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Timing of Developmental Reduction in Epithelial Glutathione Redox Potential is Associated with Increased Epithelial Proliferation in the Immature Murine Intestine

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Abstract

Background—The intracellular redox potential of the glutathione (GSH)/glutathione disulfide (GSSG) couple regulates cellular processes. *In vitro* studies indicate that a reduced GSH/GSSG redox potential favors proliferation, while a more oxidized redox potential favors differentiation. Intestinal growth depends upon an appropriate balance between the two. However, how the ontogeny of intestinal epithelial cellular (IEC) GSH/GSSG redox regulates these processes in the developing intestine has not been fully characterized *in vivo*.

Methods—Ontogeny of intestinal GSH redox potential and growth were measured in neonatal mice.

Results—We show that IEC GSH/GSSG redox potential becomes increasingly reduced (primarily driven by increased GSH concentration) over the first 3 weeks of life. Increased intracellular GSH has been shown to drive proliferation through increased poly-ADP-ribose polymerase (PARP) activity. We show that increasing IEC poly-ADP-ribose chains can be measured over the first 3 weeks of life indicating an increase in IEC PARP activity. These changes are accompanied by increased intestinal growth and IEC proliferation as assessed by villus height/crypt depth, intestinal length, and Ki67 staining.

Conclusion—Understanding how IEC GSH/GSSG redox potential is developmentally regulated may provide insight into how premature human intestinal redox states can be manipulated to optimize intestinal growth and adaptation.

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Introduction

Oxidative stress has been implicated in many diseases affecting premature infants including retinopathy of prematurity, chronic lung disease, intraventricular hemorrhage, periventricular leukomalacia and necrotizing enterocolitis (NEC) (1). However, clinical studies have failed to demonstrate improved outcomes with antioxidant administration to premature infants (2–4). This is likely because physiologic reactive oxygen species (ROS) signaling regulates many necessary, homeostatic processes, which are negatively affected by global ROS suppression through massive antioxidant supplementation approaches(5). Intracellular ROS signaling and redox potential has been implicated in regulating developmental processes in the fetus and premature newborn and depends upon tightly regulated changes in cellular localization and concentration(6–8). In particular, the intracellular redox potential (E_h), as determined by the Nernst equation using glutathione (GSH) and glutathione disulfide (GSSG) concentrations, plays a key role in regulating important developmental cellular processes, such as proliferation and differentiation(9–11).

In vitro studies indicate that a reduced GSH/GSSG redox potential favors proliferation while an oxidized redox potential favors differentiation(10, 12). Developmental regulation of intestinal growth and adaptation depends upon an appropriate balance between the two. However, how the ontogeny of intestinal epithelial cellular (IEC) GSH/GSSG redox regulates these processes in the developing intestine has not been characterized *in vivo*. Here, we show that IEC GSH/GSSG redox potential becomes increasingly reduced (primarily driven by a more than 4-fold increase in IEC GSH concentration) over the first 3 weeks of life in all regions of the murine intestine. Consistent with the idea that reduced intracellular GSH/GSSG redox potential increases cellular proliferation, we found an increase in intestinal growth and IEC proliferation as assessed by villus height/crypt depth, intestinal length, and Ki67 staining over the first 3 weeks of murine life.

IEC proliferation in the developing gut is an important host defense mechanism. The premature infant intestine is expected to double in length postnatally in order to match *in utero* growth rates(13). Further, IEC proliferation in response to injury is critical to promote wound healing and recovery from disease. This defense mechanism not only plays a key role in the postnatal maturation of gut function in the preterm neonate, but also in how neonates and children recover from intestinal injury that may be chemically-induced (ie prostaglandin inhibitors), infection-induced, or surgically-induced (for NEC or congenital bowel defects) (14). Thus, understanding the potential mechanisms behind GSH-induced IEC proliferation is important in order to provide insight into how neonatal intestinal redox states can be manipulated to optimize intestinal growth and adaptation.

One mechanism by which increases in intracellular GSH have been shown to drive cellular proliferation through increased poly-ADP-ribose polymerase (PARP) activity(15). To determine whether the developmental increase in intracellular GSH increased IEC proliferation is accompanied by increasing PARP activity, we measured the amount of intracellular poly-ADP-ribose chains in the neonatal intestine over the first 3 weeks of life. We found that IEC poly-ADP-ribose chains increased over the first 3 weeks of life in the immature murine intestine indicating an increase in IEC PARP activity. These studies

indicate that developmentally-regulated increases in intracellular GSH concentration may promote IEC proliferation by inducing IEC PARP activity in the developing murine gut. Thus, nutritional or pharmacologic strategies that encourage a more reduced IEC intracellular GSH/GSSG state in the immature intestine may be critical to maintaining intestinal health during development and following intestinal injury in premature infants.

Materials and Methods

Animal care

C57BL/6J mice were bred at an animal facility at Emory University and all studies were approved by the Institutional Animal Care and Use Committee. Neonatal and adult mice were anesthetized with CO₂ and euthanized by cervical dislocation at the ages indicated. Whole proximal small intestine, distal small intestine, and colon were isolated and fixed in 10% formalin (for histologic staining) or frozen in Trizol (for qRT-PCR); or freshly collected intestinal epithelial cells were scraped into ice cold perchloric acid (PCA) solution (for GSH analysis) or ice cold RIPA lysis buffer (for PAR Western blot analysis).

GSH assay

To assay the GSH antioxidant pool, we measured both GSH and GSSG concentrations by HPLC as S-carboxymethyl, N-dansyl derivatives using γ -glutamyl-glutamate as an internal standard as previously described(38, 39). Intestinal epithelial cell scrapings were immediately placed in cold 5% PCA buffer containing 10 μ M γ -glutamyl-glutamate. Samples were subsequently derivatized, analyzed by HPLC, and intracellular GSH and GSSG concentrations were determined (normalized to protein concentration) and used in the Nernst equation to determine the redox potential(40).

qRT-PCR

Proximal small intestine, distal small intestine and colon were isolated from mice of the desired ages into Trizol (Invitrogen, Grand Island, NY). Samples were briefly sonicated and total RNA isolated and reverse transcribed from random hexamer primers using the QuantiTect Reverse Transcription Kit (Qiagen, Carol Stream, IL). The resulting cDNA products were analyzed by real-time quantitative RT-PCR (iQ SYBR Green Supermix on MyiQ real time PCR detection system, Biorad, Hercules, CA) for GCLC and 18s ribosomal RNA. Level of GCLC expression was normalized to the 18s rRNA of the same sample. Fold difference was the ratio of the normalized value of each sample compared to the average 2day results.

18s Forward Primers: GGC GCC GGC GGC TTG GTG ACT CTA

18s Reverse Primers: CGC GCC TGC TGC CTT CCT TGG ATG T

GCLC Forward Primers: AGC ATC TGG AGA ACT AAT GAC TG

GCLC Reverse Primers: CAA GTA ACT CTG GAC ATT CAC AC

Primer Source: Ungvari Z, *et al.* Adaptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. *Am J Physiol Heart Circ Physiol April 2011 300:H1133–H1140*

Histologic staining

Proximal small intestine, distal small intestine and colon were isolated from mice of the desired ages and fixed in 10% formalin. Samples were paraffin embedded and cut into 5µm sections. Standard hematoxylin and eosin staining was done and images taken using a Nikon Eclipse 50i microscope. Slides stained for Ki67 were deparaffinized in xylene, then treated with 0.9% hydrogen peroxide in methanol and rehydrated. Antigen retrieval was done in a 10 mM citrate buffer (pH 6.0) in a pressure cooker. Slides were blocked using 2% dry milk in PBS-T. Slides were probed with anti-Ki-67 (Abcam, Cambridge, MA), followed by biotin conjugated anti-rabbit IgG(H+L) (Pierce Antibodies, Rockford, IL) and streptavidin-HRP (Abcam). Slides were developed using DAB Substrate Kit (Invitrogen, Grand Island, NY) and counterstained with hematoxylin (Invitrogen). Images were taken using a Nikon Eclipse 50i microscope and Ki-67 positive cells were quantified.

PAR ELISA

For ELISA analysis of PAR, murine intestinal epithelial cells were scraped into ice cold RIPA lysis buffer containing protease inhibitors (Roche Applied Science, Indianapolis, IN), sonicated, and centrifuged to remove debris. Protein concentration was assayed by DC Protein Assay Kit II (Bio-Rad, Hercules, CA) and measured by a SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA). PAR was detected by PAR ELISA kit 'PARP in vivo Pharmacodynamic Assay 2nd Generation (PDA II)' according to manufacturer's instructions (Trevigen, Gaithersburg, MD). Luminescence was measured by a FLUOstar OPTIMA (BMG Labtech, Ortenburg, Germany). Samples were all run in triplicate. PAR concentrations were normalized to protein concentration for each sample.

L-Buthionine-Sulfoximine (BSO) Treatment

Two week old animals were given 0.22mg BSO or an equivalent volume of sterile PBS by oral gavage daily for 1 week. Animals were subsequently sacrificed 4 hours after the final administration. Small intestine and colon were harvested into 10% formalin for histology or intestinal epithelial cell scrapings were collected into ice-cold 5% PCA buffer containing 10 GM γ -glutamyl-glutamate for GSH assay by HPLC analysis as described above.

Statistical analysis

For comparison among multiple groups, data were first tested for normality using Kolmogorov-Smirnov test. If normality passed, statistical differences were determined by parametric one-way analysis of variance (Dunnett's multiple comparison post-test). If normality was not passed or N determined to be too small for normality test, statistical differences were determined by Krusal-Wallis test (non-parametric test) and Dunns multiple comparison test. For comparison between 2 groups, a Student's T-test was performed. A $p < 0.05$ was considered statistically significant.

Results

Intestinal epithelial cell GSH/GSSG redox potential reduces over the first 3 weeks of postnatal murine life

To characterize the ontogeny of IEC GSH/GSSG redox potential, we isolated intestinal epithelia from the proximal small intestine, distal small intestine, and colon of neonatal mice over the first 3 weeks of life and we measured GSH and GSSG concentrations by HPLC. The IEC GSH/GSSG redox potential became increasingly reduced over the first 3 weeks of life in all regions of the murine intestine (from -202mV at 2 days to -265mV at 3 weeks in the proximal small intestine; from -165mV at 2 days to -246mV at 3 weeks in the distal small intestine; and from -174mV at 2 days to -244mV at 3 weeks in the colon, Figure 1). This change in IEC GSH/GSSG redox potential was driven primarily by an increase of more than 9-fold in IEC GSH concentration (1.5mM at 2 days to 18.3mM at 3 weeks in the proximal small intestine; 1.5mM at 2 days to 13.5mM at 3 weeks in the distal small intestine; and 1.1mM at 2 days to 12.6mM at 3 weeks in the colon (Figure 2A). This change was accompanied by an increase in gamma-glutamylcysteinyl synthetase catalytic subunit (*gclc*, the catalytic subunit of the rate limiting enzyme for *de novo* GSH synthesis) mRNA expression (Figure 2B). IEC GSSG concentration remained stable over the first 3 weeks of life (Figure 2C).

Intestinal epithelial cell proliferation increases over the first 3 weeks of postnatal murine life resulting in increased villus height, crypt depth, and intestinal length

Since previous *in vitro* studies indicate that a reduced GSH/GSSG redox potential favors cellular proliferation, we investigated the ontogeny of IEC proliferation rates by measuring Ki67 positive cells in the proximal small intestine, distal small intestine, and colon of neonatal mice over the first 3 weeks of life. As expected, IEC proliferation, as measured by positive Ki67 staining, increased in all regions of the intestine over the first 3 weeks of life (from 10 to 20 positive cells per crypt from 2 days to 3 weeks in the proximal small intestine; from 11 to 14 positive cells per crypt from 2 days to 3 weeks in the distal small intestine; and from 7 to 11 positive cells per crypt from 2 days to 3 weeks in the colon, Figure 3). This was accompanied by an increase in villus height, crypt depth (from 164 to 295 microns from 2 days to 3 weeks in the proximal small intestine; from 112 to 179 microns from 2 days to 3 weeks in the distal small intestine; and from 95 to 250 microns from 2 days to 3 weeks in the colon, Figure 4A); and intestinal length in both the small and large intestine (from 8 to 20 cm from 2 days to 3 weeks in the small intestine and from 2 to 4 cm from 2 days to 3 weeks in the colon, Figure 4B).

Intestinal epithelial cell intracellular concentration of poly-ADP-ribose chains increases over the first 3 weeks of postnatal murine life indicating increased PARP activity

Increases in intracellular GSH have been shown to drive cellular proliferation through increased poly-ADP-ribose polymerase (PARP) activity(15). To determine whether increased IEC proliferation in the developing neonatal mouse could also be driven by a similar mechanism, we assessed PARP activity by measuring intracellular epithelial poly-ADP-ribose (PAR) chains in the proximal small intestine, distal small intestine, and colon by ELISA. Increasing intracellular epithelial PAR chains were detectable in all regions of the

intestine over the first 3 weeks of life indicating increasing PARP activity (from 5 to 919 pg/g from 2 days to 3 weeks in the proximal small intestine; from 231 to 1279 pg/g from 2 days to 3 weeks in the distal small intestine; and from 812 to 1855 pg/g from 2 days to 3 weeks in the colon, Figure 5).

Inhibition of GSH synthesis prevents developmental reduction in glutathione redox potential, prevents developmental increase in IEC glutathione, and reduces IEC proliferation in the neonatal murine colon

Finally, we sought to determine whether some of the developmental changes observed over the first 3 weeks of neonatal murine life can be inhibited by inhibiting GSH synthesis. To do so, we treated 2 week old mice with carrier control or L-Buthionine-Sulfoximine (BSO), an inhibitor of gamma-glutamylcysteinyl synthetase (the rate limiting enzyme of GSH synthesis) daily by oral gavage for one week. We subsequently measured IEC glutathione redox potential, IEC GSH concentration, and IEC proliferation in the small intestine and colon as described above.

Unexpectedly, BSO treatment had no effect on IEC glutathione redox potential in the small intestine, while it did prevent developmental reduction in IEC glutathione redox potential in the colon (Figure 6A). Similarly, BSO treatment had no effect on IEC GSH concentration, while it did block the developmental increase in IEC GSH concentration in the colon (Figure 6B). We speculate that the more proximal portions of the intestine (small intestine) was able to use dietary intake of GSH to regulate GSH concentrations and compensate for lack of GSH synthesis. Finally, we demonstrated a reduction in Ki67 positive cells in the colon of neonatal mice treated with a week of BSO, while we demonstrated no reduction in Ki67 positive cells in the small intestine of neonatal mice treated with a week of BSO (Figure 6C). These results indicate that BSO can reduce intracellular GSH and IEC proliferation in the developing colon and are consistent with our idea that increased intracellular GSH may drive increased IEC proliferation developmentally in the neonatal murine intestine.

Discussion

The immature intestine of the premature infant may be at greater risk for intestinal injury and inflammation (antecedents of NEC) due to defects in barrier function, innate immunity, motility, digestion, and circulatory regulation(16, 17). Thus, developing strategies to promote maturation of the premature gut has become a stated priority(18). In particular, the intracellular redox potential of GSH/GSSG is known to play a key role in regulating proliferation and differentiation(9). In an effort to determine how intracellular redox states in the neonatal intestine may be targeted to promote intestinal homeostasis in the premature infant, this study aimed to characterize the ontogeny of intestinal epithelial intracellular GSH/GSSG redox potential using a murine model of immature intestine.

Using this model, we demonstrated that the IEC GSH/GSSG redox potential becomes increasingly reduced during postnatal intestinal maturation. This change was primarily driven by an increase in intracellular GSH concentration in the second week of murine life. Developmentally, the two-week old murine intestine is thought to correspond to a 24-week old premature human intestine(19). Thus, significant increases in IEC GSH production may

influence appropriate development of premature human intestine at this time. Disruptions to this process may negatively affect important developmental processes and potentially reduce the infant's ability to respond to such injury. As the premature intestine adapts to extrauterine life, it is at risk for injury from multiple sources, such as microbes, nutrients, and medications. Since reduced intracellular GSH/GSSG may be important to drive IEC proliferation, disruption in the maturation of the IEC GSH/GSSG redox potential, may hinder the ability of the immature intestine to respond to epithelial injury with proliferation. Epithelial injury may further reduce intestinal barrier function leading to invasion with toxins or microbes causing inflammation and additional injury, resulting in a vicious cycle that could have serious consequences(17).

The premature infant is at particularly high risk to develop diseases related to oxidative stress because the preterm infant has prematurely transitioned from the hypoxic *in utero* environment (in utero) to the relatively hyperoxic extrauterine environment. Furthermore, premature infants have underdeveloped antioxidant systems to counteract exogenous and endogenous sources of oxidative stress(20–22). Our study indicates that maturation of the GSH antioxidant system may be important for maturation and growth of the neonatal intestine. This may be occurring *in utero* in the fetus and postnatally in the premature neonate. Thus, oxidative stress in the preterm neonate may negatively affect continued postnatal maturation and growth of the immature intestine. *In vitro* studies indicate that very reduced GSH redox states (-250mV) favor cellular proliferation, slightly oxidized GSH redox states (-220mV) favor cellular differentiation, while further oxidation promotes cellular apoptosis or necrosis ($<-190\text{mV}$)(23, 24). In our study, IEC GSH redox potentials seen at 2–3 weeks in the neonatal murine intestine were consistent with an environment promoting cellular proliferation (-240 to -265mV range), while IEC GSH redox potential seen at 1 week were consistent with an environment promoting cellular differentiation (-200 to -215mV range). In the more distal portions of the intestine (distal SI and colon), 2-day-old murine IEC exhibited GSH redox potentials consistent with an environment promoting apoptosis or necrosis (-165 to -175mV range). Thus, preterm neonates not able to induce more robust IEC GSH synthesis may exhibit prolonged periods of more oxidized IEC redox potentials ($<-190\text{mV}$) and may potentially be predisposed to IEC apoptosis or necrosis. This may ultimately result in preterm intestinal diseases such as spontaneous intestinal perforation (SIP) and NEC. Consistent with this idea, hyperoxia causes NEC-like injury in neonatal animals(25); animal and human studies suggest that apoptosis may be an early pathogenic factor in NEC development(26–28); and distal SI and colon are commonly affected in both SIP and NEC(29–31). Further, hyperoxic injury has been used as an animal model of NEC(25).

Inflammatory processes are also known to lead to increased ROS production that may further influence GSH/GSSG redox imbalance in the IEC, causing impaired healing in response to injury. Future studies investigating how to restore this balance in the preterm intestine may lead to better preventive and therapeutic strategies for neonatal intestinal diseases such as SIP and NEC. Since ROS are known to function as messenger molecules that serve to regulate homeostatic cellular functions, global normalization of ROS production via antioxidant therapies will likely not be an ideal strategy. Indeed, clinical trials of antioxidant therapy in preterm infants have largely failed to yield improved outcomes(2–

4). In this study, changes in reduction of GSH redox potentials were primarily driven by the increased GSH concentrations. Studies in adult mice confirm that blocking GSH production causes intestinal epithelial cell damage(32). Enteral GSH administration can restore intestinal GSH levels and reverse epithelial damage(33, 34). Further, dietary restriction of sulfur amino acid intake has also been shown to negatively influence intestinal GSH levels(35) while sulfur amino acid supplementation has been shown to improve intestinal adaptation after small bowel resection *in vivo* via effects on intestinal mucosal redox potential(36). Keratinocyte growth factor has also been shown to improve intestinal mucosal growth by increasing its GSH content(37). This suggests that nutritional or pharmacologic strategies to optimize GSH levels or production may be a viable strategy to normalize GSH redox potential to promote homeostasis in the preterm intestine without suppressing physiologic ROS function.

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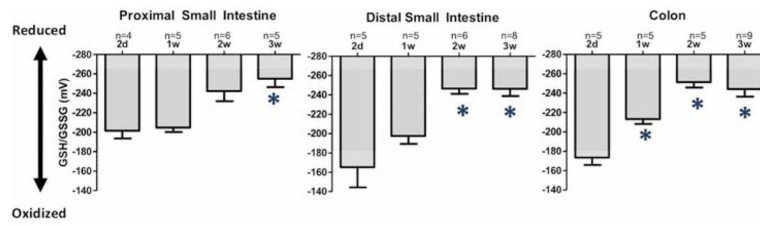


Figure 1. IEC GSH/GSSG redox potential reduces over the first 3 weeks of postnatal murine life GSH/GSSG redox potential in epithelial cells isolated from different regions of the murine intestine at various postnatal ages. Data are means \pm SEM. * $P < 0.05$ when compared to 2d condition.

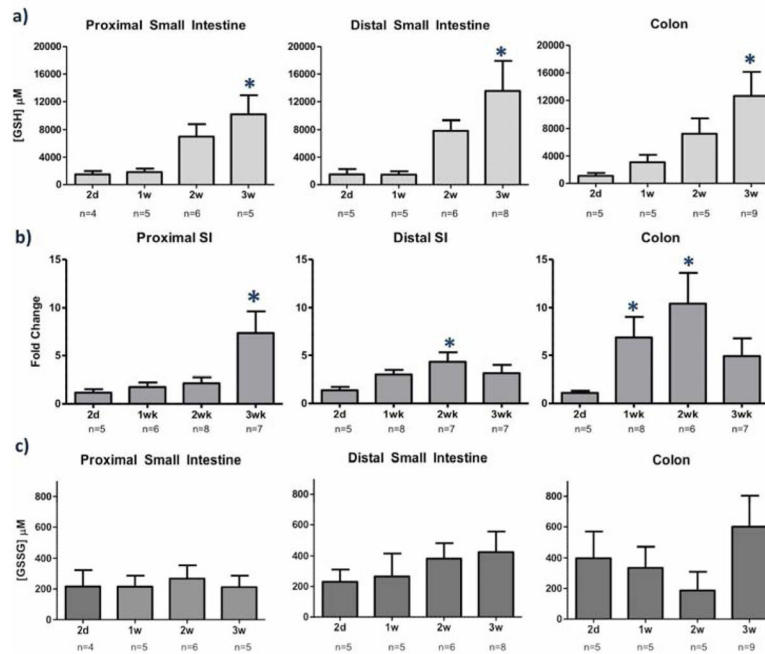


Figure 2. IEC [GSH] increases while [GSSG] remains stable over the first 3 weeks of postnatal murine life

A) Glutathione concentration [GSH] in epithelial cells isolated from different regions of the murine intestine at various postnatal ages. B) Fold change in *gclc* (gamma-glutamylcysteinyl synthetase catalytic subunit of the rate limiting enzyme for *de novo* GSH synthesis) mRNA expression in epithelial cells isolated from different regions of the murine intestine at various postnatal ages as assessed by qRT-PCR. C) Glutathione disulfide concentration [GSSG] in epithelial cells isolated from different regions of the murine intestine at various postnatal ages. Data are means \pm SEM. * $P < 0.05$ when compared to 2d condition.

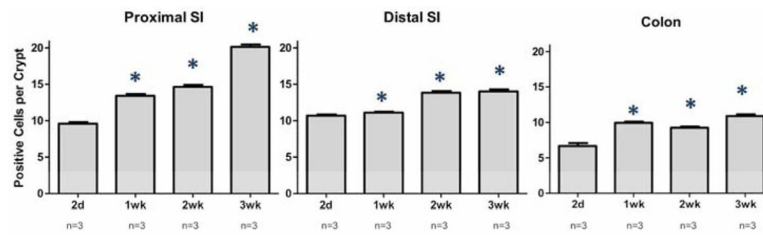


Figure 3. IEC proliferation as assessed by Ki67 staining increases over the first 3 weeks of postnatal murine life

Number of Ki67 positive cells per crypt in different regions of the murine intestine over the first 3 weeks of postnatal life. Data are means \pm SEM. * $P < 0.05$ when compared to 2d condition.

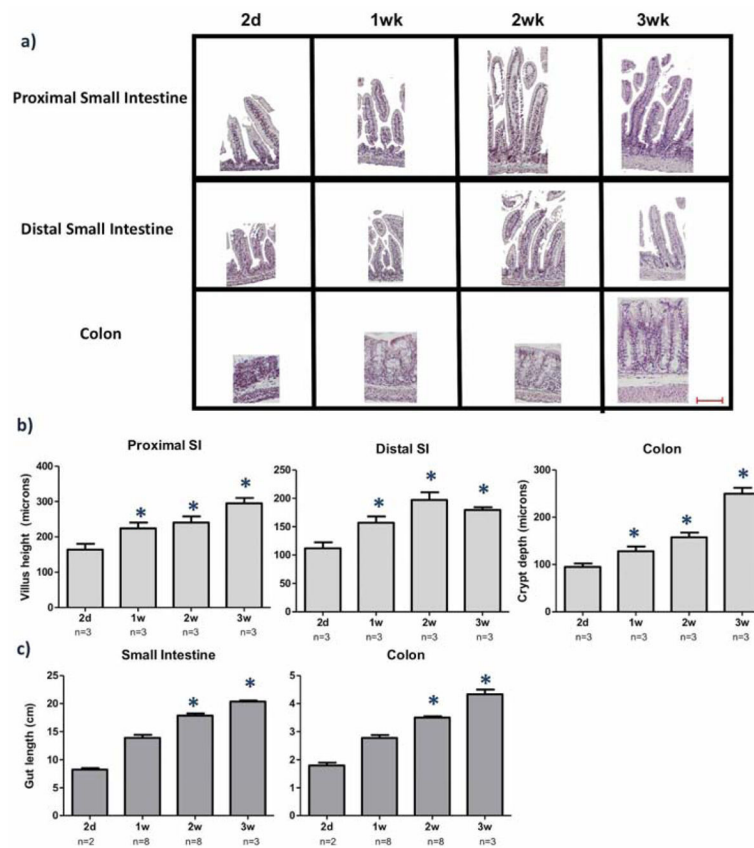


Figure 4. Intestinal villus height, crypt depth, and intestinal length increases over the first 3 weeks of postnatal murine life

A) H&E staining of murine intestine over the first 3 weeks of postnatal life. Scale bar represents 100 μ M. B) Villus height/crypt depth (mean \pm SEM) in different regions of the murine intestine over the first 3 weeks of postnatal life. C) Intestinal length of small intestine and colon (mean \pm SEM) over the first 3 weeks of postnatal life. * $P < 0.05$ when compared to 2d condition.

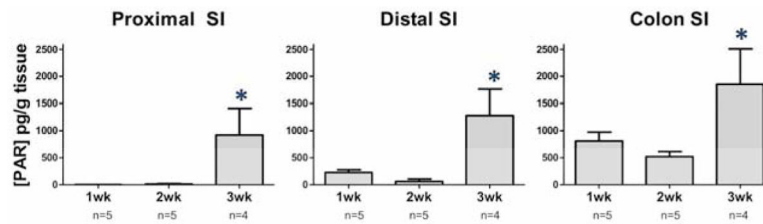


Figure 5. IEC PARP activity increases over the first 3 weeks of murine life

Concentration of intracellular epithelial PAR chains as assessed by ELISA from IEC isolated from different regions of the murine intestine at various postnatal ages (normalized to protein concentration of each sample). Data are means \pm SEM. * $P < 0.05$ when compared to 1wk condition.

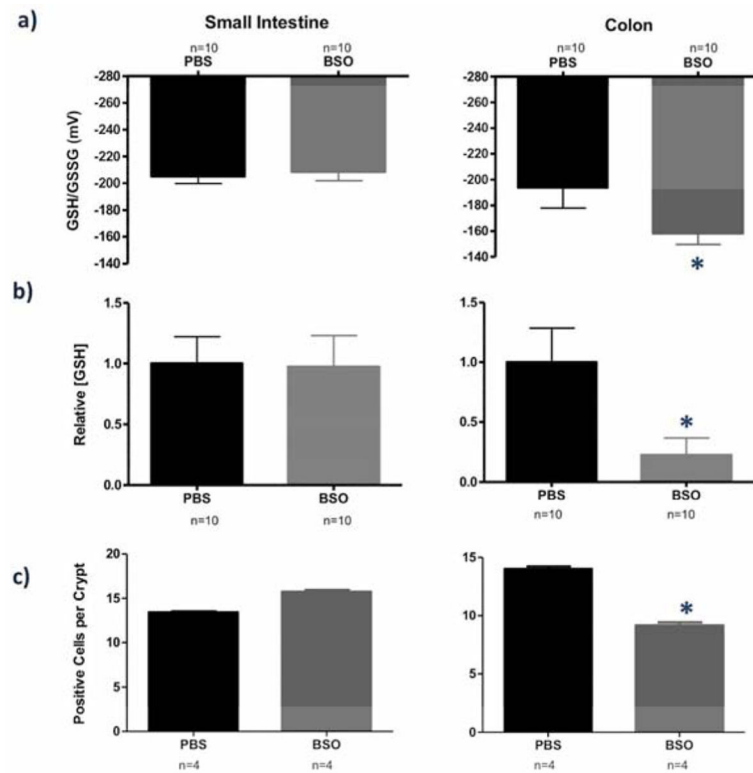


Figure 6. Reduction in IEC glutathione reduces IEC proliferation in neonatal mice

A) GSH/GSSG redox potential in 3 week old neonatal mice treated with PBS +/- BSO once daily for a week. B) Relative IEC glutathione concentration in 3 week old neonatal mice treated with PBS +/- BSO for a week. C) Number of Ki67 positive cells per crypt in 3 week old neonatal mice treated with PBS +/- BSO for a week. Data are means \pm SEM. * $P < 0.05$.