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Short Communication

Histopathological evaluation of crypt fission during intestinal development in neonatal mice

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Abstract: Pathological evaluation of juvenile toxicity studies requires the understanding of normal tissue development at different ages. Here, we report the morphological features of the neonatal mouse intestine, focusing on crypt fission. Postnatal day (PND) 7 and 14 mice showed fewer crypts and less mature epithelial morphology compared to PND 21 and 28. Crypt fission occurred in three stages: 1) flattening of the crypt base into a skirt shape, 2) penetration of myofibroblasts into the crypt base center, and 3) complete separation of a single crypt into two daughter crypts. The ratio of crypt fission to total number of crypts was the highest at PND 14 and 7 in the jejunum and colon, respectively. Crypt fission, a key phenomenon for balance or imbalance in epithelial cell hierarchy, including stem and differentiated cells, is related to tissue injury repair and tumorigenesis. Therefore, examining crypt fission can provide valuable insights into current conditions of intestine. (DOI: 10.1293/tox.2019-0032; J Toxicol Pathol 2020; 33: 39–46)

Key words: juvenile toxicity, crypt fission, intestine, development

Recently, juvenile toxicity testing has become a topic of interest for pathologists, and the challenges in evaluating juvenile toxicity studies have been discussed[1]. These challenges include how to assess and interpret histopathological changes in tissues from juvenile animals. Although trained pathologists are familiar with changes in adult tissues, they often lack experience in studying neonatal tissues; therefore, information on normal tissue development at different ages will be helpful for pathologists.

Few reports are available on the detailed morphological features in intestinal development[2, 3]. One well-known phenomenon is the process of crypt fission, which is important for increasing the number of crypts in neonates but is a rare event in adults[4–6]. An important aspect of understanding this process is to comprehend the process histopathologically. Here, we report the morphological features of crypt fission in the intestine of neonatal mice.

Twelve pregnant mice (C57BL/6J) were purchased from Charles River Laboratories (Kanagawa, Japan). Infants were obtained from each parent (n=2–9 each). These parents and their infants were then divided into 4 groups. Three parent mice and their infants were sacrificed by exsanguination under deep anesthesia with isoflurane at the following time points: 7, 14, 21, and 28 days after birth. Samples of the intestine were acquired as cross-sections from the middle of the jejunum, defined as the first half of the small intestine from the stomach side, and from the colon immediately adjacent to the cecum. For adults, 4% paraformaldehyde (PFA) was injected into the intestinal lumen for fixation before obtaining cross-sections. The samples were then fixed in 4% PFA at 4°C for 16–24 h, followed by processing and embedding in paraffin using the AMeX method[7]. Sections from each block were stained with hematoxylin and eosin (HE) whereas some representative blocks were stained with Alcian Blue-Periodic acid-Schiff (AB-PAS). Immunohistochemical staining with primary antibodies against lysozyme (GTX62819, GeneTex, Irvine, CA, USA, ×1,000), Ki67 (#12202, Cell Signaling Technology, Danvers, MA, USA, 0.5 µg/mL), and alpha smooth muscle actin (α-SMA) (#19245, Cell Signaling Technology, 0.1 µg/mL) was also performed. Briefly, the primary antibodies were incubated after antigen retrieval in Target Retrieval Solution (S1699, Agilent, Santa Clara, CA, USA). A labeled polymer reagent (EnVision+ Single Reagents, HRP. Rabbit, K4003, Agilent) was applied as the secondary antibody, and the reaction was visualized using 3, 3'-diaminobenzidine (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) solution. The slides were counterstained with hematoxylin and observed under a light microscope.

For histopathological evaluation, 6 infants were select-

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ed at each time point to include mice of both sexes (range of ratio to male and female, 1:5–4:2) and at least one infant from each of the 3 parents. HE slides were scanned using the Aperio scan scope AT2 (Leica Biosystems, Nussloch, Germany), and the maximum diameter of the jejunum and colon was measured using the system's measurement function (one sample per mouse). The number of crypts and fissions in the total circumference of the jejunum and colon cross-sections were counted (one sample per mouse), and the distribution of crypt fission was evaluated using light microscopy. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Chugai Pharmaceutical Co. Ltd. (Shizuoka, Japan), and all experimental protocols were approved by the Institutional Animal Care and Use Committee.

The maximum diameter of both the jejunum and colon increased from postnatal day (PND) 7 to 28 (Fig. 1. A–D). At the tip of the villi in the jejunum on PND 7 and 14, ambiguous cell boundaries and irregularly aligned nuclei were observed (Fig. 2A). Moreover, vacuoles were found in the cytoplasm of epithelial cells at these time points. At PND 21 and 28, the shape of epithelial cells had become a typical simple columnar type. At these latter time points, the brush border was more obvious, and the nuclei were basically aligned on the basal side of the epithelium, but looked like stratified cells in some areas depending on the section level. Similar findings were observed in the colon (Fig. 2B). In addition to the above findings in the colon at PND 14, when the mucosa was less thick, goblet cells were located at the tip of the mucosa. However, when the thickness of the mucosa increased on PND 28, the main type of cells located at the tip of the mucosa were enterocytes, and goblet cells were located in the middle or at the base of the mucosa (Fig. 2B). These morphological changes in the epithelium are thought to be caused by the change in diet from suckling to weaning[8, 9]. Ki67-positive cells (proliferating cells) were moderately observed on PND 14, whereas the number of proliferating cells increased on PND 28, and was almost equivalent to those in adults in both the jejunum and colon (Fig. 3A and B). Lysozyme-positive cells (Paneth cells) were not observed at PND 7, but a few were observed at the crypt base on PND 14, and this number increased by PND 21 (Fig. 3C). Thus, we found several morphological changes in the intestine at different postnatal time points.

The characteristic structure of crypt fission is well-known in the neonatal phase in the intestine of rodents and

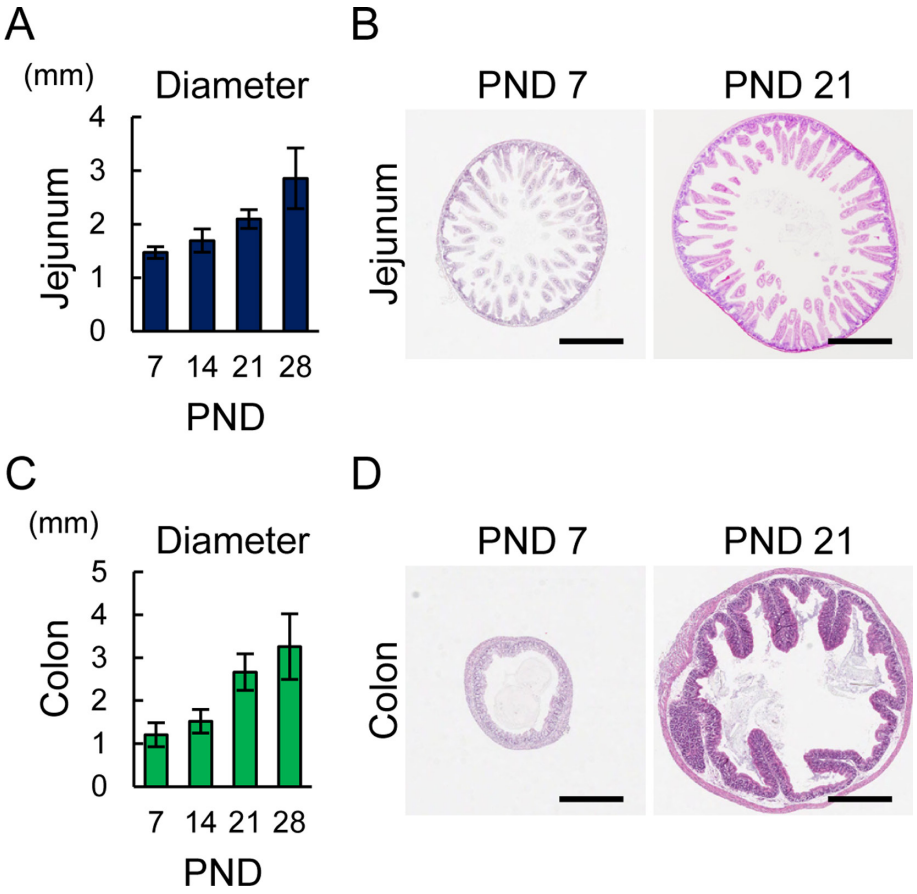

Fig. 1. (A) Diameter of the jejunum from postnatal day (PND) 7 to 28 (n=6). (B) Histopathological images of cross-sections of the jejunum at PND 7 and 21. (C) Diameter of the colon from PND 7 to 28 (n=6). (D) Histopathological images of cross-sections of the colon at PND 7 and 21. Values are the mean ± SD. Hematoxylin and eosin (HE) staining. Bar = 500 µm.

humans[4, 5]. Crypt fission, the process through which one parent crypt separates into two daughter crypts, is also an important phenomenon for increasing intestinal stem cells in the developmental phase[10]. Crypt fission observed in the current study could be categorized into 3 stages. First, the crypt base flattened and shaped like a skirt; second, stromal cells penetrated vertically into the center of the crypt base; and finally, one crypt completely separated into two crypts (Fig. 4A). The number of crypts in both the jejunum and colon increased from PND 7 to 28 (Fig. 4B and C), but crypt fission in the jejunum and colon reached a peak at different times (Fig. 4B and C). In the jejunum, the number of

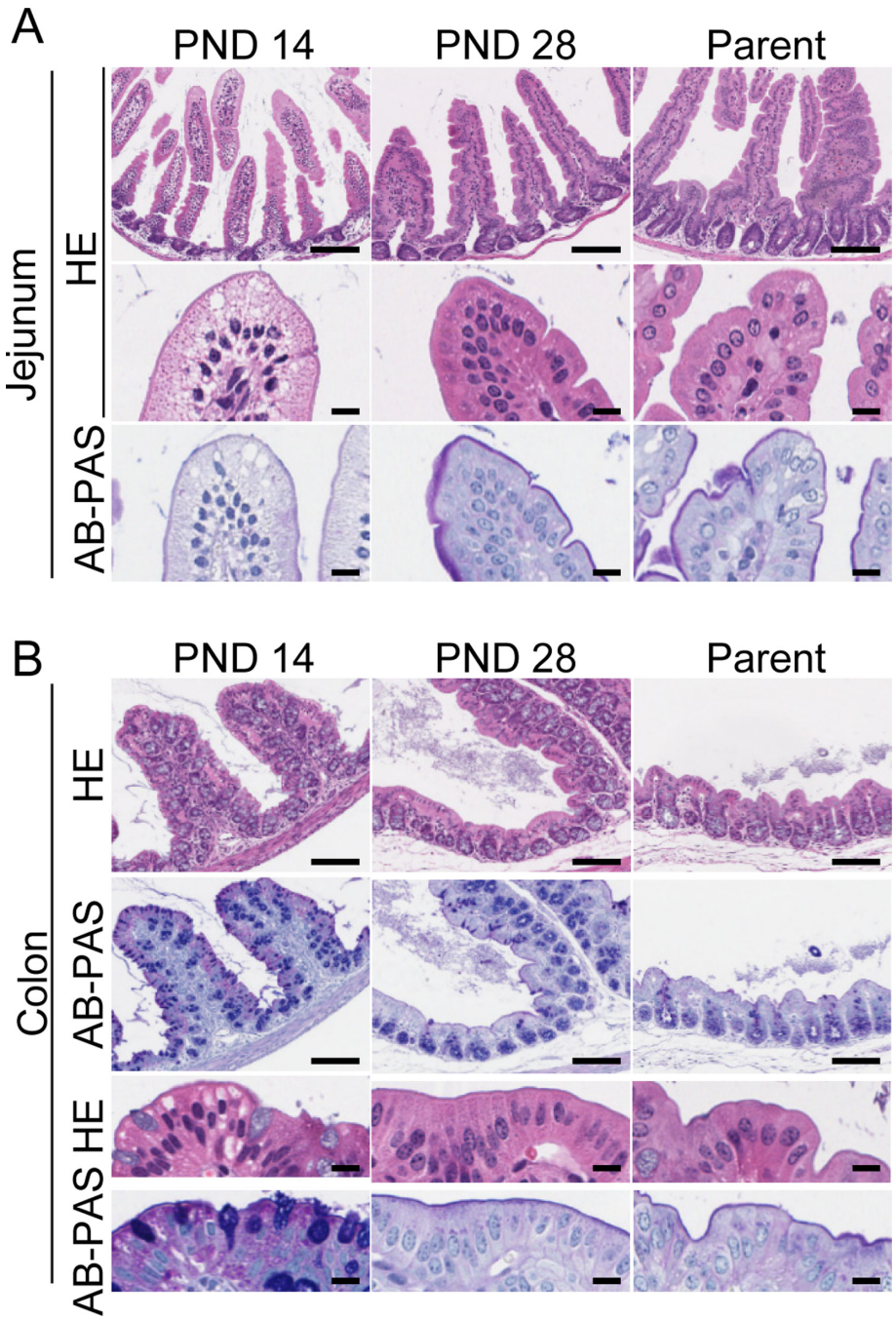

Fig. 2. (A) Histopathological images of the jejunum at postnatal day (PND) 14, 28, and parent. Hematoxylin and eosin (HE) and Alcian Blue-Periodic acid-Schiff (AB-PAS) staining. In lower rows of the pictures for HE and AB-PAS staining, the surface of mucosa is shown at high magnification. Bar=100 μm for upper images at lower magnification and 10 μm for middle and lower images at higher magnification. (B) Histopathological images of the colon at PND 14, 28, and in the parent. HE and AB-PAS staining. In lower rows of the pictures for HE and AB-PAS staining, the surface of the mucosa is shown at high magnification. Bar=100 μm for images of the upper two lines at lower magnification and 10 μm for images in the lower two lines at higher magnification.

crypt fissions was very low at PND 7 but had approximately tripled at PND 14, followed by a decrease, whereas in the colon, the number of crypt fissions observed at PND 7 was almost the same as that on PND 21 and showed a decrease at PND 28. In the jejunum, the ratio of crypt fissions to the total number of crypts peaked at PND 14 and decreased after PND 21 (Fig.4B), whereas in the colon, the ratio of fissions was the highest at PND 7 and decreased after PND 14 (Fig. 4C). The distribution of crypt fission along the entire circumference of the intestine is described in Fig. 4D and E. Crypt fission was diffused along the entire circumference in both the jejunum and colon. The number of crypt fissions is thought to be influenced by suckling and weaning in rodents and humans[8]. During the milk feeding period, crypt fission is observed frequently, but is markedly reduced after weaning, when crypt hyperplasia starts to predominate over crypt fission. A difference in the incidence of crypt fission between the small and large intestines in rats has been reported[11], but the underlying reason remains unclear.

In crypt bases during crypt fission, AB-PAS-positive cells (Paneth cells or goblet cells) were not specifically located and Ki67-positive cells were diffuse, unlike in the crypt bases seen in adults (Fig. 5A). This suggests that crypt fission occurs regardless of the cell type, including differentiated and proliferating cells, which are rarely observed in the crypt base of adults (refer to Fig. 3A, parent). This could be indispensable to crypt fission. Almost all stromal cells were positive for α-SMA, and these stromal cells penetrated the crypt base during crypt fission on PND 14 (Fig. 5B). In addition, the α-SMA-positive stromal cells surrounding the crypt base were plumper at PND 14 than in the parent (Fig. 5C). Further, there were fewer lymphocytes in the lamina propria on PND 14 than those in the parent, which makes α-SMA-positive stromal cells the most common components of the lamina propria in neonatal mice. These observations suggest that the dynamic re-arrangement of the intestinal structure involved in crypt fission is caused by cooperation between epithelial cells and their surrounding stromal cells.

The stroma act as a physical support for the intesti-

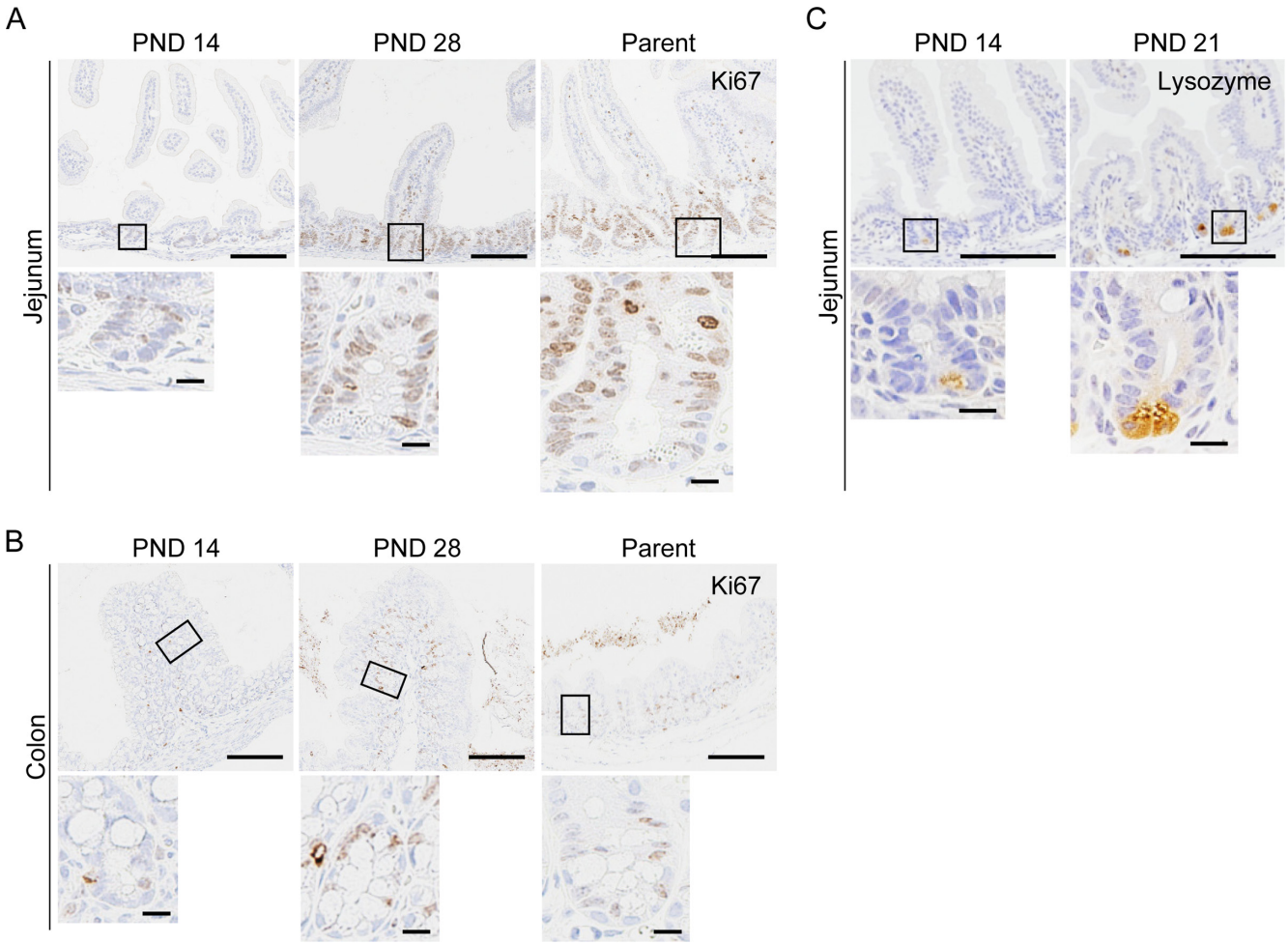

Fig. 3. (A) Images of immunohistochemical staining for Ki67 at postnatal day (PND) 14, 28, and parent in the jejunum. (B) in the colon. (C) Images of immunohistochemical staining for the lysozyme in the jejunum at PND 14 and 21. Rectangles indicate the area for higher magnification images under the pictures. Bar=100 μm for the upper images at lower magnification and 10 μm for the lower images at higher magnification.

nal structure as well as a source of growth factors for the development and proliferation of epithelial cells[12]. In this study, we evaluated the relationship of crypt fission with the surrounding stromal cells in intestinal development and

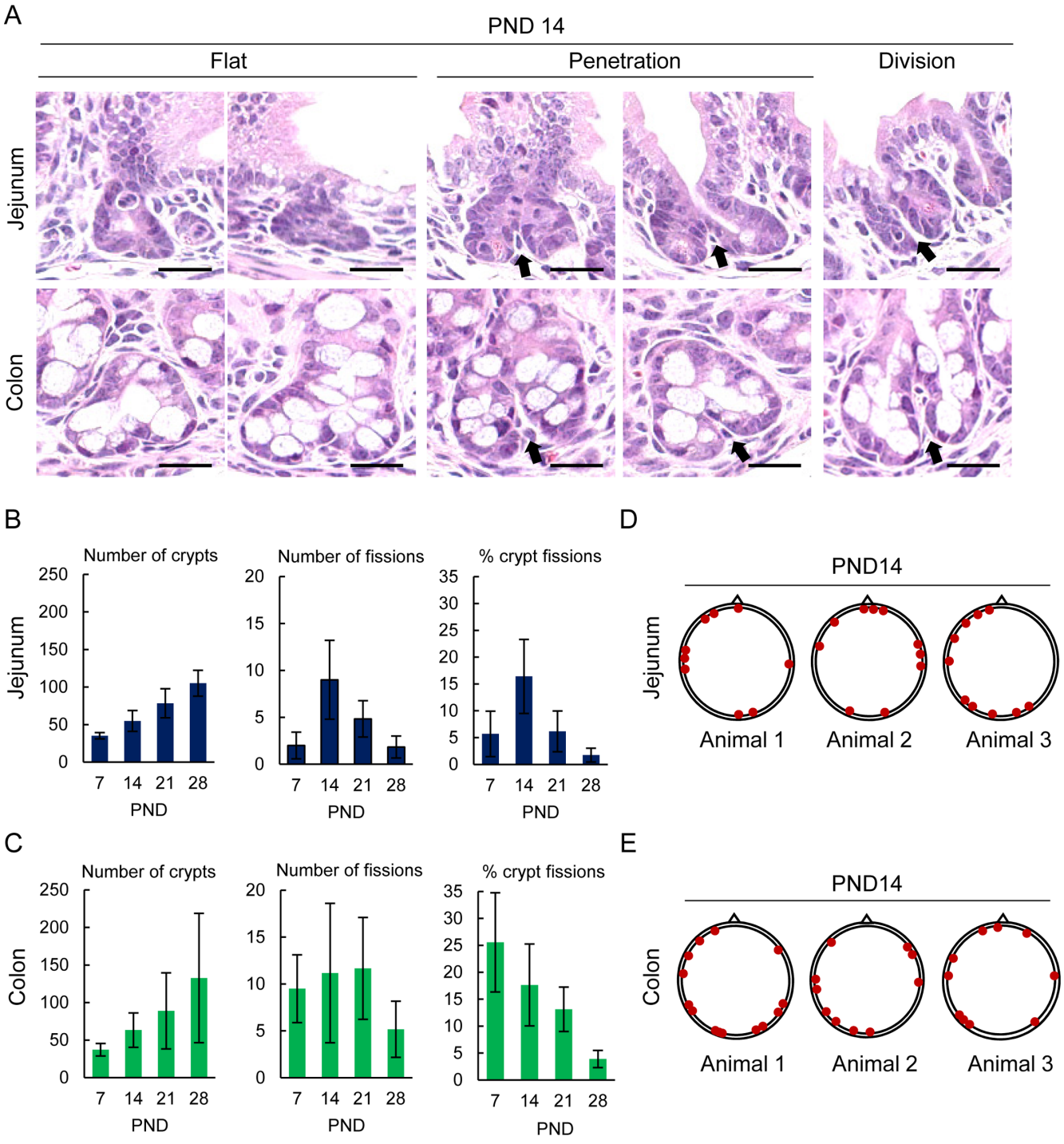

Fig. 4. (A) Different stages of crypt fission in the jejunum and colon. The left two images of two lines show the first stage of crypt fission, in which the crypt base becomes "flat", shaped like a skirt. The middle two images of two lines show the second stage of crypt fission, when some stromal cells "penetrate" the center of the crypt base. The right images show the final stage of crypt fission, which shows complete "division" into two daughter crypts. Arrows indicate crypt fission. Hematoxylin and eosin (HE) staining. Bar=20 μm. (B) Number of crypts, number of fissions, and percentage of fissions to crypts in the jejunum at postnatal day (PND) 7 to 28. Values are the mean ± SD (n=6). (C) Number of crypts, number of fissions, and percentage of fissions to crypts in the colon at PND 7 to 28. Values are the mean ± SD (n=6). (D) Distribution of crypt fission in the jejunum at PND 14. Red dots indicate crypt fission. Triangle indicates the mesentery. Three representative animals were analyzed. (E) Distribution of crypt fission in the colon at PND 14.

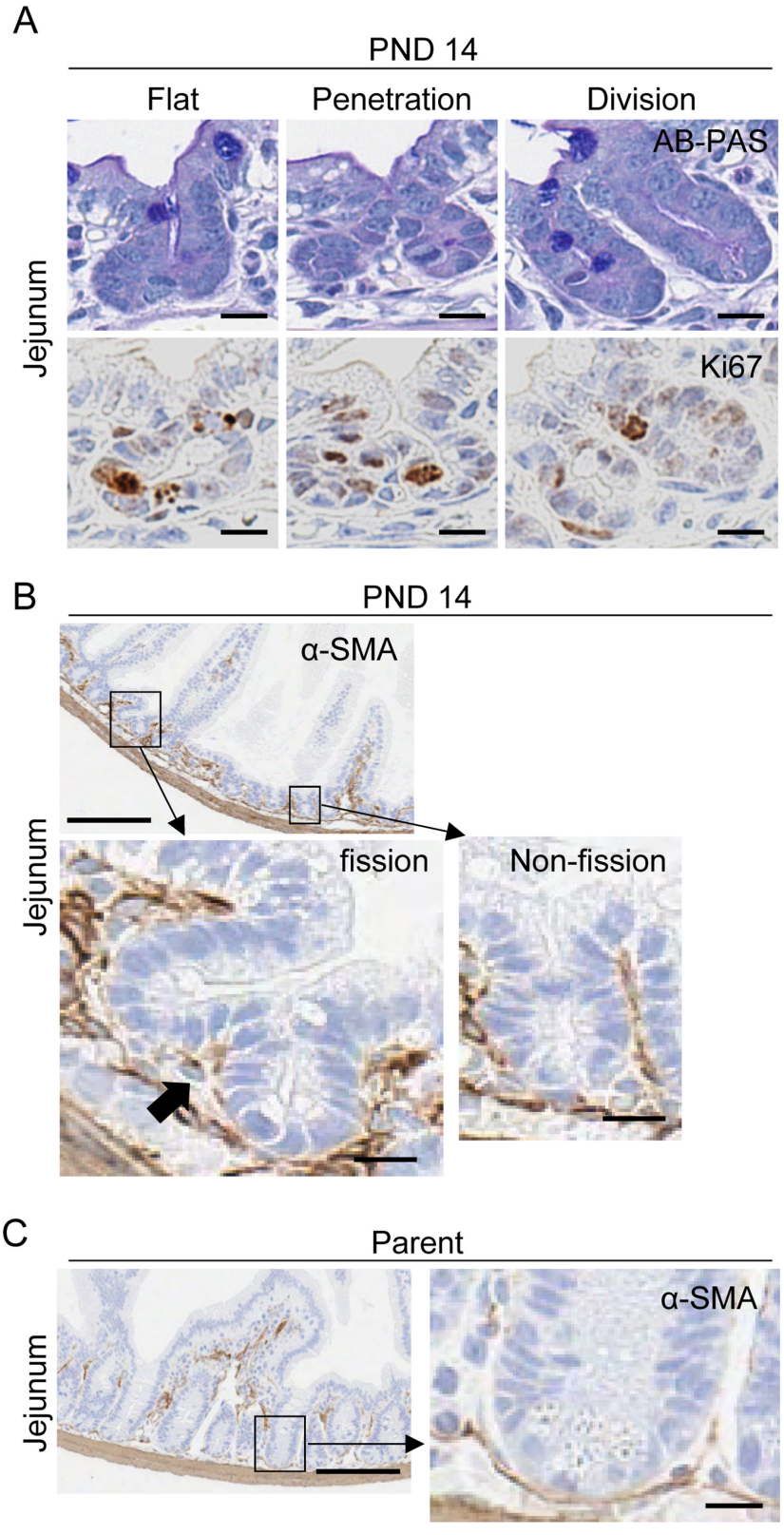

Fig. 5. (A) Histopathological images of crypt fission in the jejunum stained with Alcian Blue-Periodic acid-Schiff (AB-PAS) and anti-Ki67 antibody at postnatal day (PND) 14. Bar=10 μm. (B) Histopathological images for the interstitium surrounding crypt fission in the jejunum at PND 14 stained with anti-alpha smooth muscle actin (α-SMA) antibody. α-SMA-positive stromal cells (myofibroblasts) penetrate the center of the crypt base in crypt fission. As a reference, images for the non-crypt fission area at PND 14 are also shown. The arrow indicates the crypt fission. Bar=100 μm for upper images at lower magnification and 10 μm for lower images at higher magnification. (C) Histopathological images for the interstitium surrounding the crypt of parents in the jejunum stained with an anti-α-SMA antibody. Bar=100 μm for the left image at lower magnification and 10 μm for the right image at higher magnification.

found that cells penetrating the crypt were always α-SMA-positive (myofibroblasts). The surrounding stromal cells are also thought to contribute to the increase in epithelial cells by acting as a scaffold that expands the surface area of the villi-crypt axis. The evidence supporting this notion is that when intestinal epithelial stem cells are co-cultured *in vitro* with intestinal subepithelial myofibroblasts, the enteroids are larger and have improved viability[13]. In addition, although the source of these factors is not always limited to the stroma, there are reports concerning the relationship of crypt fission with differentiation/proliferation signals[5, 14–16] and inflammatory diseases[17] in rodents and humans. In summary, these indications of mutual dependence suggest that physical interaction between epithelial cells and stromal cells is needed to complete the intestinal structure.

Crypt fission is observed both at the developmental phase and during crypt regeneration after intestinal injury, such as with ischemia, infection, irradiation, inflammatory bowel disease[18], or chemotherapy[19]. Moreover, crypt fission is a potential candidate to explain how the number of tumor cells expands during early tumorigenesis[19]. Once the number of proliferating cells reaches a threshold, crypts undergo fission, and an adenoma or carcinoma is generated. Fission is also recognized as the mechanism through which a mutated clone of stem cells expands in the gastrointestinal tract[20]. Based on these studies, crypt fission is a key phenomenon that causes balance or imbalance in the epithelial hierarchy of both stem and differentiated cells in these conditions. Examination of crypt fission provides important clues to evaluate the condition of the intestine. Therefore, a greater understanding of the detailed morphology of crypt fission in neonates is important to evaluate juvenile toxicity studies more accurately. Such knowledge may also facilitate more appropriate evaluation in stages when other dynamic intestinal changes occur, including adult toxicity or disease. For example, it has been reported that the number of crypts increase following incremental crypt fission during the regeneration phase after doxorubicin treatment[21].

Disclosure of Potential Conflicts of Interest: The authors declare that they have no competing interests.

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