inhibition of H3K27 trimethylation in mouse ileal organoids suppressed crypt formation and Paneth cell maturation, until around P10. Overall, our findings show that post-transcriptional modification of histones, particularly H3K27 trimethylation, leads to the structural and functional maturation of Paneth cells during postnatal development.

Keywords Paneth cells · Crypts · Organoids · Histone modifications · Epigenetic processes

Introduction
The small-intestinal mucosa has numerous villi and crypts and, therefore, can considerably expand its surface for function. The epithelium covering the mucosal surface is renewed continuously and rapidly. The epithelium consists of absorptive cells (or enterocytes), goblet cells, enteroendocrine cells, tuft cells, and Paneth cells. All intestinal epithelial cells (IECs) originate from intestinal stem cells (ISCs) that reside in niches of the lower crypt. ISCs give rise to transient amplifying cells that become progenitor cells positioned at the bottom two-third of the crypts (Barker and Clevers 2007). The activation of Notch signaling targeting Hes-1 and Math-1 results in the differentiation of the early progenitor cells into absorptive cells and secretory cell lineages, respectively, in IECs (Fre et al. 2005). Furthermore, in secretory cell lineages, Gfi1 functions to select goblet/Paneth versus enteroendocrine progenitors (Shroyer et al. 2005). Mucus-secreting goblet cells migrate toward the villus tip, whereas antimicrobial peptide-secreting Paneth cells move to the base of the crypts. In addition, Paneth cells provide a niche for leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5)-expressing stem cells in the crypts (Sato et al. 2011).

In mice, morphogenesis of the intestinal epithelium begins around embryonic day 14 (E14), followed by the reshaping of the endodermal pseudostratified epithelium to organize the villi and intervillous region (Grosse et al. 2011). The villi begin to form from E15. At the beginning of intestinal development, proliferating progenitors that express ISC markers are ubiquitously distributed throughout the epithelium. In the villus form, the villus cluster emits
bone morphogenetic protein (BMP) signals, acting opposite to Wnt signaling; hence, putative ISC s are restricted to the villus base (Shyer et al. 2015). In contrast, the crypts begin to form approximately on P7. Paneth-cell-specific mRNAs, including cryptdin-4, cryptdin-5, lysozyme, matrilysin, and defensin-related sequences, have been detected in P1 mouse intestine (Darmoul et al. 1997). Within P14, Paneth cells mature and express cryptdin proteins as specific markers (Bry et al. 1994; Inoue et al. 2008). Lgr5 deficiency leads to premature Paneth cell differentiation in the small intestine without detectable effects on the differentiation of other cell lineages, or on proliferation or migration (Garcia et al. 2009). However, the relationship between the localization of Paneth cells at the bottom of the crypt and their maturation remains to be elucidated.

In our previous study, we showed that the absorptive cells of the jejum and ileum change rapidly and dramatically from suckling to adult type during the weaning period (Fujita et al. 2007). It has been suggested that the intestinal transcription factor Blimp-1 is a critical driver of the postnatal epithelial phenotype, and its expression loss in the third postnatal week is a likely requirement for the maturation of the neonatal epithelium to adult epithelium (Harper et al. 2011; Muncan et al. 2011). Organoid culture studies revealed that suckling–weaning transition is intrinsically programmed (Navis et al. 2019). However, the factors driving Blimp-1 expression and loss remain unknown.

Several epigenetic regulation events, including DNA methylation and histone modifications, are known to occur during early embryonic development. In chick embryos and neonates, it has been shown that spatiotemporal-specific epigenetic alterations could be critical for the late development of the liver, jejunum, and breast skeletal muscles (Li et al. 2015). In contrast, abrupt changes in gene expression frequently occur in differentiating cells. These changes are accompanied by major chromatin structural changes that are triggered by modifications of the histone tail, such as acetylation, methylation, and phosphorylation (Rice et al. 2003; Schubeler et al. 2004). Among several histone modifications that have been identified, acetylation and methylation of histone H3 have been extensively studied; regulation of H3 modifications is related to on/off switching in transcription. In the small intestine, induction of sucrase-isomaltase (SI) gene expression during epithelial cell translocation from the crypt to the vill is associated with changes in histone H3 modifications from methylation at lysine 9 to di-acetylation at lysine 9 and 14 residues, as well as increased binding of Cdx-2 to the SI promoter region (Suzuki et al. 2008). In the murine small intestine, enhancer of zeste homolog 2 (EZH2), which selectively catalyzes H3 lysine 27 trimethylation, was expressed in nondifferentiated proliferative crypt IECs. It was found to be involved in the regulation of intestinal homeostasis, maintaining progenitor cell proliferation and an optimal balance between secretory and absorptive lineage differentiation programs (Chiacchiera et al. 2016; Koppens et al. 2016). The reduction in or suppression of histone H3K27 trimethylation has also been reported to be related to intestinal tumorigenesis and cancer (McCleland et al. 2015). However, the epigenetic reprogramming event in IECs during neonatal–weaning transition is unknown.

In this study, we investigated the temporal histological changes that include epigenetic modification in the ileum and the effect of methyltransferase inhibitors on epithelial development using an organoid culture to elucidate the relationship between Paneth cell maturation and crypt formation.

Materials and methods

Animals

In this study, three adult male C57BL/6 J mice (CLEA Japan) were used. They were housed under conventional laboratory conditions at a constant temperature of 22 °C, with a 12 h light/dark cycle. Data of postnatal studies were assessed on P0, P7, P14, P21, and P28. Three mice were used at each age. After weaning, P28 male mice were used. The mice were anesthetized via an intraperitoneal injection of a mixture of medetomidine, midazolam, and butorphanol, and then the intact ileal tissues were dissected. The research protocol was approved (AE08-004) by the ethics committee of the University of Occupational and Environmental Health and was conducted in accordance with the provisions of the Declaration of Helsinki, 1995 (as revised in Edinburgh 2000).

Immunohistochemistry of tissue and organoids

For light microscopy, the tissues and organoids were fixed for 16 h in 10% neutral buffered formalin at 4 °C. The tissues were embedded in paraffin and cut into 5-μm-thick sections. The sections were stained with hematoxylin and eosin (H and E) or subjected to immunohistochemistry. The organoids were embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, USA), frozen at −20 °C, and cut into 10-μm-thick sections. For immunohistochemistry, paraffin-embedded sections were prepared by boiling them in 10 mM citrate buffer pH 6.0 for 15 min. After blocking with 1% bovine serum albumin, the sections were incubated for 16 h at 4 °C with the following primary antibodies: anti-lysozyme C (1:200, goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-proliferating cell nuclear antigen (PCNA, 1:100, mouse monoclonal; Agilent Dako, Santa Clara, CA, USA); anti-5-mC (1:300, rabbit monoclonal; Cell
Signaling Technology, Boston, MA, USA); and anti-
H3K4, anti-H3K9, anti-H3K27, anti-H3K36, and anti-
H3K79me3 (1:300, rabbit monoclonal; Cell Signaling
Technology). Before incubation with the secondary anti-
bodies, the tissues were washed to remove the unbound
antibodies. The sections were then incubated with the
secondary antibodies (1:1000 dilution; Alexa Fluor 488
and/or Alexa Fluor 555; Invitrogen, Carlsbad, CA, USA) for 1 h at 25 °C and mounted using UltraCruz Mount-
ing Medium with 4',6-diamidino-2-phenylindole (DAPI)
(Santa Cruz Biotechnology). The samples were observed
under a fluorescence microscope (Axioskop 2 plus or
Apotome.2, Carl Zeiss, Göttingen, Germany).

Electron microscopy

For electron microscopy, the tissues and organoids were
fixed for 16 h in 2.0% paraformaldehyde and 2.5% glu-
taraldehyde in 0.1 M phosphate buffer (PB) at 4 °C, and
post-fixed in 1% osmium tetroxide in 0.1 M PB for 2 h at
4 °C. The specimens were dehydrated and embedded in
epoxy resin. Semi-thin sections were stained with Tolui-
dine Blue O (Sigma-Aldrich, St. Louis, MO, USA) and
observed under a light microscope (Axioskop 2 plus). Thin
sections were stained with uranyl acetate and lead citrate
and observed under a transmission electron microscope
(TEM) at 80 kV (1400Plus; JEOL, Tokyo, Japan).

Organoid culture

The intestinal organoids were cultured according to a
previously reported protocol (Sugimoto and Sato 2017).
C57BL/6 J mice were used on P4, P7, and P10 (n = 10
each). The terminal ileum was harvested, and the tissues
were sliced into segments. The crypts were isolated in
2.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS
for 30 min at 4 °C and pelleted by centrifugation at 400g
for 3 min at 4 °C. The crypts were then resuspended in
Matrigel (Corning, Corning, NY, USA) and transferred to
48-well plates. After polymerization, mouse IntestiCult
organoid growth medium (Stem Cell Technologies, British
Columbia, Canada) supplemented with penicillin–strepto-
tomycin (100 U/mL) was added to each well, and then
the plates were incubated at 37 °C. Three days after crypt
isolation, 0.2 µM 3-deazaneplanocin A hydrochloride
(DZNep, Abcam, Cambridge, UK) or 20 µM GSK126 (Bio
Vision, Waltham, MA, USA) was added to the medium
to inhibit the activity of histone methyltransferase EZH2,
which mediates H3K27 methylation. After 3 days of
incubation with the EZH2 inhibitor, the organoids were
observed under a light microscope.

Statistical analysis

Results are expressed as mean ± standard error of the mean
(SEM). Statistical differences were assessed using Dunnett’s
test. Statistical significance was set at p < 0.05.

Results

Morphological changes in the ileum
during neonatal–weaning transition

It remains unclear whether crypt formation is related to
Paneth cell maturation. To test this, we histologically
observed the mouse ileum from the neonatal to adult
stages (Fig. 1a–f). The villi were present in the P0 ileum,
and a time-dependent increase in length and width was
observed. Between the villi, shallow crypts were present
on P14 (Fig. 1c), and a time-dependent increase in depth
was observed (Fig. 1c–f). Goblet cells were dispersed in
IECs from the crypts to villi. The supranuclear lysosomes
of absorptive cells were enlarged on P7 and P14 (Fig. 1b,
c). However, there were no large lysosomes in the absorp-
tive cells of P21 mice. Paneth cells, including eosinophilic
granules, were observed at the bottom of the shallow crypts
on P14 (Fig. 1c). The number of Paneth cells and gran-
ules within the cell increased in a time-dependent manner
(Fig. 1d–f).

For a detailed structural analysis of IECs, we next
observed the ileum under a TEM (Fig. 2a–f). In P0 mice,
absorptive cells, goblet cells, enteroendocrine cells,

![Fig. 1 Histological changes in the mouse ileum from neonatal to adult stages. Morphological analysis was performed on 5-µm-thick paraffin-embedded sections stained with H and E. a P0, b P7, c P14, d P21, e P28, f adult. Paneth cells, including eosinophilic granules (arrowhead), were observed at the bottom of the ileal crypts after P14. Bar, 20 µm](image-url)
undifferentiated cells, and dividing cells were observed (Fig. 2a). In addition to typical goblet cells (Fig. 2d), some goblet cells containing secretory granules with a small dense core, called granular goblet cells, were observed (Fig. 2e). Typical Paneth cells were not observed in the epithelium. The cell types lining the P7 epithelium were similar to those in the P0 epithelium. In addition to P0 granular goblet cells, another type of granular goblet cells was observed in the primordia of the crypt (Fig. 2b, f). The core of cells was larger than that of the first type, and varied in size in the secretory granules. Several dense core secretory granules enriched with a clear halo were observed in Paneth cells positioned at the bottom of the P14 crypts (Fig. 2c). Subsequently, the number of Paneth cells increased.

Epigenetic modification in the ileum during neonatal–weaning transition

To examine the epigenetic event, we attempted to detect 5-methylcytosine (5-mC) for DNA methylation and H3K4, H3K9, H3K27, H3K36, and H3K79me3 for histone modifications in the epithelium of the developing ileum. There was no significant alteration in the expression of 5-mC, H3K4, H3K9, H3K36, or H3K79me3 in the IECs of the intervillous region or crypts from neonatal to weaning mice (data not shown). However, IECs located at the base of the crypt were positive for H3K27me3 after P14 (Fig. 3a–d). H3K27me3-positive cells in the crypts were negative for PCNA showing proliferative cells (Fig. 3e–h) and positive for lysozyme C, a Paneth cell marker (Fig. 4a–d).

Effect of the EZH2 inhibitor on ileal organoid culture

To examine the effect of H3K27 trimethylation on the development of the ileal epithelium, we used two types of EZH2 inhibitors, DZNep and GSK126, to inhibit the histone methyltransferase EZH2, which mediates the trimethylation of H3K27. The ileal crypts were isolated from the ileum of P4, P7, or P10 mice and cultured as organoids. DZNep or GSK126 was added to the culture medium after 3 days of culture. Subsequently, we observed the organoids under a light microscope (Fig. 5a). In the culture isolated from P4 and P7, the crypt budding was significantly inhibited, and organoids appeared in a spheroid form in the DZNep- and GSK126-treated groups compared...
with those in the control group (Fig. 5b–d). H3K27me3-positive cells were observed in the organoids of the control group, but not in those of the DZNep- and GSK126-treated groups (Fig. 5e–g). However, regardless of EZH2 inhibition, new crypt budding occurred in most organoids isolated from P10 mouse ileum, although the number and depth of the crypts in the DZNep- and GSK126-treated organoids were less than those of the crypts in the control group (Fig. 6a–c). In the organoids isolated from P7 mouse ileum, there were Paneth cells in addition to goblet cells and two types of granular goblet cells (Fig. 7a). However, Paneth cells were rarely observed in the DZNep- and GSK126-treated groups, although goblet cells and two types of granular goblet cells were observed (Fig. 7b, c). In the organoids derived from P10 mouse ileum (Fig. 7d–f), mature Paneth cells were observed regardless of EZH2 inhibition, although the cell number in EZH2-inhibited organoids was fewer than that in the control group.

**Discussion**

In this study, the second type of granular goblet cells with secretory granules enclosing a larger and irregular dense core appeared in the ileal epithelium, later than the first
granular goblet cells and earlier than Paneth cells. The cells were located in the ileal crypt, deeper than the first granular goblet cells and shallower than Paneth cells.

Granular goblet cells in the upper part of the crypts, which we call the first type, differ from other goblet cells because of the presence of small dense granules embedded within the mucus globules (Calvert et al. 1988; Cheng 1974a). In addition, the presence of intermediate cells between Paneth and goblet cells is considered to probably occur via intermediate cells (Mantani et al. 2014). In our study, the first granular goblet cells appeared in the epithelium of the villi and intervillous region. The second granular goblet cells that were similar to intermediate cells appeared below the first granular goblet cells. Moreover, Paneth cells appeared at the bottom of the crypt after
P14. The appearance of these three cell types differs temporally and spatially. In addition, we observed another cell type that had a few granules without cores (data not shown). Recently, a study revealed that the phenotype of intermediate cells is consistent with that of immature Paneth cells (Dekaney et al. 2019). Our findings indicate that two types of granular goblet cells, especially in the second type, are precursors of Paneth cells; however, further research on the expression of specific marker genes and proteins is required.

In the present study, Paneth cells located at the bottom of the crypt in P14 mouse ilea had trimethylated histone H3 lysine 27, although methylation of DNA and other H3 lysine residues, 4, 9, 36, and 79, was not significant during neonatal–weaning transition. Inhibition of EZH2 in the organoid culture suppressed stage-specific crypt budding. Post-transcriptional modification of histones plays an essential role in the regulation of chromatin structure and gene transcription. Histone methylation occurs in histone H3 at lysine 4, 9, 14, 27, 36, and 79 residues, and in histone H4 at lysine 20 and 59 residues. Generally, H3 methylation at lysine 4, 36, and 79 is correlated with euchromatin and transcriptional activation, whereas H3 methylation at lysine 9 and 27, and H4 at lysine 20 is associated with heterochromatin and transcriptional repression. However, post-transcriptional modification of histones fluctuates with the developmental stage (Lee et al. 2005). The reduction or suppression of histone H3K27 trimethylation is also related to intestinal tumorigenesis and cancer (McCleland et al. 2015).

Enhancer of zeste homolog 2 (EZH2), as a part of polycomb repressive complex 2 (PRC2), selectively catalyzes H3 lysine 27 trimethylation. In the murine jejunum and ileum, EZH2 and suppressor of zeste-12 (SUZ12) are expressed in nondifferentiated proliferative crypt IECs, and the PRC2 complex ensures the proper response of IECs to cell density (Turgeon et al. 2013). PRC2 regulates intestinal homeostasis, maintaining progenitor cell proliferation and an optimal balance between secretory and absorptive lineage differentiation programs (Chiacchiera et al. 2016; Koppens et al. 2016). PRC2 activity is required to maintain cell plasticity at the bottom of the intestinal crypt and the repression of Atoh1 and Gfi1, which are master regulators of goblet cells (Chiacchiera et al. 2016). However, cell-specific trimethylation at H3K27 has not been reported in the developing small intestine.

In this study, H3K27 trimethylation was detected in Paneth cells at the bottom of the crypts during the second week in postnatal mice. In the culture of organoids from mice until around P7, the EZH2 inhibitor suppressed crypt budding and Paneth cell maturation, unlike that from mice around P10. These results suggest that H3K27 trimethylation in Paneth cells at the bottom of the crypts began early in the second postnatal week and was almost complete during the second week, and it was related to its functional and morphological maturation. In our study, the transition of the absorptive cells from suckling to weaning was assumed to be completed after Paneth cell maturation. Paneth cell maturation, which is involved in not only the secretion of antimicrobial peptides but also the construction of the ISC niche, is related to the formation of the crypt and its localization. However, the crypts have been reported to form independent of Paneth cells in mice lacking lysine-specific demethylase 1A (Zwiggelaar et al. 2020). Consistent with our observations, organoids derived from fetal IECs undergo suckling–weaning transition, and organoids with crypts gradually increase, contrary to decreasing spheroids (Navis et al. 2019). However, the authors of that study concluded that spheroids transition to organoids and do not reflect the maturation stages. Further research is needed to determine whether all cell types mature or not. The intestinal transcription factor Blimp-1, which is selectively expressed in mouse IECs during embryonic and postnatal development, lost its expression during suckling–weaning transition (Muncan et al. 2011). Although it was assumed that H3 trimethylation at lysine 27 participates in the suppression of Blimp-1, the genes suppressed by H3K27 trimethylation remain unknown. To address this, it is necessary to understand the mechanism by which intermediate cells that are regarded as premature Paneth cells appear aberrantly in intestinal diseases such as IBD. It is difficult to demonstrate the detailed mechanisms that underlie the relationship between H3K27 trimethylation and crypt formation. However, it is estimated that H3K27 trimethylation might be involved in Paneth cell maturation and that Paneth cell maturation could lead to stem cell niche formation, that is, accelerated crypt formation.

In conclusion, our results showed that post-transcriptional modification of histones, particularly H3 at lysine 27, induced structural and functional maturation of Paneth cells during postnatal development in mice.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors of this article declare no conflict of interest.
Sugimoto S, Sato T (2017) Establishment of 3D intestinal organoid cultures from intestinal stem cells. Methods Mol Biol 1612:97–105. https://doi.org/10.1007/978-1-4939-7021-6_7

Suzuki T, Mochizuki K, Goda T (2008) Histone H3 modifications and Cdx-2 binding to the sucrase-isomaltase (SI) gene is involved in induction of the gene in the transition from the crypt to villus in the small intestine of rats. Biochem Biophys Res Commun 369(2):788–793. https://doi.org/10.1016/j.bbrc.2008.02.101

Troughton WD, Trier JS (1969) Paneth and goblet cell renewal in mouse duodenal crypts. J Cell Biol 41(1):251–268. https://doi.org/10.1083/jcb.41.1.251

Turgeon N, Blais M, Delabre JF, Asselin C (2013) The histone H3K27 methylation mark regulates intestinal epithelial cell density-dependent proliferation and the inflammatory response. J Cell Biochem 114(5):1203–1215. https://doi.org/10.1002/jcb.24463

Watanabe N, Mashima H, Miura K, Goto T, Yoshida M, Goto A, Ohnishi H (2016) Requirement of Goq/Gq11 signaling in the preservation of mouse intestinal epithelial homeostasis. Cell Mol Gastroenterol Hepatol 2(6):767–782. https://doi.org/10.1016/j.jcmgh.2016.08.001

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