Paneth cells expand from newly created and preexisting cells during repair after doxorubicin-induced damage

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Paneth cells expand from newly created and preexisting cells during repair after doxorubicin-induced damage. Am J Physiol Gastrointest Liver Physiol 305: G151–G162, 2013. First published May 9, 2013; doi:10.1152/ajpgi.00441.2012.—Paneth cell numbers increase following intestinal damage, but mechanisms driving this process are not understood. We hypothesized that the increase in Paneth cell numbers is due to recruitment of cells from a preexisting pool of secretory progenitors. Mice were given a single injection of doxorubicin (Dox), and intestinal tissue was collected 0–168 h after treatment. Paneth, goblet, and intermediate cells were counted and evaluated for cell morphology. Quantitative RT-PCR was used to measure expression of various genes associated with Paneth cell allocation and maturation. Paneth cells were birth dated using incorporation of thymidine analogs before or after Dox. Staining revealed “intermediate” cells, which were rarely observed in control crypts but increased significantly in number 96 and 120 h after Dox treatment. Birth dating of intermediate cells 5 days after Dox treatment revealed that 24% of these cells took up thymidine analog given prior to Dox treatment and 36% took up thymidine analog given after Dox treatment. Quantitative RT-PCR demonstrated a significant increase in Sppdef, Atoh1, Sox9, EphB3, Mist, Wnt5a, FGF-9, and FGF-18 mRNAs and a significant decrease in Indian hedgehog mRNA. Expansion of the Paneth cell compartment after Dox treatment is due to generation of new cells and recruitment of cells from an existing pool. These cells express Paneth and goblet biomarkers and are found only during repair. Expansion of these cells correlates temporally with reduced Indian hedgehog and increased FGF and Wnt mRNA. These findings are significant, as they provide a first step in understanding mechanisms of Paneth cell expansion during mucosal repair.

Paneth cell; doxorubicin; intermediate cell

Paneth cells are one of four differentiated cell lineages that arise from intestinal stem cells (ISC), and unlike the other three cell lineages found in small intestinal epithelium, they migrate toward and reside in the base of intestinal crypts (5, 6). Paneth cells are part of the innate mucosal immune system in the intestine and protect against microbial infection and overgrowth. Antimicrobial peptides, including cryptdins (α-defensins), lysozyme, secretory phospholipase A2, and matrilysin [matrix metalloproteinase 7 (MMP-7)], are secreted from granules localized to the apical membrane of Paneth cells upon bacterial stimulation (2, 25). At the crypt base, Paneth cells are adjacent to and intercalated among ISC and are, therefore, in an advantageous position to influence the stem cell microenvironment (5, 6). In addition to secreting numerous antimicrobial factors, Paneth cells synthesize and secrete factors that are capable of influencing proliferation and migration of intestinal epithelial cells, including EGF, granulocyte-macrophage colony-stimulating factor, R-spondin, Wnt3a, TGFβ, and TNFα (8, 13, 27, 29, 30, 45). However, the role of Paneth cells in defining and influencing the ISC niche in vivo remains unclear (14, 18, 29).

Doxorubicin (Dox) is a chemotherapeutic agent used alone or in combination with other drugs to treat various types of cancers, including bladder, breast, lung, and ovarian cancer, as well as Hodgkin’s lymphoma, non-Hodgkin’s lymphoma, and leukemia. Associated with Dox treatment are numerous gastrointestinal side effects, including nausea, vomiting, and mucositis, which present significant difficulties for patients and may limit the dosage of chemotherapy. Preclinical studies in mice have demonstrated that Dox rapidly induces apoptosis in the stem cell positions at the base of intestinal crypts, causing crypt loss, cell cycle arrest, and decreased numbers of cells migrating onto existing villi, resulting in villus atrophy. In C57BL/6 mice, by 96–120 h after Dox exposure, we demonstrated crypt fission (a surrogate for ISC expansion) preceding total repair of the epithelium (9). After Dox-induced damage, expansion of Paneth cell number and size occurs in this same time frame. The mechanism(s) that drive(s) Paneth cell expansion after Dox-induced injury is unclear. Numerous trophic factors, including Wnts, FGFs, serotonin, Indian hedgehog (Ihh), and colony-stimulating factor, are known to influence the number of Paneth cells residing at the base of intestinal crypts (7, 11, 16, 34, 37). However, little is known about the mechanisms by which damage to intestinal epithelium influences Paneth cell numbers or their differentiation.

Despite numerous reports of Paneth cell expansion in intestinal metaplasia, in inflammatory bowel disease, after administration of methotrexate to rats, after intestinal resection, and in response to mucosal damage from Dox, we understand very little about specification and maturation of these cells in damaged epithelium (9, 15, 31, 38, 43). The current study tested the hypothesis that expansion of the Paneth cell zone after Dox-induced damage was due to recruitment of cells from a preexisting pool of secretory progenitors. In this study, we demonstrate that expansion of the Paneth cell zone involves increases in cell size and number. Mature Paneth cells experience an increase in the number and size of secretory granules. Furthermore, we demonstrate that expansion of the Paneth cell zone is also due to an increase in the number of intermediate cells (cells maintaining Paneth and goblet cell characteristics). Intermediate cells are rare in the untreated small intestine and, after Dox-induced damage, arise from two sources: 1) epithelial cells that exist prior to and survive Dox treatment and 2) cells generated after Dox treatment. We also demonstrate that expansion of these cells coincides with regulation of mRNAs associated with lineage allocation and maturation of Paneth cells and mRNAs indicative of activation of the Wnt and FGF pathways and reduced Ihh expression.
MATERIALS AND METHODS

Animals. Adult female C57BL/6 mice (Charles River) were housed under a 12:12-h light-dark cycle and allowed unlimited access to food (rodent laboratory chow 5001, Purina Mills, St. Louis, MO) and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Mice were given a single intraperitoneal injection of Dox (Pharmacia & Upjohn, Kalamazoo, MI) at 20 mg/kg body wt, which was previously demonstrated to induce rapid apoptosis and subsequent intestinal damage (9). Animals were killed at time points after Dox treatment chosen to span early apoptosis, peak damage, and mucosal repair: 0, 6, 24, 48, 72, 96, 120, 168, and 240 h. At each time point, mice were anesthetized and euthanized, and the small intestine was dissected. The small intestine was flushed with ice-cold PBS (pH 7.4) and divided into segments for fixation or exposure to freezing liquid nitrogen for later isolation of protein and RNA. Tissue harvested for histology was fixed in 10% zinc-buffered formalin and embedded in paraffin.

Histology. Paraaffin-embedded tissues were cut into 5-μm-thick sections and stained with hematoxylin and eosin, the goblet cell marker Alcian blue (AB), or the Paneth cell marker phoxine-tartrazine (PT)-AB (PTAB). Hematoxylin-eosin-stained jejunal sections from 3 mice per time point were used to count granules in 20 clearly delineated Paneth cells. PTAB-stained sections from three mice per time point were used to calculate granule diameter in PT+AB− and PT+AB+ cells. For PT+AB− cells, ≥130 granules were measured, with an average of 195 granules measured per section. As PT+AB+ cells were rare at early time points (0 and 6 h), an average of 35 granules were measured per section. An average of 173 granules from PT+AB+ cells were measured per section. Granules were measured under oil emersion at x1,600 magnification using AxioVision software (Zeiss), which allowed the measurement of granule diameter. PTAB-stained sections were also used for scoring of PT+AB−, PT+AB+, and PT+AB+ cell numbers. A total of 25 crypts were evaluated per mouse per time point, and cells were scored only if the crypt had a single layer of continuous epithelium and was well oriented. The above-described parameters were scored in a blinded fashion.

Immunohistochemistry. Paraaffin-embedded sections were deparaffinized by incubation in a series of graded alcohol: twice for 7.5 min, then twice for 4.5 min 100% ethanol, once for 3 min in 95% ethanol, once for 3 min in 80% ethanol, and once for 3 min in distilled water. Antigen retrieval was performed using 10 mM sodium citrate (0.05% Tween 20, pH 6.0) and heating at 100°C for 30 min. After they were cooled to room temperature, the slides were washed twice for 3 min each in 1× PBS-0.3% Triton X-100. If necessary, slides were blocked using avidin/biotin block according to the manufacturer’s instructions (catalog no. SP-2001, Vector Laboratories, Burlingame, CA). The antibodies were as follows: anti-β-catenin (catalog no. 610153, BD Biosciences, San Jose, CA; 1:500 dilution), anti-lysozyme (catalog no. sc-27958, Santa Cruz Biotechnology, Dallas, TX; 1:100 dilution), anti-mucin 2 (Muc2; catalog no. sc-15334, Santa Cruz Biotechnology; 1:200 dilution), anti-Sox9 (catalog no. AB5535, EMD Millipore, Billerica, MA; 1:500 dilution), anti-EphB3 (catalog no. AF432, R & D Systems, Minneapolis, MN; 1:40 dilution), anti-Mist1 (catalog no. sc-80984, Santa Cruz Biotechnology; 1:100 dilution), anti-5-chloro-2′-deoxyuridine (CldU; catalog no. NB500-169, Novus Biologicals, Littleton, CO; 1:200 dilution), and anti-5-iodo-2′-deoxyuridine (IodU; catalog no. 347580, BD Biosciences, San Jose, CA; 1:200 dilution). When mouse monoclonal antibodies were used, slides were washed and blocked with mouse-on-mouse IgG block (catalog no. BMK-2202, Vector Laboratories) for 1 h at room temperature. After the slides were washed, primary antibodies, diluted in mouse-on-mouse primary antibody diluents, were added for 1 h at room temperature. The slides were washed and incubated in secondary antibody at room temperature in darkness for 1 h. They were washed again, and a nuclear stain, DRAQ5 (catalog no. 62254, Thermo Scientific; 1:4,000 dilution in 1× PBS), was added to the slide for 10 min at room temperature in darkness. The slides were washed, dotted with Hydromount (catalog no. HS-106, National Diagnostics), and sealed with a coverslip. The slides were imaged using a Zeiss Axio Imager A1 microscope with a mercury arc lamp and analyzed using AxioVision 4.6.3.

Transmission electron microscopy. Transmission electron microscopy (TEM) was used to verify Paneth cell phenotype. For TEM, jejunal tissue pieces were fixed overnight in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.15 M sodium phosphate (pH 7.4), washed in sodium phosphate buffer, postfixed for 1 h in potassium ferrocyanide-reduced osmium, and embedded in PolysBed 812 epoxy resin (Polysciences, Warrington, PA). Cross sections (1 μm) were cut, stained with 1% toluidine blue, and examined by light microscopy to isolate the area of interest. Ultrathin (70- to 80-nm-thick) sections were cut, mounted on 200-mesh copper grids, and stained with 4% aqueous uranyl acetate for 15 min and then with Reynolds’ lead citrate for 8 min. The sections were observed using a transmission electron microscope (model EM-910, LEO Electron Microscopy, Thornwood, NY), with accelerating voltage of 80 kV, and digital images were taken with a charge-coupled device camera (Orius SC 1000, Gatan, Pleasanton, CA).

Flow cytometry. Flow cytometry of Paneth cells was performed using the method described by von Furstenberg et al. (41). Briefly, jejunal epithelial cells were isolated using the EDTA-dispase method described by Formeister et al. (12). Cells were fixed for 15 min in 4% paraformaldehyde. After they were washed, the cells were resuspended in saponin permeabilization buffer (catalog no. PB001, Invitrogen, Carlsbad, CA) with lysozyme-FITC antibody (catalog no. F0372, Dako Cytomation; 1:10 dilution) and incubated for 30 min at room temperature. Cells were washed again and analyzed by flow cytometry.

Gene expression analysis. Total RNA was isolated from jejunal tissue using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer’s directions. Lack of contamination with genomic DNA was verified by running 1 μg of total RNA on a 0.9% agarose gel. The TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA) and TaqMan Gene Expression assays for PPARβ/δ (Mm00803186_g1), Atoh1 (Mm00476035_s1), Spdef (Mm00600221_m1), Gfi1 (Mm00515855_m1), Sox9 (Mm00448840_m1), Fgf-9 (Mm01319105_m1), Fgf-18 (Mm00433286_m1), Ihh (Mm01259021_m1), Wnt5a (Mm00437347_m1), and β-actin (Mm00607939_s1) were used for real-time RT-PCR of 100 ng of total RNA. Relative changes in expression levels were calculated by the ΔΔCt method utilizing the 0-h total RNA as the baseline.

Paneth cell birth dating. To determine if expansion of the Paneth cell zone resulted from cells that existed prior to Dox-induced damage or from cells derived from cell division during epithelial repair, we birth-dated cells by exposing mice via their drinking water to two distinguishable thymidine analogs, IdU and CldU, before and after Dox treatment, respectively. Mice were maintained for 10 days on water supplemented with 0.8 mg/mL IdU. On day 10, all mice were switched to and maintained on water supplemented with 0.8 mg/mL CldU, treated with a single injection of Dox or left untreated, and killed 5 days later. Jejunal tissues from both groups were fixed overnight in 10% zinc-buffered formalin and embedded in paraffin. Sections were cut and stained with anti-IdU, anti-CldU, anti-Muc2, and anti-lysozyme antibodies, as outlined above.

Statistical analysis. All quantitative results are presented as means ± SE. RNA was isolated from three mice at each time point after Dox. All data were subjected to one-way ANOVA with correction for multiple comparisons using Fisher’s procedure. For all comparisons, P < 0.05 was considered significant.
RESULTS

Dox-induced damage alters number and size of lysozyme-positive cells within crypts. We previously reported significant increases in the number of Paneth cells per crypt within the intestinal epithelium after Dox-induced damage (9). In the current study, staining with hematoxylin-eosin confirmed an increase in the number of cells showing the typical eosinophilic staining of Paneth cells within crypts of Dox-treated mice during the repair phase (Fig. 1A). Using flow cytometry, we verified histological findings by demonstrating a significant increase in lysozyme-positive cells 120 h after Dox treatment compared with control mice (Fig. 1, B–D). Histological analysis at high power revealed that, in addition to an increase in the number of lysozyme-positive cells, there was also an increase in the number of secretory granules per Paneth cell (Fig. 1E). This increase was observed as early as 24 h and was maintained to 168 h after Dox treatment (data not shown). Furthermore, we observed a significant increase in the size of Paneth cell granules after Dox-induced damage (Fig. 1F). These data suggest an increase in the number and size of Paneth cells after Dox-induced damage.

Lysozyme staining of jejunal tissue after Dox-induced damage confirmed that the expanded cells at the crypt base expressed a key Paneth cell biomarker and also revealed the abnormal presence of lysozyme-positive cells above the crypt base (Fig. 2A). Quantification of the number of lysozyme-positive cells per crypt corroborated our flow cytometry data showing a significant increase after Dox-induced damage (Fig. 2B). Staining with AB revealed intensely stained cells toward the crypt-villus junction in control jejunum. After Dox-induced damage, intensely stained AB-positive cells were also observed at the crypt base and at higher levels in the crypt (Fig. 2C).
Quantification demonstrated an increase in these cells within crypts after Dox-induced damage (Fig. 2C). The increase in AB-positive cells and lysozyme-positive cells within crypt epithelium after Dox-induced injury led us to consider that at least some of these cells were dual-positive cells, expressing both lysozyme and goblet cell markers, which have been found following small intestinal infection and termed “intermediate cells” in the literature (17, 42).

Expansion of intermediate cells in crypt epithelium during repair. Using TEM, we corroborated the increase in the number of Paneth cells residing in the crypts, as well as the increase in the number of secretory granules per cell (Fig. 3, A and B). After Dox treatment, we further observed two morphologies among the Paneth cells: the first exhibited similar characteristics to Paneth cells from control intestine, with dense secretory granules (P1 in Fig. 4B); the second exhibited increased numbers of granules compared with Paneth cells in control intestine, but the granules of these cells had a lattice-like appearance (P2 in Fig. 3B). Costaining for lysozyme to identify Paneth cells and Muc2 to identify goblet cells (Fig. 3) revealed that, in control animals, dual-positive cells were rare and lysozyme-positive cells were distinct from Muc2-positive cells within crypts. In contrast, at 168 h after Dox-induced damage, we observed the presence of 1) lysozyme-positive, Muc2-negative, 2) lysozyme-negative, Muc2-positive, and 3) lysozyme-positive, Muc2-positive cells within regenerating crypts.

To quantify the number of dual-positive cells within crypt epithelium after Dox injection, we utilized a combinatorial staining technique (PTAB) that allowed us to distinguish between 1) highly granulated, mucin-negative (PT+AB−) cells, 2) mucin-positive, minimally granulated (PT−AB+) cells, and 3) cells that are both granulated and mucin-positive (PT+AB+). As seen in Fig. 4A, PT+AB− and PT−AB+ cells were found in normal and regenerative crypts. However, PT+AB+ cells were
rare in normal crypt epithelium but were observed in crypt epithelium during repair. PT$^+$AB$^+$ cells increased in number per crypt 96 and 120 h after Dox injection (Fig. 4B). Concomitantly, we observed a decrease in PT$^+$AB$^-$ cell numbers (Fig. 4C) with minimal change in the number of PT$^-$AB$^+$ cells (Fig. 4D). These changes in secretory cell allocation were accompanied by significant increases in granule size (Fig. 4E). Throughout the time course, secretory granules in PT$^+$AB$^+$ cells were significantly smaller than those in PT$^+$AB$^-$ cells.

Fig. 3. Dox treatment alters crypt size and crypt cell lineage allocation. A and B: electron micrographs of jejunal crypts from control (A) and Dox-treated (B) mice. Magnification $\times$3,150. P, Paneth cell; P1, similar to Paneth cells from control intestine; P2, more granules than Paneth cells from control intestine; P3, more granules but fewer electron-dense than Paneth cells from control intestine; L, lumen. C–H: immunofluorescent staining for lysozyme (Lys) and mucin 2 (Muc2) in jejunal tissue from control (C–E) and Dox-treated (F–H) mice. Gray arrows, Muc2-positive Paneth cells (C, E, F, and H); white arrows, lysozyme-positive cells (D, E, G, and H); white arrowheads, Muc2-positive/lysozyme-positive cells (H).
Dox-induced damage activates Wnt signaling/nuclear β-catenin in Paneth-like cells. Others have demonstrated that Wnt signaling plays an important role in Paneth cell maturation (34). We, therefore, evaluated nuclear localization of β-catenin, a key outcome of activated Wnt signaling, in crypt epithelium after Dox damage as one biomarker of Wnt activation and found a significant increase in the number of β-catenin-positive nuclei in granulated cells 96 h after damage (Fig. 5).

Paneth cell zone expansion is associated with changes in factors linked to secretory cell lineage allocation and maturation. Specification of cells to the goblet and Paneth cell lineages involves numerous transcription factors, including Spdef, Atoh1, and Sox9. Significant increases in Spdef mRNA were observed 96, 120, and 168 h after Dox treatment, coinciding with expansion of intermediate cells (Fig. 6A). Little change in Atoh1 mRNA was observed after Dox treatment, with a significant increase observed only at 168 h (Fig. 6B). We observed a significant increase in expression of Sox9 mRNA, which is necessary for Paneth cell differentiation (3, 22), 24, 48, 72, and 168 h after Dox treatment (Fig. 6C). Immunofluorescent staining of Sox9 revealed an increase in nuclear expression of the transcription factor in crypt epithelial cells, both Paneth and non-Paneth, 120 h after Dox treatment relative to control tissue (Fig. 6C). Expression of EphB3, a surface protein that plays a role in

Fig. 4. Expansion of crypt secretory cells is due to increase in intermediate cell number. A: micrograph of phloxine-tartrazine (PT)-Alcian blue (AB) staining of jejunal sections 0 and 120 after Dox treatment. Gray arrows, Paneth cells (PT⁺AB⁻); black arrows, goblet cells (PT⁻AB⁺); white arrows, intermediate cells (PT⁺AB⁺). Scale bars, 25 μm. B–D: quantification of the number of PT⁺AB⁺, PT⁺AB⁻, and PT⁻AB⁺ cells per crypt 0–168 h after Dox treatment. E: granule size in PT⁺AB⁺ and PT⁺AB⁻ cells 0–168 h after Dox treatment. Values are means ± SE; n = 3. *P < 0.05 vs. respective 0-h time point; †P < 0.05 vs. PT⁺AB⁻ cells at the same time point.
Paneth cell maturation, was significantly increased at the mRNA level 24 and 48 h after Dox treatment (Fig. 6D). In untreated mice, EphB3 protein colocalized with lysozyme at the base of intestinal crypts. While EphB3 expression remained confined to the crypt base in mice treated with Dox, the extent of EphB3-positive cells appeared to broaden. Furthermore, we observed EphB3-negative/lysozyme-positive cells within regenerating crypts (Fig. 6D”). Expression of Mist1 mRNA, a transcription factor thought to function as a scaling factor to control secretory potential of exocrine cells (21), was significantly increased 24 h after Dox treatment but was otherwise unchanged during regeneration (Fig. 6E). As shown in Fig. 6E”, Mist1 expression was confined to the lysozyme-positive cells located at the crypt base in untreated and Dox-treated mice. In jejunal tissue from Dox-treated mice, we detected lysozyme-positive cells that were negative for Mist1.

Dox treatment alters mRNAs linked to Paneth cell specification and maturation. Studies by Vidrich et al. (40) demonstrated that signaling through FGF receptor 3 (FGFR3) is critical for specification and maturation of Paneth cells. Therefore, we examined whether treatment with Dox altered levels of mRNA encoding FGF-9 and FGF-18, which are ligands for FGFR3. FGF-9 mRNA significantly increased 48 h after Dox treatment and significantly decreased 96 and 168 h after Dox treatment (Fig. 7A). FGF-18 mRNA expression also significantly increased 6–72 h after Dox treatment (Fig. 7B). The increases in FGF-9 and FGF-18 mRNA preceded Paneth cell expansion. Varma et al. (37) reported that Paneth cell specification was regulated by Ihh signaling, a finding substantiated in mice with intestinal epithelial-specific knockout of Ihh (19, 33). Therefore, we evaluated Ihh mRNA expression after Dox treatment and found significant decreases in Ihh from 24 to 168 h, concomitant with Paneth cell expansion (Fig. 7C). The Wnt receptor Frizzled 5 (Fz5) plays a critical role in Wnt-dependent Paneth cell maturation and binds Wnt5a as a ligand (34). We observed a significant increase in Wnt5a mRNA expression between 24 and 72 h after Dox treatment, time points that precede Paneth cell expansion (Fig. 7D).

Expanded crypt secretory cells derive from cells present prior to and generated after Dox treatment. We next wanted to gain insight into the source of expanded intermediate cells and Paneth cells after Dox-induced damage. On the basis of the premise that expanded cells could originate from cells that existed within the epithelium prior to Dox treatment or from cells derived from cell proliferation after Dox-induced damage, we treated mice with the thymidine analogs IdU and CldU before and after Dox treatment, respectively. Mice were given IdU in drinking water for 10 days prior to Dox treatment (Fig. 8A). At the time of injection with Dox, mice were switched to CldU in drinking water for an additional 5 days (Fig. 8A), which allowed us to label epithelial cells in the S phase that were generated after Dox injection. As shown in Fig. 8A, extended inclusion of IdU in drinking water of control mice resulted in nuclear labeling of all non-Paneth epithelial cells and occasional Paneth cells. Similarly, 5 days of exposure of control mice to CldU in drinking water resulted in labeling of the entire epithelium with the exception of most Paneth cells (Fig. 8A). Mice treated with Dox were killed 5 days after injection for tissue collection. Using immunofluorescence, we evaluated staining for Muc2, lysozyme, IdU, and CldU in jejunal tissue and identified lysozyme-positive and lysozyme-positive/Muc2-positive cells that were negative for IdU and from cells derived from cell proliferation after Dox-induced damage. Quantification of lysozyme-positive cells (Fig. 8B) demonstrated that 92% of these cells were present in the intestinal epithelium prior to injection with Dox (neg or IdU”), while only 8% of these cells took up CldU after Dox treatment, confirming that expansion of the Paneth cell zone after Dox treatment is not due to an increase in Paneth cells. Quantification of lysozyme-positive/Muc2-positive cells (Fig. 8C) demonstrated that 36% of these cells took up CldU after Dox treatment, while 64% of these cells were derived from cells that were present in the epithelium prior to Dox (neg or IdU”). These data suggest that the increase in intermediate cells during epithelial repair after Dox-induced damage is due to
Fig. 6. Changes in expression of factors involved in secretory lineage allocation and Paneth cell differentiation. A–E: expression of Spdef, Atoh1, Sox9, EphB3, and Mist1 mRNAs 0–168 h after Dox treatment. *P < 0.05 vs. 0 h. C′–E′: immunofluorescence staining for Sox9, EphB3, and Mist1 from control (0 h) and Dox-treated (120 h) mice. Arrows in E′ point to Mist1-positive nuclei. Scale bars, 30 μm.
reappropriation of existing cells, as well as to cells that recently exited the cell cycle.

**DISCUSSION**

We previously demonstrated substantial changes in allocation of secretory lineages after Dox-induced damage, particularly during the repair phase (9). In the present study, we sought to validate and assess mechanisms underlying Paneth cell expansion within regenerating intestinal crypts. Our current work used flow cytometry as independent confirmation of previous histological findings of an increase in lysozyme-positive cells after Dox-induced damage. Furthermore, we demonstrate, in addition to increased cell number, increases in size and number of Paneth cells and size of granules within Paneth cells. After Dox treatment, concomitant with the increase in the number of lysozyme-positive Paneth cells in the intestinal crypts, there was an increase in AB-positive cells within the same area. Simultaneous staining for Paneth and goblet cell markers, specifically lysozyme and Muc2, revealed that the expanding cell population at the crypt base was composed of lysozyme-positive/Muc2-positive, as well as lysozyme-positive/Muc2-negative and lysozyme-negative/Muc2-negative cells after Dox-induced damage.

![Fig. 7. Dox treatment alters expression of members of pathways that influence Paneth cell specification and maturation. A–D: relative mRNA expression 0–168 h after Dox treatment for FGF-9, FGF-18, Indian hedgehog (Ihh), and Wnt5a (n = 3). *P < 0.05 vs. 0 h.](http://ajpgi.physiology.org/)

**Fig. 8. Birth dating of Paneth/intermediate cells after Dox treatment.**

A: mice were exposed to 5-iodo-2'-deoxyuridine (IdU) in drinking water before and 5-chloro-2'-deoxyuridine (CldU) after Dox treatment, and jejunal tissue was collected 5 days after Dox injection. A': immunostaining of jejunal tissue with anti-IdU antibody after 10 days of IdU in drinking water showing positive-staining nuclei throughout the epithelium. Scale bars, 50 μm. A'': immunostaining of jejunal tissue with anti-CldU antibody after 5 days of CldU in drinking water showing positive-staining nuclei throughout the epithelium. Scale bars, 50 μm. B: quantification of the percentage of lysozyme+/Muc2+, lysozyme+/Muc2+/IdU+, and lysozyme+/Muc2+/CldU+ intermediate cells in the epithelium 5 days after Dox treatment. C: quantification of the percentage of lysozyme+, lysozyme+/IdU+, and lysozyme+/CldU+ Paneth cells 5 days after Dox treatment.
positive cells. We quantified this finding with PTAB staining and found that the increase in lysozyme-positive cells was primarily due to an increase in the number lysozyme-positive/Muc2-positive or intermediate cells.

Intermediate cells are rarely seen in normal adult intestinal epithelium, but they have been reported in cases of intestinal infection, inflammation, and inhibition of Notch signaling (36, 39, 42). The function of these secretory cells is not clear. However, their presence at times of pathogenic insult suggests that they may play a role in managing or defense against changes in luminal microbiota. Alternatively, these cells may reflect a secretory cell progenitor or “transitional cell” present at times of substantial changes or acceleration of lineage allocation, such as during epithelial repair. Our PT-labeling studies suggest that these cells may indeed be respecified Paneth cells, as we observed a concomitant decrease in numbers of (PT+AB-) Paneth cells accompanying the increase in intermediate cell number during epithelial repair. Furthermore, our IdU-CldU-labeling studies suggest that these newly generated intermediate cells may be derived from cells that incorporated IdU prior to Dox-induced damage and from cells that incorporated CldU after Dox-induced damage.

The mechanisms that drive expansion of secretory cells within intestinal crypts are not completely understood. More specifically, mechanisms that regulate specification and maturation of the Paneth cell lineage are not well defined, especially during repair. After damage and during repair, specification and maturation may represent distinct processes. Recent studies by Varnat et al. (37) suggest that, in adult small intestine, mature Paneth cells may play a role in specification by steering progenitor cells away from the Paneth cell lineage through Ihh signaling, thereby controlling Paneth cell number. Conversely, conditional knockout of Ihh in mouse small intestine resulted in mispositioned Paneth cells due to an increased number of Paneth cells (19, 33). Our current observations that Ihh mRNA is decreased during the Paneth/ intermediate cell expansion phase of epithelial repair are consistent with a model where reduced Ihh signaling during repair after Dox-induced injury may recruit new progenitor cells to the Paneth cell lineage.

Evaluation of several transcription factors associated with secretory cell lineage allocation in intestinal epithelium revealed varied responses to Dox-induced damage. The significant increase in expression of Spdef mRNA from 96 to 168 h after Dox treatment coincided with the peak times of expansion of intermediate cells. Noah et al. (23) reported a similar observation: induced overexpression of Spdef in intestinal epithelial cells resulted in increased goblet cell numbers at the expense of Paneth cells, suggesting that the increase in Spdef expression reported in our study might play a role in the increase in intermediate cells. Interestingly, we saw a significant change in mRNA expression of Atoh1, which is necessary for allocation of cells to the secretory lineage (44), only at 168 h after Dox treatment, suggesting that the changes in Paneth cell/intermediate cell numbers are not due to Atoh1 transcription.

In the small intestine, Sox9 is necessary for control of proliferation, as well as differentiation, of Paneth cells. Formeister et al. (12) demonstrated that differential expression of Sox9 within cells of the intestinal crypt could delineate enteroendocrine cells, transit-amplifying cells, and intestinal stem cells based on Sox9-EGFP intensity and demonstrated a correlation with enhanced green fluorescent protein (EGFP) and endogenous Sox9 expression. We observed a significant increase in Sox9 mRNA after Dox treatment that was paralleled by an increase in Sox9 nuclear staining in Paneth and non-Paneth cells. Van Landeghem et al. (35) demonstrated that Sox9-EGFPlow and Sox9-EGFPhigh cells were activated in small intestinal epithelium following irradiation. Although we did not quantify Sox9-EGFP levels in this study, our data suggest that the increase in Sox9 expression observed in this study is associated with expansion of intestinal stem cells, as well as expansion of the Paneth cell zone.

As part of the expansion of the Paneth cell zone after Dox-induced damage, we observed an increase in the number and size of Paneth cell secretory granules. Mist1, a basic helix-loop-helix transcription factor found primarily in exocrine secretory cells such as pancreatic acinar cells, zymogenic cells of the stomach, and Paneth cells, regulates downstream genes that control secretory vesicle maintenance and trafficking (24). Recently, Mist1 was described as a scaling factor that can be utilized by specialized secretory cells to scale up secretion (21). Nuclear localization of Mist1 protein was found in lysozyme-positive cells at the crypt base in untreated mice. In Dox-treated mice, Mist1 expression was confined to lysozyme-positive cells at the base of the crypts, while most lysozyme-positive cells higher up in the crypt were Mist1-negative. This distribution of expression is reminiscent of spasmolytic polypeptide-expressing metaplasia, where Mist1 expression is lost, resulting in decreased secretory granule size associated with increased mucus granules in metaplastic cells (20). Furthermore, loss of Mist1 expression in murine gastric epithelium results in decreased granule size, abundance, and electron density (28). Together, these data point to potential roles for Mist1 in modulating Paneth cell secretory capacity and allocation of intermediate cells during epithelial repair after Dox treatment.

The Wnt pathway plays a critical role in Paneth cell specification and maturation (34). High levels of β-catenin/transcription factor 4 (Tcf4) have been reported in Paneth cells and are linked to synthesis of several Paneth cell secretory products, including cryptdins and MMP-7 (1, 32, 34). In other studies, conditional deletion of Fz5, a Wnt receptor, in intestinal epithelium resulted in mispositioned Paneth cells due to a loss of β-catenin/Tcf4 activity and EphB3 expression (4, 34). In this study our findings of significant increases in nuclear localization of β-catenin within granulated epithelial cells after Dox-induced injury are consistent with increased Wnt signaling. Furthermore, the increases in mRNA expression of Wnt5a, a Fz5 ligand, suggest potential activation of the Wnt5a/Fz5 pathway preceding peak expansion of intermediate cells and the repair phase after Dox.

Recent evidence also suggests that signaling through FGFR3 plays a critical role in Paneth cell maturation (7, 40). Furthermore, signaling through FGFR3 in Paneth cells activates β-catenin/Tcf4-dependent and -independent mechanisms necessary for Paneth cell maturation (7). After Dox-induced damage, we found significant increases in the FGFR3 ligands FGF-9 and FGF-18, suggesting that expansion of Paneth/intermediate cells may involve FGFR3 signaling.

The functional significance of Paneth/intermediate cell expansion in the small intestine following damage is not completely understood. Current data strongly implicate Paneth...
cells as part of the innate mucosal immunity in the intestine protecting against microbial infection and overgrowth by secreting antimicrobial peptides, including cryptidins (α-defensins), lysozyme, secretory phospholipase A₂, and matrilysin (MMP-7) (25). Indeed, Paneth cells sense bacteria and release microbialic secretions into the intestinal lumen upon bacterial stimulation, presumably to control the intraluminal bacterial population (2). Dox initiates the damage cascade by rapidly inducing apoptosis within the intestinal epithelium. This, in turn, may provide a permissive environment for penetration of bacteria through the epithelial barrier, which may induce expansion of Paneth/intermediate cells.

Additionally, the position of Paneth cells at the base of intestinal crypts adjacent to the putative stem cell region and their expression of various growth factors, including TGFβ (30), EGF (26), Wnt3a (29), and R-spondin (45), place these cells in an advantageous position to act as sentinels of ISC and to influence the stem cell microenvironment. We know from recently reported culture experiments that inclusion of Paneth cells with isolated ISC increases the efficiency of enteroid formation (by isolated ISC) by an order of magnitude and that the Paneth cell product Wnt3a is responsible for this increase in efficiency (10, 29). We hypothesize that, following damage, Paneth cells play a critical role in ISC expansion as part of the repair phase. This could be of clinical importance, particularly in the context of managing the gastrointestinal side effects associated with treatments including Dox and other chemotherapeutic agents. Our current findings set the stage for future investigations of the functional role of Paneth cells following damage and during epithelial repair.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.L.K. and J.J.M. performed the experiments; S.L.K. and C.M.D. analyzed the data; S.L.K., J.J.M., and C.M.D. approved the final version of the manuscript; J.J.M. and C.M.D. are responsible for conception and design of the research; C.M.D. interpreted the results of the experiments; C.M.D. prepared the figures; C.M.D. drafted the manuscript; C.M.D. edited and revised the manuscript.

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