ESTROGEN ENHANCES FEMALE SMALL INTESTINE EPITHELIAL REGENERATION

by

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ABSTRACT

Promoting intestinal epithelial regeneration remains a major medical challenge. Interestingly, there is a sexually distinct wound healing response to consumption of non-steroidal anti-inflammatories (NSAIDs). We mined the literature for NSAID patient data finding that female patients taking NSAIDs are less likely to have acute damage to the intestine than males. We verified in vivo that female mice recover faster than male mice following drug-induced acute intestinal epithelial damage by scoring enteritis symptoms and performing histological staining on sectioned tissue. Using an ex vivo organoid system we showed that estrogen is necessary and sufficient in enhancing female organoid formation via estrogen receptor beta. Thus, estrogen promotes female intestinal epithelial organoid regeneration to lower the incidence of intestinal bleeding and ulceration.

The intensive and prolonged usage of NSAIDs remains a dominant treatment strategy for many diseases including cancer and arthritis. With distillation of dosage, mechanistic explanation and approved clinical trials, estrogen supplementation holds promise to minimize gastrointestinal side effects for patient groups subject to chronic NSAID treatment. It is paramount to separate these beneficial, protective intestinal effects from the feminizing effects of estrogen to make this treatment viable for all patient populations. In total, these experiments demonstrate estrogen to be partially responsible for a more robust healing of the female intestinal epithelium.

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are perhaps the most widely consumed medicines, comprising 5-10% of all prescribed drugs [1]. However, NSAIDs have many adverse side effects [2]. These drugs decrease intestinal mucosal secretions and destabilize the epithelial integrity of the gastrointestinal (GI) tract [3]. This damage results in intestinal bleeding and ulceration, which may be observed in up to 62% of patients who consume NSAIDs [4]. Male patients using NSAIDs are at a higher risk of being diagnosed with intestinal epithelial damage, including hemorrhages and ulcers [4], [5]. Varied hormone levels between sexes may affect the regenerative ability of the intestinal epithelium accounting for NSAID susceptibility disparities.

Estrogen is known to promote regeneration after acute injury via interactions with the stem cell niche [6], [7]. Tissue regeneration is initiated by molecular and protein binding to receptors in the stem cell niche. Each tissue system is affected by different factor subgroups which are not mutually exclusive. In female reproductive organs, estrogen is known to induce cell proliferation and promote tissue regeneration through estrogen receptor alpha (ERα) [6]. In certain non-reproductive organs, estrogen promotes the self-renewal and/or differentiation of several types of somatic stem cells via estrogen receptor beta (ERβ) [7], [8]. As such, there is evidence that estrogen plays a significant regenerative role in specific tissues.

Intestinal healing is heavily dependent on the migration, proliferation, and differentiation of stem cells housed in the villi crypt [9]. After damage, it is important to reestablish intestinal epithelial barrier integrity to ensure nutrient absorption and antigen

protection are maintained. ERα and ERβ are both expressed in the intestine alongside another recently discovered G-protein coupled estrogen receptor (GPER) [10]. Despite observed sexual differences in NSAID–induced acute wound healing ability and estrogen receptor expression, little is known about the relation between estrogen and intestinal wound healing.

Our aim was to isolate the role and mechanism of estrogen in intestinal wound healing. Mice of both sexes, challenged with sustained NSAID dosage, were used to confirm sex-based intestinal healing differences in vivo. The mechanism of action that orchestrates wound healing was carefully studied using a controlled, ex vivo, intestinal organoid system [11]. The results of these experiments elucidate estrogen's possible role in intestinal regeneration from acute injury. Greater understanding of intestinal wound healing benefits those patients who chronically use NSAIDs and/or have degenerative upper intestinal epithelial conditions. Furthermore, an increased understanding of estrogen's role in healing will enable development of new, specific regenerative treatments for acute intestinal injuries.

BACKGROUND

Non-steroidal anti-inflammatory drugs (NSAIDs) are a widely utilized class of drugs used to treat inflammation. Their inexpensive nature and effective composition secure their position as the third most consumed drug globally [3]. NSAID's function through inhibition of cyclooxygenase (COX1 and COX2). By neutralizing these enzymes, cells cannot convert arachidonic acid into thromboxanes, and prostaglandins [12]. Reduction of these lipid molecules, known as eicosanoids, plays a critical role in decreasing inflammation as thromboxanes are involved in clotting and prostaglandins are necessary for vasodilation and nociception (pain sensing). As this mechanism works exceedingly well, these drugs are consumed widely to treat muscle pain and migraines [12]. NSAIDs are not perfect though, as heavy usage induces adverse effects which predominantly affect the gastro-intestinal tract and cardiovascular system [13].

The gastrointestinal system is primarily responsible for digestion, absorption, excretion of nutrients and waste [13]. It comprises many unique organs, mouth to anus, which serve unique roles in the extraction of nutrients. The human small intestine spans approximately 20 feet in length and is the primary site of nutrient absorption [14]. The surface of the small intestine is composed of villi, or cellular protrusions made of epithelial cells [15]. Villi maximize surface area within the small intestine allowing for rapid absorption of nutrients, necessary for sustained vital processes. Food degradation and waste elimination harm intestinal epithelium through sustained chemical degradation and mechanical abrasion. In order to maintain absorption, villi must be shed and replaced every three days [16]. The anatomy of the villi allows for sustained growth and epithelial barrier

maintenance. At the base of each protrusion, intestinal stem cells are interspersed with Paneth cells in an area known as the crypt. Paneth cells produce critical signaling molecules that promote continued differentiation in the stem cell niche. Thereafter, cells begin to amplify and differentiate towards the villi tip [15]. Once the cells reach the top of the intestinal lumen, they undergo a process of anoikis (cell death) [16]. The result is a conveyor belt which transports cells through stages of differentiation to maintain maximum nutrient intake (Fig.1).

When consumed, NSAIDs are believed to heavily damage the intestinal epithelium by decreasing the mucosal levels produced by the goblet cells. Particularly, the reduction in mucosal prostaglandins via cyclooxygenase inhibition (COX) reduces the ability of the small intestine to produce protective secretions. This results in higher acidity and decreased bicarbonate secretion, blood flow, and proliferation in the small and large intestine [2]. Particularly important is prostaglandin receptor 2 (PGE2) which has been subclassified into four specific G-protein coupled receptor subtypes: ER1-4 [17]. The distribution of these

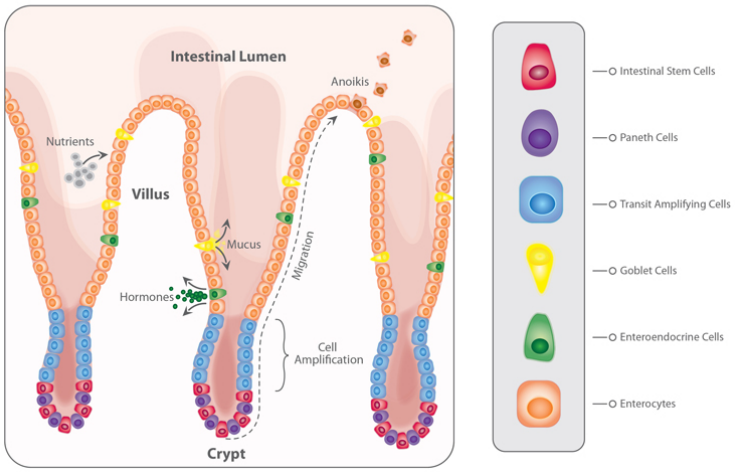

Figure 1: Diagram of the intestinal epithelium showcasing relevant cell types [11]. The intestinal crypt, made up of paneth, intestinal stem cells and transit amplifying cells, is responsible for the robust growth and regeneration of the intestinal epithelium following injury.

receptors is critical in understanding NSAID susceptibility. Within the small and large intestine PGE2-EP3 and EP4 activation has been demonstrated to be critical for secretion, motility, and healing [17]. When NSAIDs are administered, COX inhibition disrupts this activation of PGE2 effectively damaging the ability of the intestine to maintain epithelial secretion, causing ulceration and bleeding. Prostaglandins are created through the metabolism of arachidonic acid, which is released by phospholipases. COX couples with thromboxane synthase, prostaglandin F synthase, and cytosolic prostaglandin E synthase isozymes to transform arachidonic acid into prostaglandin through biosynthesis [18]. When COX is inhibited with an NSAID, a reduction in PGE2 is observed, harming the mucosa and compromising the epithelial integrity of the GI tract.

Following damage, the unique intestinal anatomy supports quick tissue remodeling and rebuilding to ensure the epithelial barrier is maintained. To repair a wounded area, a precise balance of migration, proliferation, and differentiation is necessary. First, epithelial cells must take on a flattened morphology and migrate to the damaged tissue. The formation of a cellular barrier is necessary to prevent antigens from entering the blood stream. This process happens within minutes of injury. Next, proliferation of stem cells begins to increase the total pool of cells available for resurfacing. Stem cell differentiation provides a variety of cell types, including secretory and absorptive cells, to the surrounding tissue. Once matured, these new cells exit the intestinal crypt and travel to the site of injury to restore functionality [9]. This process takes approximately three to four days [19]. The underlying chemical signaling that orchestrates wound healing in the small intestine is poorly understood. Previous studies indicate that various peptides, toll-like receptors and dietary factors modulate intestinal epithelial wound healing [9].

When seeded into a gelatinous, three-dimensional extracellular matrix; freshly harvested mammalian intestinal crypts form organoids which recapitulate the wound healing response of the GI tract [11]. Once placed in gel, cells self-assemble into an epithelial monolayer surrounded by a central lumen reminiscent of the intestinal architecture. All cell types of the intestinal niche are present (Fig 1). The robust nature of stem cells allows growth to occur quickly, with mature organoids showing by day seven [4]. This system enables the careful study of intestinal epithelial wound healing in a controlled environment, where healing may be quantified as the size and number of organoids grown from seeded crypts.

Hormonal variation is a hallmark of sexual differentiation with estrogen often representing a defining character of the female physiology [20]. There exist three defined estrogen receptors: estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and a G-protein coupled estrogen receptor (GPER) [10]. For clarity, the ERα receptor is encoded by the ESR1 gene, the ERβ receptor is encoded by the ESR2 gene and the GPER receptor by the GPER gene. Interestingly, estrogen has been shown to play a significant role in wound healing and regeneration. Functioning through ERα, ERβ, and GPER receptors, estrogen is known to induce proliferative behavior in the mammary glands, endometrium, neural stem cells and hematopoietic stem cells [6], [7], [8]. Little is known about the role of estrogen in intestinal epithelial regeneration, despite the expression ERβ and GPER in the small intestine.

METHODS

Experimental approach:

This study aims to explore how physiological sex-differences affect wound stimulated epithelial regeneration. Specifically, we hope to understand the mechanism that enables female patients to sustain less prolonged intestinal damage following acute injury. The results of these experiments may be applied to better treat NSAID damage alongside other degenerative upper intestinal epithelial conditions.

A top down approach was utilized where analysis began with the clinical data and concluded with exploratory ex vivo experiments. This methodology was utilized to ensure all ex vivo experiments were applicable to human physiological systems. This research was performed in the following manner:

1. Patient validation that females experience fewer GI complications following acute intestinal injury from prolonged NSAID consumption.
2. Confirmation that female mice recover faster from NSAID-induced intestinal wounds.
3. Ex vivo organoid experiments to isolate the specific contribution of estrogen to epithelial regeneration.

Clinical Study Analysis

A literature review of clinical studies involving NSAID use and upper GI incidents from 1985 to 2016 was conducted [21-27]. This analysis only includes studies involving patients receiving NSAID treatment who had adverse upper GI events and reported risk

assessments based on patient sex. Studies that did not draw a conclusion about the relative risks between male and female patients were not included. Data are shown as an odds ratios (OR) with the exception of the study of Lanas et al. [26], which is reported as relative risk (RR). Error bars represent 95% confidence intervals whenever given.

Indomethacin-Induced Intestinal Damage Model

Cohorts of male and female adult C57BL/6J mice were subcutaneously injected with indomethacin (Sigma Aldrich) dissolved in 5% sodium bicarbonate at a dosage of 1.5 mg/kg on days 1 and 2 [34]. At this concentration indomethacin inhibits release of COX1 and PGE2 causing the mucus lining of the intestine to erode. This leads to bleeding and epithelium damage from ulceration [51]. Over the following 8-day acute phase, mice were weighed and scored for clinical symptoms daily. Fecal matter was collected each day from the cages as well. Clinical symptoms of intestinal damage and enteritis include [35]:

1. Behavioral: hunched posture, shivering, and lack of spontaneous locomotion
2. Fecal: wet stool, light yellow stool, bloody/black stool, and constipation

If any symptoms were present, the mice were scored "positive". If no symptoms were present they scored "negative". After 8 days, the mice were continuously monitored for body weight changes until completely recovered.

Detection of Intestinal Bleeding Using Modified Guaiac Fecal Occult Blood Test

Intestinal bleeding was detected by analyzing the fecal hemoglobin content, using a modified guaiac fecal occult blood test [36] (Santa Cruz Biotechnology). Guaiac gum powder was dissolved in ethanol at a concentration of 100 mg/mL. The dissolved solution

(10ml) was added to each well in a 96-well plate and air dried. Stool samples were gathered throughout the experiment and stored at 20°C. After completion of clinical scoring, the fecal samples were weighed and resuspended in 300 mL of 70% ethanol to extract heme groups. The samples were incubated at room temperature for 30 minutes and then homogenized. After a 10-minute incubation samples were centrifuged at 21,000x g for 5 minutes. The supernatant was collected for analysis where 50 μL of the fecal extracts were added, in triplicates, into a pre-coated 96-well plate. A gradient of concentrations of commercial hemoglobin standards (Sigma-Aldrich) was used to generate a standard curve. Developing solution was made with 40% methanol, 40% ethanol, 6% hydrogen peroxide, and 14% H_2O. Developing solution (200 mL) was added to each sample and absorbance was measured at 630 nm with a SpectraMax spectrometer. Fecal blood hemoglobin concentration was calculated using the standard curve, and normalized to the weight of collected feces.

Histology Staining of Jejunal and Duodenal Sections of Small Intestines

All mice studies were approved by University of Utah Institutional Animal Care and Use Committee under protocol number 16-05012 and 18-02010. All experiments have been conducted in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals [29]. Mice were euthanized using a CO_2 chamber with a flow rate of 3 L/min.

The jejunal and duodenal sections of small intestines of indomethacin-treated euthanized mice were surgically removed and fixed in zinc formalin for 2 hours followed by overnight incubation in 70% ethanol. After, the tissues were dehydrated with

incrementing gradient of ethanol and cleared with CitriSolv (VWR). Tissues were processed with molten paraffin at 65°C under 381-mmHg vacuum for 30 minutes. Processed sections were then embedded in paraffin and sectioned at 8 μm with a microtome. Standard hematoxylin eosin staining was performed [37]. The slides were imaged using EVOS^{TM} Auto Imaging System.

Mouse Intestinal Epithelial Crypt Isolation

C57BL/6J (wildtype) and estrogen receptor beta (ERβ) knockout breeder mice ($Esr2^{+/-}$) were purchased from Jackson Laboratories. Crossing of $Esr2^{+/-}$ parent mice allowed for generation of $Esr2^{-/-}$ mice, validated by western blotting. All male and female mice used for intestinal crypt isolation were adults (8–10 weeks old). The isolation procedure was modified from an established protocol [28]. The jejunal sections of the mouse's small intestine were harvested, opened longitudinally and washed with ice cold 1x phosphate buffer saline (PBS) (Sigma-Aldrich). Mucus and villi were removed using a thin glass coverslip and tissue was cut into 1 cm sections. The tissue pieces were washed in cold PBS and incubated in 30 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) dissolved in PBS for 8 minutes on ice. Under harsh stripping conditions, dithiothreitol (DTT) (Thermo-Fisher) was added to this solution at a concentration of 1.5 mM. Tissue pieces were transferred to PBS and incubated for 15 minutes on ice. Crypts were liberated by vigorously shaking for 3 minutes and isolated by passage through a 70 mm cell strainer. Purified crypts were pelleted by centrifugation (300 g for 5 minutes at 23°C) and washed twice with PBS to remove any remaining contaminants.

Organoid Culture

Isolated crypts were counted then pelleted by centrifugation (300 g for 5 minutes at 23˚C). 1000 crypts were suspended in 80 mL of 50% growth-factor-reduced phenol-red-free Matrigel (Corning) diluted in conditioned LWRN culture medium containing WNT3a, R-spondin, and Noggin (preparation previously described [30]). This suspension was pipetted onto the center of a 24 well plate to create a dome. Each suspension gelled at 37°C for 20 minutes. 750 mL of media containing a treatment (singularly including β-estradiol (Sigma-Aldrich), 4-hydroxytamoxifen (Cayman), and G15 (Tocris)) was added to fully submerge the gel. Variable concentrations were added depending on experiment. β-estradiol concentrations ranged from 1, 100, 500 to 1000 pg/ml. 4-hydroxytamoxifen concentrations used were 0.1 and 1 μM. For details of which concentration were used, please reference the experimental figures (7C, 8B, 9A, 9B and 9C). G15 was added at a concentration of 1 μM exclusively. Media was changed every 3 to 4 days, with treatment maintenance throughout. Organoids were counted under 4x magnification with images captured on the EVOS™ Auto Imaging System 4 and 7 days after plating. The number of organoids was normalized per each experiment. Quantification of size and number was performed by a custom program written in Matlab (Appendix 1).

Estrogen Free Media

To prepare estrogen-free LWRN media (EF-LWRN), charcoal stripped hormone-free 5% fetal bovine serum (Sigma-Aldrich) and phenol-red-free advanced DMEM/F-12

medium (Thermo-Fisher) were used to culture L-WRN cells. Conditioned LWRN medium is prepared from the culture supernatant of L-WRN cells [30].

Estradiol Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

The quantification of total estradiol and estradiol-mimics (estradiol-3-sulfate, estradiol-3-glucuronide, estrone, and estrodiol-17-glucuronide) in culturing media was performed using a commercial estradiol enzyme-linked immunosorbent assay kit per manufacturer's protocol [31] (Cayman). This assay has a detection limit of 6.6 pg/mL as established by the manufacturer [31].

Quantitative Real-Time Polymerase Chain Reaction

Organoids were harvested as described above. Total RNA was extracted with trizol (Life Technologies) and purified with a Direct-Zol RNA Prep Kit (Zymo). Purified RNA was used to generate cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). With the resulting cDNA, quantitative real-time polymerase chain reactions were performed using Power SYBR Green Master Mix (Life Technologies) and an Applied Biosystems 7900HT Fast Real-Time PCR System. Primers were designed using the Primer3 engine seeking to obtain approximately equal gene amplification [32]. Data were analyzed by the standard $\Delta\Delta$Ct method [33]. Copy number, thereafter, was calculated based on the transcript quantity of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used include:

1. ESR1 forward – aatgaaatgggtgcttcagg
2. ESR1 reverse – atagatcatgggcggttcag

3. ESR2 forward – aactctctgagtcccgagca
4. ESR2 reverse – gttccttgtcagccagcttc
5. GPER1 forward – cctctgctactccct- catcg
6. GPER1 reverse – gagaaggctgcaaggttgac
7. GAPDH forward – tggcaaagtggagattgttg
8. GAPDH reverse – tgactgtgccgttgaatttg.

Western Blot Analysis

Organoids were harvested by removing all media and washing in cold PBS. The mixture was spun down (300 g for 5 minutes at 4° C) and the supernatant removed. This process was repeated 4-6 times or until no Matrigel could be observed by visual inspection. Organoids were lysed using Pierce IP Lysis Buffer (Thermo Fisher). The lysates were centrifuged at 15,000 g for 5 minutes at 4°C and the supernatant saved. Protein concentration was quantified by a bicinchoninic acid protein assay (Thermo Scientific). Protein concentration for each sample was equalized and mixed with equal volume of Laemmle buffer. The proteins were denatured at 90° C for 10 minutes then loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis (15V, 80 minutes) proteins were transferred onto methanol activated, polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat dry milk in 1X TBS and 0.1% Tween-20 then incubated overnight with primary antibody in 1X TBS, 0.1% Tween-20 with 5% non-fat dry milk at 4° C. The next day, membranes were washed 3 times with 1X TBS, 0.1% Tween-20 then incubated with secondary antibody in 1X TBS, 0.1% Tween-20 with 5% non-fat dry milk for 1 hour at 23° C. After 3 washes in 1X TBS, 0.1% Tween-20, the

labeled bands were detected using Immobilon enhanced chemiluminescent horseradish peroxidase substrate according to manufactures instructions (Millipore) and visualized using a ChemiDoc Touch Imaging System.

Statistical Analysis

Welch t-tests were used to compare means of 2 groups. One-way analysis of variance (ANOVA) with Welch's correction was used to compare means among more than 2 groups. These tests were selected to ensure valid comparisons even in the presence of unequal variances between the groups [38]. For female organoid formation, relative organoid numbers were log transformed. Thereafter, one-way randomized block ANOVA with Tukey's multiple comparisons was performed. This methodology was utilized to pair data within a single experiment to their corresponding control when multiple experiments were performed. This allows for normalization of experiments even when great variability exists between the experiments [39]. As multiple organoid experiments were plated and run in parallel, this analysis was optimal. With all interpretations, if two groups are significantly different, the null hypothesis was rejected. If statistically significant differences between the group was not observed, we could not reject the null hypothesis.

RESULTS

Females Experience Less NSAID-Associated Intestinal Epithelial Damage

An analysis of clinical studies from 1985 to 2016, which investigated the sex differences of NSAID- associated upper GI bleeding and ulceration, revealed that female patients taking NSAIDs are less likely to present upper GI bleeding or ulcers than male patients (Fig. 2). However, post-menopausal females are more likely to present upper GI symptoms than male patients of the same age (Fig. 2).

Additionally, male patients with NSAID-induced injury are less likely to heal following treatment (Fig. 3). No data are published regarding the analogous healing ability of females. These findings suggest that sexual dimorphisms may play an important role in enhancing female intestinal epithelial regeneration after injury.

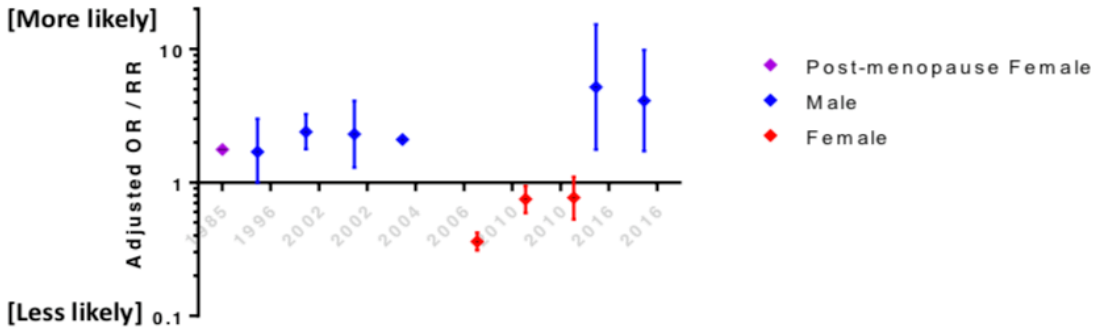

Figure 2: Females showcase reduced risk of GI bleeding or ulceration. NSAID induced injury risk ratios from GI bleeding or ulceration were calculated based on studies from 1985 – 2016. Error bars represent a 95% confidence interval.

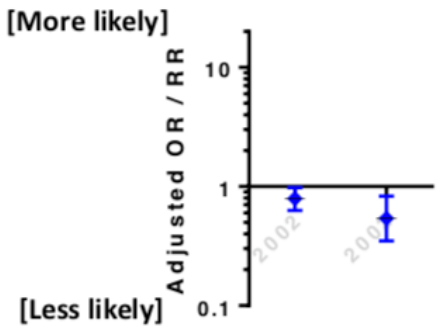

Figure 3: Male NSAID consuming patients are less likely to heal following treatment. This plot showcases the adjusted risk ratio for healing for males in years 2002 and 2008. Error bars represent 95% confidence intervals.

Female Mice are Protected from NSAID-Induced Intestinal Damage

Male and female C57BL/6J mice were given indomethacin (an NSAID) by subcutaneous injection, which induced intestinal epithelial damage (Fig. 4A), bleeding (Fig. 4B), and body weight loss (Fig. 4C). Female mice recovered from the intestinal damage substantially faster than male mice – demonstrating an increased rate of weight recovery (Fig. 4C) and faster elimination of enteritis symptoms (Fig. 4D). Combined, this shows that the female mouse intestinal epithelium more robustly regenerates following acute injury.

Estrogen Enhances Female Mouse Intestinal Organoid Regeneration

Organoid formation from mouse intestinal epithelium crypts is an ex vivo assay which reflects the robustness of the intestinal stem cells to support epithelial regeneration and wound repair [40]. Breached crypts may heal into intact villus-like intestinal organoids in three-dimensional Matrigel cultures when supplemented with necessary growth factors [11] (Fig. 5). By using a harsh crypt stripping method [28], a drastic difference in

regeneration between male and female intestinal crypts was observed (Fig. 6A). Female crypts are more likely to grow and form organoids than male crypts (Fig. 6B).

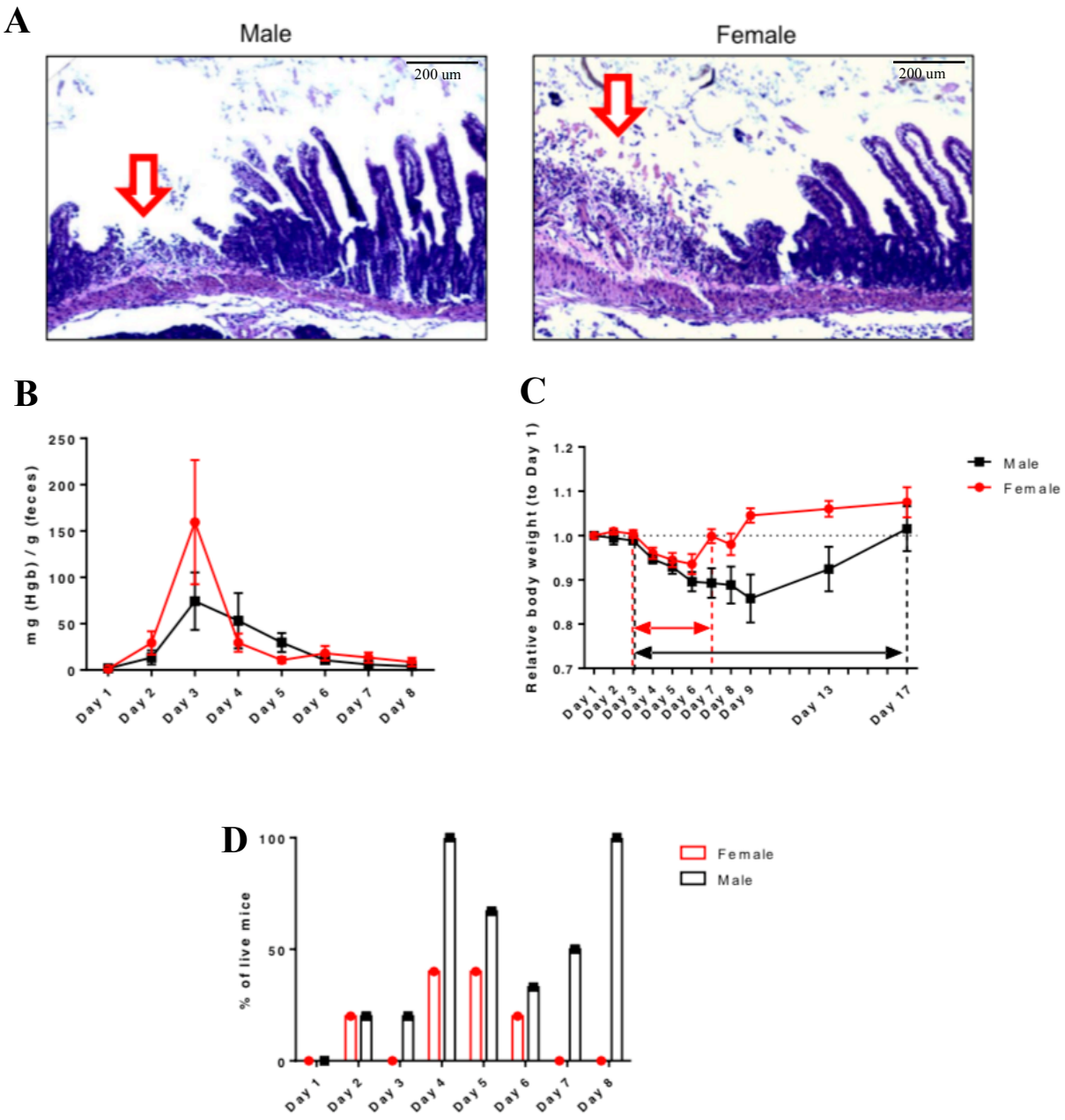

Figure 4: Female mice recover faster than males after NSAID-induced damage. A) Hematoxylin-eosin staining of male and female mouse intestine one day after injection where arrows indicate epithelial lesions. B) Fecal occult blood analysis for mice treated with indomethacin (n=10). C) Body weight changes for mice treated with indomethacin (n=10) where dashed lines define ranges of weight deficiency. D) Percentage of live mice showcasing enteritis symptoms following NSAID treatment.

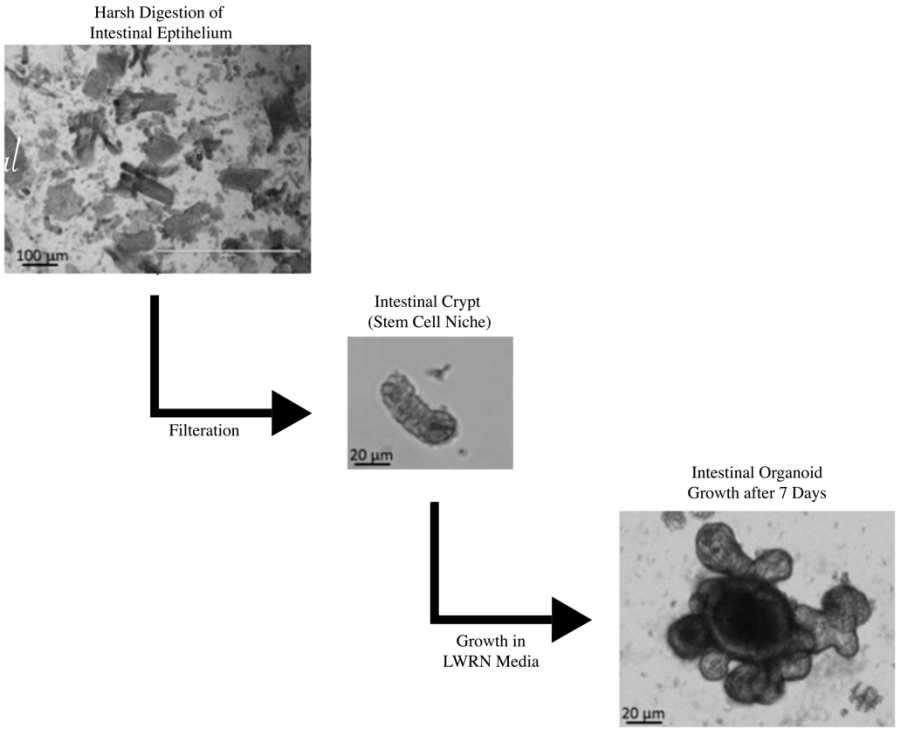

Figure 5: The regeneration of stripped intestinal epithelial crypts into intact organoids in 3-D Matrigel cultures. Mouse intestinal epithelium was stripped and dissociated with 30 mM EDTA and 1.5 mM DTT. Crypts were isolated via filtration. Isolated crypts were plated in Matrigel and cultured in conditioned LWRN media. Seven days later, crypts form intestinal organoids.

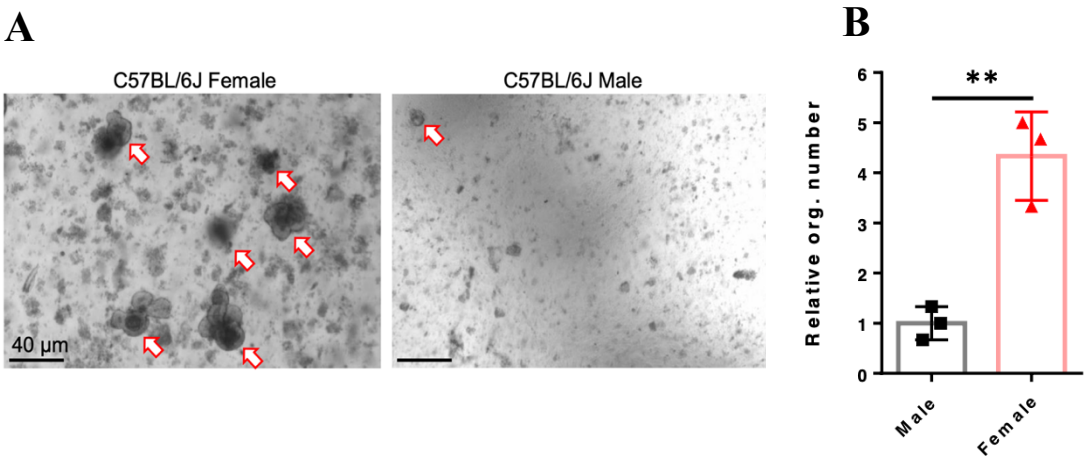

Figure 6: Female crypts form organoids more robustly than male crypts. A) Representative images of organoid formation in LWRN media taken seven days after seeding. B) Number of organoids formed (n=100) in LWRN medium from the same number of seeded crypts where error bars represent standard deviation. ** $P < 0.01$

When cultured with a hormone-free media, stripped of lipophilic content (Fig. 7A), however, female crypts lose their organoid formation advantage (Fig. 7B). The addition of a physiologically relevant concentration of β-estradiol to EF-LWRN cultures significantly increased the number of organoids formed from female crypts (Fig. 7C) [41].

Estrogen Enhances Female Mouse Intestinal Organoid Regeneration via ERβ Receptor

Female mouse intestinal epithelium expresses detectable levels of ERβ and GPER, but does not express ERα as shown by mRNA transcript level (Fig. 8A). In female ERβ knockout mice, β-estradiol did not enhance organoid formation (Fig. 8B). Furthermore, in estrogen-containing LWRN medium, the addition of 4-hydroxytamoxifen inhibited female intestinal organoid formation (Fig. 9A).

Blocking GPER signaling (indicated by p-ERK inhibition, Fig. 9C) using a highly specific inhibitor [41], did not affect the β-estradiol-induced enhancement of organoid formation (Fig. 9B). These data indicate that the enhancing effect of estrogen in female intestinal epithelial regeneration is mediated through the ERβ receptor.

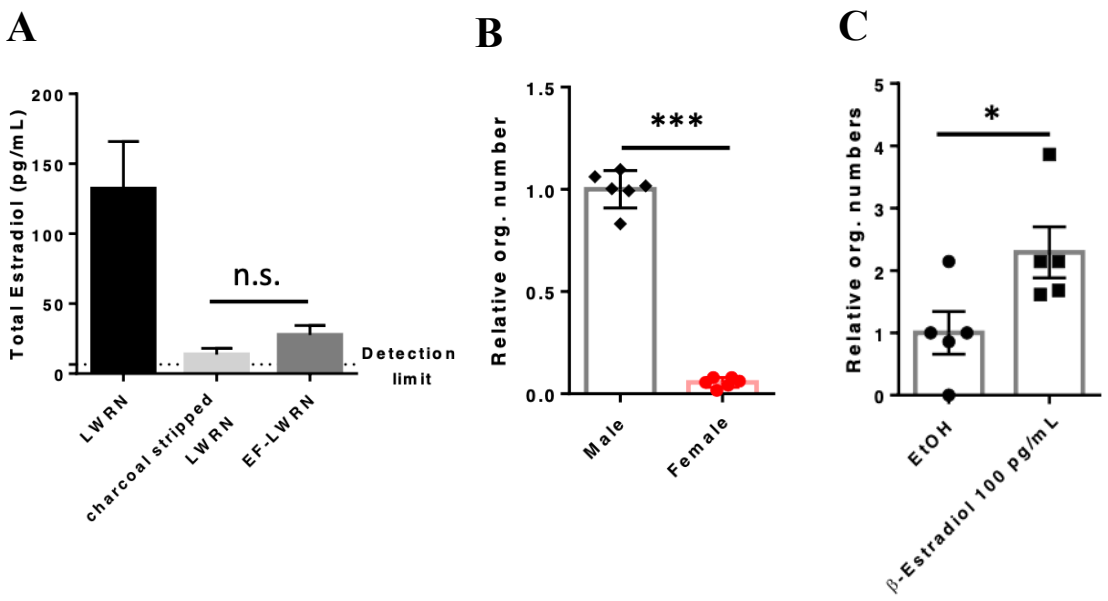

Figure 7: Female organoid forming advantage is lost in estrogen free media and is partially rescued by addition of synthetic estrogen. A) ELISA quantification of total estradiol content in LWRN media. B) Number of organoids formed in estrogen free media with equal seeded crypts. C) Quantifications of female organoid formation with indicated treatments. * P< 0.05, *** P<0.001, EtOH = ethyl alcohol

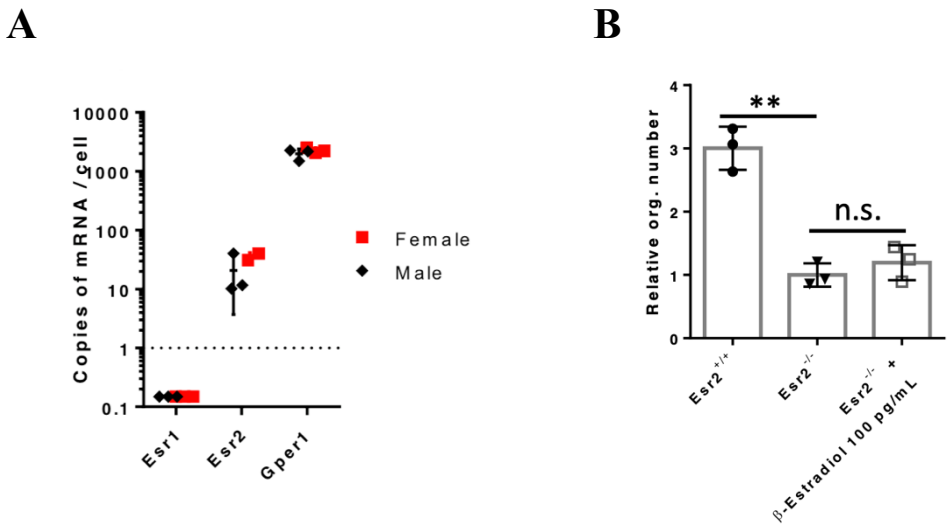

Figure 8: ESR2 inhibition does not affect female organoid growth. A) Quantitative RT-PCR for gene expression of three estrogen receptors. B) Organoids grown from ESR2 knockout mouse with addition of estradiol. ** P < 0.01

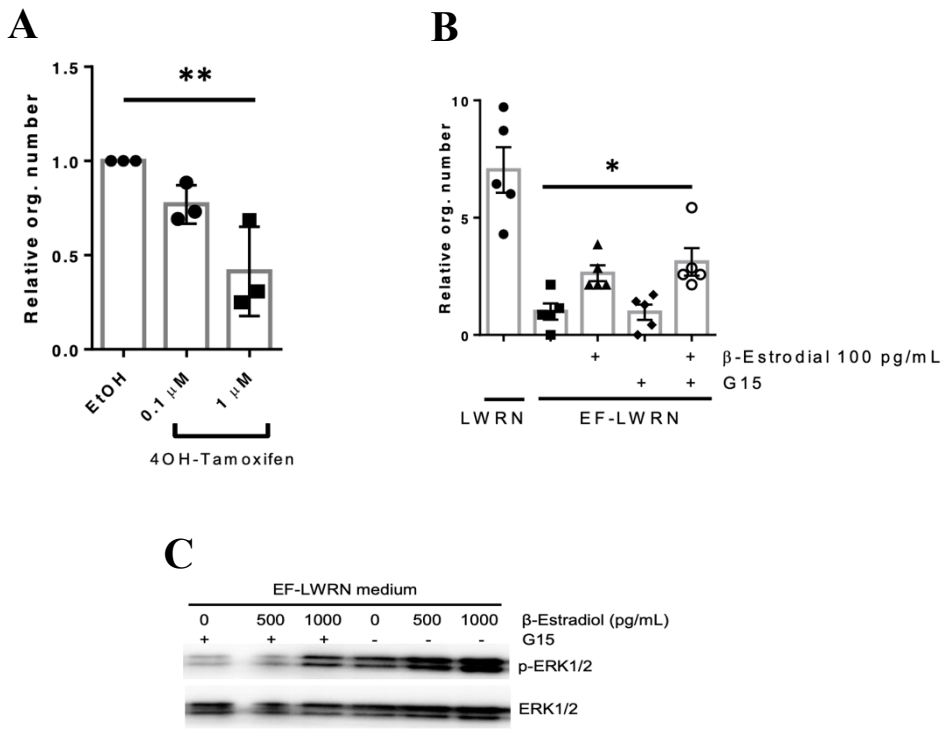

Figure 9: The GPER estrogen receptor is partially responsible for increased in vivo regeneration. A) Organoids in EF-LWRN seeded with 4OH-Tamoxifen. B) Utilization of highly specific GPER inhibitor on organoid growth. All seeding densities are equal. C) Immunoblot of extracellular signal-regulated kinase phosphorylation (p-ERK ½), downstream of GPER. * $p < 0.05$, ** $p < 0.01$

DISCUSSION

This study aimed to isolate and understand the role of estrogen in intestinal epithelial wound healing. Literature mining was used to distill risk of adverse gastro-intestinal (GI) damage following acute epithelial injury from human NSAID use. In vivo mice studies were performed to confirm a sex-defined, differential intestinal-epithelial healing ability. Finally, ex vivo experiments were performed to demonstrate estrogen is partially responsible for the more robust regeneration of murine female intestinal epithelium compared to murine males. This, in part, contributes to the relative resistance of females to chronic intestinal bleeding and ulceration.

In the clinic, female patients were less likely to present GI tract injuries when taking NSAIDs when compared to men (Fig. 2). After menopause, females lose their advantage to withstand prolonged NSAID usage without developing GI bleeding or ulceration (Fig. 2,3). This trend suggests that estrogen could partially contribute to the increased resilience and robust healing response of female intestinal epithelium to acute injury. It is important to note that during menopause many sex-specific hormones vary in expression. This study examines the specific role of estrogen. Other hormones may play a role in regulating GI-related wound healing.

When applied to an in vivo mouse model, female intestinal epithelia are more resistant to prolonged NSAID exposure. Histology of the intestinal epithelium shows greater damage to the outermost layer of the intestinal epithelium in male mice (Fig. 4A). Female mice exhibited more fecal blood content demonstrating increased efficacy of indomethacin to induce ulceration and bleeding within the intestine when compared to

males. A greater percentage of live female mice showcased enteritis symptoms following acute NSAID dosage. Interestingly, females are able to recover lost weight faster than males, despite increased fecal blood content (Fig. 4B-D). These results demonstrate that despite higher fecal blood content, female mouse intestinal epithelium recovers faster following acute injury. These in vivo data mirror clinical trends, which show similar correlations in intestinal bleeding (Fig. 2). In total, the female mouse intestine can withstand acute NSAID exposure with more robustness than the male intestine.

Utilizing an ex vivo organoid system, estrogen was demonstrated to be necessary and sufficient for the enhanced robustness in female intestinal epithelium regeneration (Fig 5-9). However, as β-estradiol could not fully restore female organoid growth, other hormones or lipophilic factors may be required for maximal female intestinal epithelial regeneration (Fig. 9). All observed effects appear to be functioning through ERβ as inhibition of the GPER had minimal effect on organoid growth.

This work suggests a potential role of estrogen in wound healing mediated through the ERβ receptor. Recent publications suggest a potential role of estrogen and related receptors in maintaining homeostatic ion concentrations within the small and large intestine. Estrogen receptors are shown to regulate certain ions, HCO_3^- and Cl^-, which are critical for intestinal secretion production [44]. Among a variety of physiological roles, intestinal secretions are critical to epithelial barrier maintenance [45]. Damage to intestinal secretion production compromises the epithelium, often resulting in enteritis and/or ulceration [9]. Heavy NSAID usage at high doses reduces prostaglandin production impairing production of vital secretions [1]. This suggests that the observed difference in healing ability between sexes, observed in vitro and in vivo, may be attributable to a better

maintenance of the intestinal epithelial barrier when challenged with acute injury. As females exhibit higher levels of estrogen within the circulatory system and are able to tolerate higher dosages of NSAIDs, the observed results support the possibility of estrogen acting as a regulator of intestinal secretion.

An additional difference between males and females are the maintained microbes within the small and large intestine. Interestingly, the gut microbial flora has been shown to play a key role in intestinal wound healing. Studies demonstrate how gut microbiota regulate estrogens via release of β-glucuronidase, an enzyme capable of estrogen activation. When the production of estrogen is reduced by harm to the microbiome, less estrogen is activated within the intestine [46]. The gut flora between males and females is not identical [47]. As such, there is potential for estrogen to be differentially regulated affecting the healing ability of the intestinal niche. Additional studies would need to be performed to ensure intrinsic estrogen levels within the females were not confounding the role of gut microbiota in wound healing.

Organoids are a core component of this work as this ex vivo system enabled controlled study of the intestinal stem cell niche. No previous studies using organoids have reported similar findings with regards to differential organoid growth from male or female hosts. Our study posits a new position, that the presence of estrogen within media may alter observed organoid experiments when crypts are removed with harsh isolation. This differential growth may affect future studies choosing to explore sexual differences with an ex vivo system of study.

Several statistical incongruencies should be noted with the interpretation of these data. Since clinical data came pre-processed from a variety of sources, no standardization

on male and female data was performed. Furthermore, not all studies adjusted for patient characteristics, such as smoking, alcohol drinking, and H. pylori infection. Hence, the male-to-female ratio of intestinal bleeding prevalence appears to be quite variable. Although the trend of higher prevalence of intestinal bleeding and ulceration in males is pronounced, the interpretation of these conclusions should be supported by additional experimentation to increase confidence.

This study utilized a less traditional, more harsh, intestinal crypt stripping method. This strategy was selected to observe the relative resistance of intestinal stem cells to damage [28]. Harsh stripping involved using high concentrations of EDTA (30 mM) and DTT (1.5mM) alongside mechanical shaking force to dissociate stem cells from the surrounding tissue. Compared to other gentle isolation techniques, our methodology may yield fewer organoids when plating the same number of crypts in Matrigel [46]; thus, gentle isolation procedures may lead to different observations.

These findings imply a novel strategy for encouraging intestinal epithelial wound regeneration in patients suffering from small intestinal bleeding. With the widespread chronic usage of NSAIDs, estrogen supplementation may minimize GI side effects [3]. We suspect estrogen to play a critical role in epithelial barrier maintenance, a core component of epithelial regeneration. Understanding the mechanisms of action driving increased healing are critical to transforming this work into actionable treatment. Currently, supplementation of estrogen may be problematic as it has been shown to increase risk of cancer and develop feminizing features [49], [50]. Isolation of dosing and localized treatment will be critical to ensuring hormonal treatment does not affect other tissue systems. This work provides a new therapeutic target for all diseases and treatments which

damage the epithelial intestinal tissue such as obscure gastrointestinal bleeding or high dose radiation [42][43].

In order to further confirm the role of estrogen in female intestinal regeneration after NSAID-induced intestinal bleeding, necessity studies should be performed in mice. These may include NSAID dosing in female mice who have undergone ovariectomy surgery or intestinal epithelium-specific ERβ knockout. Combined, these studies would potentially verify estrogen's role in intestinal wound healing. Further experimentation should explore the mechanism by which ERβ promotes wound healing in female mice and humans. Research should be performed to examine if specific factors, such as particular ions or microbiomes, play a peculiar role in maintenance of intestinal epithelium. Isolation of this pathway is critical in developing therapies and understanding the signaling orchestrating the observed phenomenon. Additionally, non-feminizing estrogens should be tested to enable an estrogen-based treatment to function for male populations [50].

These experiments demonstrate that estrogen is partially responsible for a more robust healing of the female intestinal epithelium. This work provides a novel angle for developing therapies to promote intestinal wound healing. Development of treatments should benefit those with intestinal disease and populations taking medications, such as NSAIDs, in which side effects can greatly damage the intestinal epithelia. More in vivo studies and clinical trials are necessary to develop a suitable estrogen treatment to promote intestinal wound healing.

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APPENDIX 1: Organoid Quantification Program

Matlab (2017)

```
function varargout = Organoid2(varargin)

%Excess GUI Code
% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @Organoid2_OpeningFcn, ...
                   'gui_OutputFcn',  @Organoid2_OutputFcn, ...
                   'gui_LayoutFcn',  [], ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
  gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT


% --- Executes just before Organoid2 is made visible.
function Organoid2_OpeningFcn(hObject, eventdata, handles, varargin)
%Intialization of Variables used within different functions of the GUI
%including preset values for threshold area minima and contrast strel
handles.output = hObject;
handles.areaarray=[];
handles.orgnumber=[];
handles.minimumsize=2000;
handles.streldiskvalue=5;
axis off
axes(handles.Image)
axis off

% Update handles structure
guidata(hObject, handles);


% --- Outputs from this function are returned to the command line.
```

```
function varargout = Organoid2_OutputFcn(hObject, eventdata, handles)

% Get default command line output from handles structure
varargout{1} = handles.output;


% --- Executes on button press in Organoidregionselecter.
function Organoidregionselecter_Callback(hObject, eventdata, handles)

%Region Selecter Corresponds with the button 'select organoid region':
%The goal of this button was to open a rectangle window to select an
%organoid. Once selected, the new 'cropped selection' will be evaluated for
%area using binarization, morphological identification and regionprops. The
%exact processed is outlined below.

%Creating a rectangular window:
axes(handles.Image); %selection of the image within the large axis (tagged Image)
cropBox = imrect; %imrect: embedded function which allows creation of movable rectangle. Used to select the organoid
roiPosition = wait(cropBox); %Wait for user input double click. Once a double click is detected, record the coordinates of the rectangle within the larger image as a region of intrest

% Establishing the ROI coordinates
xCoords = [roiPosition(1), roiPosition(1)+roiPosition(3), roiPosition(1)+roiPosition(3), roiPosition(1), roiPosition(1)];
yCoords = [roiPosition(2), roiPosition(2), roiPosition(2)+roiPosition(4), roiPosition(2)+roiPosition(4), roiPosition(2)];
croppingRectangle = roiPosition; %Cropping Rectangle represents the coordinates of the ROI selection within the image

% Crop out the ROI from the orginal image. Effectively establishes a
% smaller workspace which limits noise from the image. Now easy to identify
% the organoid with binarization.

%To do the crop, use the function imcrop.
handles.whitePortion = imcrop(handles.R, croppingRectangle);

%imagesc(handles.whitePortion) THIS WILL DISPLAY CROPPED PICTURE

%Extracating each layer in order to create a combined binary image instead of
%converting from greyscale to binary
r=handles.whitePortion(:,:,1);
g=handles.whitePortion(:,:,2);
b=handles.whitePortion(:,:,3);
```

```
%Setting binary threshold
rcolum=0.5;
gcolum=0.5;
bcolum=0.5;

%Binarizing the layers individually then combining
l1=imbinarize(r,rcolum);
l2=imbinarize(g,gcolum);
l3=imbinarize(b,bcolum);
Ltotal=(l1&l2&l3); %Combined Binarized image

%Inverse White and Black Pixels so that we may use morphological commands
blackimage=imcomplement(Ltotal);
filledimage=imfill(blackimage,'holes');

%Setting a basic image to structural image to get rid of excess noise
se=strel('disk',handles.streldiskvalue);
handles.cleanimage=imopen(filledimage,se);

%Display the cropped image, with binarization and morhpholical adjumestment
%made. This will allow the user to guage the effectiveness of their
%masking.
axes(handles.Image2);
imagesc(handles.cleanimage);
colormap gray
axis off

%Measurement of Area in pixels
info=regionprops('table',handles.cleanimage,'perimeter','area');

%Transfering info into an array: Allows for selected organoids area to be
%transferred from the structured table of region props to an array.
orgarea=(table2array(info(:,1)))';

%Next, within the array recieved, there will be a main large organoid area and
%lots of smaller pieces of tissue debris areas. This for loop sorts the
%the array and zeroes all portions which are under the value of minimum
%size. This is a user defined value and can be changed within the GUI

for g = 1:length(orgarea')
if orgarea(g) <=handles.minimumsize %possible button here to change value to account
for different organoid sizes
   orgarea(g)=0;
end
```

```
end

%To avoid adding in any 'zeroed' values, a simple command reshapes the
%organoid area array to only include the value of measured organoid area
orgarea(orgarea==0) = [];
handles.orgarea=orgarea;

%In order to prevent user errors, a system of while and if statments were
%combined. The first addresses if the threshold isn't large enough and
%multiple organoids are being read. This will break the loop and a suggestion will be made
to the user. If there are
%no organoid detected, the second while loop breaks the code and suggests a
%fix to the user. Finally, if the length of the organoid area is equal to
%one, the program will proceed. This step acts to protect the code from
%potential pitfalls.
while length(handles.orgarea)>1
   msgbox('More than one organoid detected. Consider increasing the minimum area
value');
   break
end

while length(handles.orgarea)<1
      msgbox('No organoids detected. Consider decreasing the minimum area value');
   break
end

if length(handles.orgarea)==1

%Within this if statment, the new organoid area is read into a table which
%is displayed on the GUI. As the user inputs more data, an increasing array
%will be created in which the data from the previous values will be
%concatenated with the previous data values.
handles.areaarray=cat(1,handles.areaarray,handles.orgarea);
handles.orgnumber=cat(1,handles.orgnumber,(length(handles.areaarray)));
handles.tablevals=[handles.orgnumber,handles.areaarray];
set(handles.tableofdata,'data',handles.tablevals);
end

guidata(hObject, handles);

% --- Executes on button press in dataexport.
function dataexport_Callback(hObject, eventdata, handles)
%The export data allows the user to take the table of saved areas and organoid numbers
and export them
%to a an excel file. Simply speaking, this is done through the commands
```

```
%uiputfile and xlswrite.
FileName = uiputfile('*.xls','Save as');
data = get(handles.tableofdata, 'data');
xlswrite(FileName,data);

% --- Executes on button press in choosepic.
function choosepic_Callback(hObject, eventdata, handles)
%The choose picture button allows the user to select a preferred image
%from a menu to analyze (utilizing uigetfile). The picture may be of png, jpg or tif format.
Once
%selected from the menu the image will be read and displayed with a
%designation to the large axes and the command imagesc.
handles.image1 = uigetfile({'*.png;*.jpg;*.tif','Supported images';...
          '*.png','Portable Network Graphics (*.png)';...
          '*.jpg','J-PEG (*.jpg)';...
          '*.*','All files (*.*)'});

handles.R = imread(handles.image1);
guidata(hObject, handles);

axes(handles.Image);
organoidpic=imagesc(handles.R);
colormap gray
axis off

function inputboxforcontrast_Callback(hObject, eventdata, handles)
%The box next to mask level is a variable textbox which allows for user input.
%Upon entering another value, the variable handle.streldiskvalue will
%change to the user input value. This may then be used to calculate a new
%mask. The comman str2double is used to ensure the value entered is turned
%into a usuable double value.
handles.streldiskvalue=str2double(get(hObject,'String'))
guidata(hObject, handles);

% --- Executes during object creation, after setting all properties.
function inputboxforcontrast_CreateFcn(hObject, eventdata, handles)

if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
   set(hObject,'BackgroundColor','white');
end

function sizethresholdvalue_Callback(hObject, eventdata, handles)
% the box next to the mask level is a variable textbox which allows for
% user input. Upon entering a value, the variable handles.minimumsize will
```

```
% change to the new user input value. This value may be used within the
% longer organoid area size. The command str2double is used to ensure the
% value eneter is turned into a usable double value.
handles.minimumsize=str2double(get(hObject,'String'))
guidata(hObject, handles);

% --- Executes during object creation, after setting all properties.
function sizethresholdvalue_CreateFcn(hObject, eventdata, handles)

if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end


% --- Executes on button press in tableclear.
function tableclear_Callback(hObject, eventdata, handles)
%The Clear Table button allows the user to clear the entered data table.
%This is simply an overwrite of any existing values. This allows the user
%to erase mistakes, or clear the table when a new file is loaded.
handles.orgarea=[];
handles.tablevals=[];
handles.areaarray=[];
handles.orgnumber=[];

set(handles.tableofdata,'data',handles.tablevals);
guidata(hObject, handles);
```

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