

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2017 July 28; 23(28): 5041-5256



### EDITORIAL

- 5041 Colorectal cancer in young adults: A difficult challenge

*Campos FG*

- 5045 Precision medicine: In need of guidance and surveillance

*Lin JZ, Long JY, Wang AQ, Zheng Y, Zhao HT*

### REVIEW

- 5051 Barrett's oesophagus: Current controversies

*Amadi C, Gatenby P*

- 5068 Gastrointestinal neuroendocrine peptides/amines in inflammatory bowel disease

*El-Salhy M, Solomon T, Hausken T, Gilja OH, Hatlebakk JG*

- 5086 Colorectal cancer screening: An updated review of the available options

*Issa IA, Noureddine M*

- 5097 Therapeutic potential of flavonoids in inflammatory bowel disease: A comprehensive review

*Salaritabar A, Darvishi B, Hadjiakhoondi F, Manayi A, Sureda A, Nabavi SF, Fitzpatrick LR, Nabavi SM, Bishayee A*

### ORIGINAL ARTICLE

#### Basic Study

- 5115 Myo-inositol reduces  $\beta$ -catenin activation in colitis

*Bradford EM, Thompson CA, Goretsky T, Yang GY, Rodriguez LM, Li L, Barrett TA*

- 5127 Involvement of CRF2 signaling in enterocyte differentiation

*Ducarouge B, Pelissier-Rota M, Powell R, Buisson A, Bonaz B, Jacquier-Sarlin M*

- 5146 Bone marrow-derived monocyte infusion improves hepatic fibrosis by decreasing osteopontin, TGF- $\beta$ 1, IL-13 and oxidative stress

*de Souza VCA, Pereira TA, Teixeira VW, Carvalho H, de Castro MCAB, D'assunção CG, de Barros AF, Carvalho CL, de Lorena VMB, Costa VMA, Teixeira AAC, Figueiredo RCBQ, de Oliveira SA*

- 5158 Single amino acid mutation of SR-BI decreases infectivity of hepatitis C virus derived from cell culture in a cell culture model

*Gao R, Gao W, Xu G, Xu J, Ren H*

- 5167** Fibroblast-derived CXCL12/SDF-1 $\alpha$  promotes CXCL6 secretion and co-operatively enhances metastatic potential through the PI3K/Akt/mTOR pathway in colon cancer

*Ma JC, Sun XW, Su H, Chen Q, Guo TK, Li Y, Chen XC, Guo J, Gong ZQ, Zhao XD, Qi JB*

**Case Control Study**

- 5179** Association between *CYP24A1* polymorphisms and the risk of colonic polyps and colon cancer in a Chinese population

*Chen XQ, Mao JY, Li WB, Li J, Yang H, Qian JM, Li JN*

**Retrospective Cohort Study**

- 5187** Clinical significance of glycemic parameters on venous thromboembolism risk prediction in gastrointestinal cancer

*Guadagni F, Riondino S, Formica V, Del Monte G, Morelli AM, Lucchetti J, Spila A, D'Alessandro R, Della-Morte D, Ferroni P, Roselli M*

- 5196** Sex-dependent difference in the effect of metformin on colorectal cancer-specific mortality of diabetic colorectal cancer patients

*Park JW, Lee JH, Park YH, Park SJ, Cheon JH, Kim WH, Kim TI*

**Retrospective Study**

- 5206** Sex-influenced association of non-alcoholic fatty liver disease with colorectal adenomatous and hyperplastic polyps

*Chen QF, Zhou XD, Sun YJ, Fang DH, Zhao Q, Huang JH, Jin Y, Wu JS*

**Observational Study**

- 5216** Development and validation of a simple and multifaceted instrument, GERD-TEST, for the clinical evaluation of gastroesophageal reflux and dyspeptic symptoms

*Nakada K, Matsuhashi N, Iwakiri K, Oshio A, Joh T, Higuchi K, Haruma K*

- 5229** Modified B-ultrasound method for measurement of antral section only to assess gastric function and guide enteral nutrition in critically ill patients

*Liu Y, Gao YK, Yao L, Li L*

**Prospective Study**

- 5237** Chronic liver failure-consortium acute-on-chronic liver failure and acute decompensation scores predict mortality in Brazilian cirrhotic patients

*Picon RV, Bertol FS, Tovo CV, de Mattos ÁZ*

**CASE REPORT**

- 5246** Refractory hepatic encephalopathy in a patient with hypothyroidism: Another element in ammonia metabolism

*Díaz-Fontenla F, Castillo-Pradillo M, Díaz-Gómez A, Ibáñez-Samaniego L, Gancedo P, Guzmán-de-Villoria JA, Fernández-García P, Bañares-Cañizares R, García-Martínez R*

- 5253** Endoscopic occlusion with silicone spigots for the closure of refractory esophago-bronchiole fistula after esophagectomy

*Uesato M, Kono T, Akutsu Y, Murakami K, Kagaya A, Muto Y, Nakano A, Aikawa M, Tamachi T, Amagai H, Arasawa T, Muto Y, Matsubara H*

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NAME OF JOURNAL  
*World Journal of Gastroenterology*

ISSN  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

LAUNCH DATE  
October 1, 1995

FREQUENCY  
Weekly

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PUBLICATION DATE  
July 28, 2017

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## Basic Study

**Myo-inositol reduces  $\beta$ -catenin activation in colitis**

Emily M Bradford, Corey A Thompson, Tatiana Goretsky, Guang-Yu Yang, Luz M Rodriguez, Linheng Li, Terrence A Barrett

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**Author contributions:** Bradford EM, Thompson CA and Goretsky T performed experiments and analyzed data; Yang GY provided assistance with pathology; Rodriguez LM and Barrett TA supported the design and execution of the clinical trial; Li L provided reagents and contributed to the experimental design; Bradford EM and Barrett TA wrote the manuscript.

**Supported by** Veterans Affairs Merit Award, No. IO1CX001353 (to Barrett TA); National Institutes of Health, No. 2R01DK095662-06A1 (to Barrett TA); the National Cancer Institute at the National Institutes of Health, No. N01-CN-35157 (to Bergan R); the Training Program in Oncogenesis and Developmental Biology through the National Cancer Institute, No. NCI T32 CA080621 (to Bradford EM); an Institutional Development Award from the National Institute of General Medical Sciences of the National Institutes of Health, No. 8 P20GM103527-05; and American Physiological Society STEP-UP Fellowship (to Thompson CA).

**Institutional review board statement:** For all human studies, informed consent was obtained from every patient and samples were coded. Collection of all material was approved by the Northwestern University or University of Kentucky Institutional Review Boards. Some biopsies were collected under the NCI Myo-Inositol Chemoprevention in Colitis-Associated Dysplasia trial, #NWU09-13-02. The ClinicalTrials.gov identifier for the Myo-Inositol Chemoprevention trial is NCT01111292.

**Institutional animal care and use committee statement:** All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

**Conflict-of-interest statement:** The authors have no conflicts-of-interest to report.

**Data sharing statement:** All patients gave informed consent to allow their samples to be used in this study. These samples were coded and no patient information is identifiable.

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**Manuscript source:** Unsolicited manuscript

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**Received:** February 6, 2017  
**Peer-review started:** February 8, 2017  
**First decision:** March 16, 2017  
**Revised:** March 31, 2017  
**Accepted:** May 9, 2017  
**Article in press:** May 9, 2017  
**Published online:** July 28, 2017

**Abstract****AIM**

To assess dietary myo-inositol in reducing stem cell

activation in colitis, and validate p $\beta$ -catenin<sup>S552</sup> as a biomarker of recurrent dysplasia.

#### METHODS

We examined the effects of dietary myo-inositol treatment on inflammation, p $\beta$ -catenin<sup>S552</sup> and pAkt levels by histology and western blot in IL-10<sup>-/-</sup> and dextran sodium sulfate-treated colitic mice. Additionally, we assessed nuclear p $\beta$ -catenin<sup>S552</sup> in patients treated with myo-inositol in a clinical trial, and in patients with and without a history of colitis-induced dysplasia.

#### RESULTS

In mice, p $\beta$ -catenin<sup>S552</sup> staining faithfully reported the effects of myo-inositol in reducing inflammation and intestinal stem cell activation. In a pilot clinical trial of myo-inositol administration in patients with a history of low grade dysplasia (LGD), two patients had reduced numbers of intestinal stem cell activation compared to the placebo control patient. In humans, p $\beta$ -catenin<sup>S552</sup> staining discriminated ulcerative colitis patients with a history of LGD from those with benign disease.

#### CONCLUSION

Enumerating crypts with increased numbers of p $\beta$ -catenin<sup>S552</sup> - positive cells can be utilized as a biomarker in colitis-associated cancer chemoprevention trials.

**Key words:** Chemoprevention; Dysplasia; Biomarker; Stem cell; Colitis-associated cancer

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**Core tip:** We report that dietary myo-inositol reduced inflammation and intestinal stem cell activation in both genetic and chemically-induced mouse models of colitis. In a limited clinical trial of colitis patients with a history of recurrent low grade dysplasia (LGD), myo-inositol reduced the number of intestinal crypts with activated stem cells. This study demonstrated for the first time that nuclear p $\beta$ -catenin<sup>S552</sup> staining discriminates between patients with inflammation and those with a history of LGD, suggesting that nuclear p $\beta$ -catenin<sup>S552</sup> staining may reflect local expansions of activated stem cells with neoplastic potential. Based on these data, we propose a more extensive clinical trial.

Bradford EM, Thompson CA, Goretsky T, Yang GY, Rodriguez LM, Li L, Barrett TA. Myo-inositol reduces  $\beta$ -catenin activation in colitis. *World J Gastroenterol* 2017; 23(28): 5115-5126 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i28/5115.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i28.5115>

## INTRODUCTION

Chronic inflammation in ulcerative colitis (UC) and Crohn's disease (CD) contributes to an elevated risk

of colitis-associated cancer (CAC)<sup>[1,2]</sup>. Patients with UC have an overall 11-fold relative risk of cancer and a 38-fold increased risk if they are diagnosed before age 30<sup>[1]</sup>. Similarly, patients with CD have an 18-fold increased risk of developing colorectal cancer<sup>[2]</sup>. In these diseases, chronic inflammation increases the risk of dysplasia through a variety of mechanisms. Oxidative stress, eicosanoid production and signaling through the cyclooxygenase/lipoxygenase pathways<sup>[3,4]</sup>, apoptosis, altered metabolism, and signaling through phosphoinositide 3-kinase (PI3K) and Akt<sup>[5-7]</sup> all contribute to the transition from inflammation to dysplasia.

$\beta$ -catenin translocation into the nucleus is a hallmark of colorectal cancer. Mutations in the genes encoding proteins in the phosphatase and tensin homolog (PTEN)/PI3K/Akt and Wnt/ $\beta$ -catenin pathways lead to colorectal cancer<sup>[8-11]</sup>. Mutations in adenomatous polyposis coli promote dysplasia and cancer by reducing  $\beta$ -catenin degradation and enhancing nuclear accumulation, detected by immunohistochemical staining. Additionally, Akt phosphorylation of  $\beta$ -catenin at serine 552 (p $\beta$ -cat) increases nuclear translocation of p $\beta$ -cat, upregulates TCF/LEF transcription, and increases *in vitro* tumor invasion<sup>[8]</sup>. Data from our group showed that mutations in PTEN enhance PI3K/Akt signaling and p $\beta$ -cat levels, leading to increased polyp formation<sup>[10]</sup>. These data suggest that alterations in PI3K and  $\beta$ -catenin signaling directly affect stem cell activation in dysplasia.

Under conditions of chronic inflammation, cells undergo neoplastic transformation after acquiring founder mutations. Vogelstein and others coined the term "field effect" to reflect the clonal expansion of mutated cells that cause areas of dysplasia to appear in the vicinity of sentinel areas of sporadic cancer or polyps<sup>[9,12]</sup>. In many cancers, including CAC, clonal fields of cells harboring DNA mutations surround visible dysplasia or carcinoma. Based on analysis of colitis-induced dysplasia, Brentnall and colleagues proposed that populations of intestinal epithelial cells<sup>[13]</sup> carrying p53 mutations spread to adjacent crypts by migrating over colonic surfaces<sup>[14]</sup>. Clevers and others suggest that the field effect originates in mutated intestinal stem cells<sup>[15,16]</sup>. We hypothesize that staining for nuclear p $\beta$ -cat provides a useful biomarker in identifying such a field effect.

Myo-inositol, a naturally abundant 6-carbon poly-alcohol isolated from cereals (*e.g.*, rough coat of rice), and its derivatives regulate a variety of intracellular pathways. Inositol polyphosphates are important as second messengers in signal transduction, act as anti-oxidants, and mediate calcium regulation in membrane signaling. Nuclear inositol signaling may also play a role in DNA repair and chromatin remodeling<sup>[11,17-21]</sup>. Due to these effects, inositol has been tested in a wide range of human clinical trials, including cancer prevention, autism, and psychiatric disorders.

Lam *et al.*<sup>[22]</sup> conducted a dose escalation trial of myo-inositol in smokers with bronchodysplasia. They

found that myo-inositol (9 g twice a day) reduced dysplasia, as well as Akt and phospho-ERK staining in dysplastic tissues<sup>[23]</sup>. Given our previous data linking PI3K signaling to  $\beta$ -catenin activation we considered the possibility that myo-inositol could reduce dysplasia by reducing ISC activation in colitis. However, before launching a large scale clinical trial, we wished to develop the means for detecting elevated numbers of activated ISC in colitis patients using p $\beta$ -cat staining. Additionally, we wished to determine whether this marker would serve as a reliable read-out of ISC chemoprevention.

## MATERIALS AND METHODS

### Animals

C57BL/6 (WT) and C57BL/6 IL-10<sup>-/-</sup> (IL-10<sup>-/-</sup>) mice were purchased from Jackson Laboratories (Bar Harbor, ME, United States). Mice were maintained under specific pathogen free conditions at the University of Kentucky. All experiments were approved by the University of Kentucky IACUC.

### Serum myo-inositol measurements

Serum myo-inositol was measured using an enzymatic spectrophotometric assay as previously described<sup>[24]</sup>. Briefly, blood was collected by cardiac perfusion into untreated 1.5 mL tubes, incubated at room temperature for 1 h, centrifuged, and serum was collected and stored at -80 °C. Serum samples were treated with glucose oxidase and catalase (Calbiochem, San Diego, CA, United States), deproteinized with perchloric acid, and subsequently neutralized. Enzymatic determination of myo-inositol was done by reading the absorbance change at 546 nm from the reduction of Fe<sup>3+</sup> - to Fe<sup>2+</sup> -bathophenanthroline disulphonic acid (VWR, Radnor, PA, United States) from the NAD<sup>+</sup>-dependent oxidation of myo-inositol by myo-inositol dehydrogenase (Sigma, St.Louis, MO, United States). Myo-inositol concentration was determined from a calibration curve prepared with aqueous standards.

### Induction of colitis in mice

Thirteen IL-10<sup>-/-</sup> mice were divided into three groups: control (IL-10<sup>-/-</sup>), piroxicam-treated (IL-10<sup>-/-</sup> Px), and piroxicam-treated plus myo-inositol (IL-10<sup>-/-</sup> Px/myo-inositol). Piroxicam-containing chow was made by Harlan-Teklad (Madison, WI, United States). As previously described, 8-10 wk old IL-10<sup>-/-</sup> mice were fed chow containing 65 mg/250 g piroxicam for one week to synchronize colitis, followed by 85 mg/250 g piroxicam to induce colitis<sup>[25]</sup>. Mice were sacrificed 14 or 42 d later. For the group treated with myo-inositol, mice were treated with 1% (w/v) myo-inositol (Sigma) in the drinking water for one week prior to piroxicam treatment and for the duration of the experiment. For experiments of dextran sodium sulfate (DSS)-induced colitis, WT mice were treated with 2% (w/v) DSS (MP

Biomedical, Santa Ana, CA, United States) for eight cycles. A single cycle of DSS consisted of 7 d of DSS in the drinking water followed by a recovery period of 14 d of regular water. Mice were treated with 1% myo-inositol during the recovery periods.

### Disease activity indices

IL-10<sup>-/-</sup> mice undergoing treatment were scored daily or every other day using a standard disease activity index (DAI) based on diarrhea, fecal blood (Beckman Coulter SENA Hemocult Test), and percent weight loss as previously described<sup>[26]</sup>. Each criterion was assigned a score from 0 (no diarrhea, fecal blood or weight loss) to 4 (severe diarrhea, visible fecal blood, and up to 20% weight loss). The daily combined DAI score is the sum of each individual criterion.

### Histological analysis

Tissues were fixed in 4% neutral buffered formalin overnight, processed through paraffin, sectioned at 5  $\mu$ m, and stained for hematoxylin and eosin (H&E). For murine tissues, colitis scores were calculated based on a graded scale of inflammation (0-3), extent (0-3), regeneration (0-4), crypt damage (0-4) and percent involvement (1-4) as previously described<sup>[27]</sup>. The combined colitis score is the sum of the scores for inflammation, extent, and crypt damage/regeneration.

### Immunohistochemistry

For human sections, standard of care biopsies were processed, stained and analyzed by the pathology departments of the University of Chicago or Northwestern University. Slides were stained for H&E,  $\beta$ -catenin, p53 or Ki67 and staining patterns were interpreted by staff pathologists. For laboratory staining of anti-p $\beta$ -catenin<sup>S522</sup> (p $\beta$ -cat) and Ki67, paraffin sections were rehydrated through graded alcohols and antigen retrieval was performed using Target Retrieval Solution (Dako, Carpinteria, CA, United States), pH 6.0, in a decloaking chamber. Sections were incubated with anti-p $\beta$ -cat (provided by Linheng Li, Stowers) or anti-Ki67 (TEC-3, Dako) followed by anti-rabbit or anti-mouse peroxidase-labeled polymer (Dako). Sections were developed using 3,3'-diaminobenzidine (DAB) tetrahydrochloride chromagen (Dako) and counter-stained with hematoxylin.

### Cell fractionation

IEC were isolated with EDTA and depleted of CD45 cells with sheep anti-rat IgG magnetic Dynabeads (Life Technologies, Grand Island, NY, United States) preloaded with rat anti-mouse CD45 antibody. For subcellular fractionation, all buffers contained ProteaseArrest<sup>™</sup> protease inhibitor cocktail (G-Biosciences), and phosphatase inhibitor cocktails I and II (Sigma) in 1:100 dilution. IEC were homogenized in Buffer I (50 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 0.01% digitonin), lysates were passed through 26G needle,

then centrifuged at 4 °C for 10 min. Pellets were resuspended in Buffer II (50 mmol/L Tris-HCl pH 7.4, 2% Triton X100, 100 mmol/L NaCl) and incubated on ice for 30 min, then centrifuged as above. Pellets were dissolved in Buffer III (50 mmol/L Tris-HCl pH 7.4, 0.25% n-Dodecyl-D-maltoside, 100 mmol/L NaCl, 2 mmol/L MgCl<sub>2</sub>) and incubated with 2U of Benzonase (Sigma) per 100  $\mu$ L of lysate for 30 min at room temperature. After centrifugation the resulting supernatant was used as the nuclear fraction.

### Immunoblotting

Proteins were transferred to Immobilon FL (Millipore) membrane by semi-dry transfer (Bio-Rad, Hercules, CA) and membranes were blocked in Pierce Protein-free T20 blocking buffer (Thermo, Rockford, IL, United States) for 1 h. Primary antibodies for anti- $\beta$ -catenin<sup>S522</sup> ( $\beta$ -cat, provided by Linheng Li, Stowers), pAkt<sup>S473</sup> (Cell Signaling, Danvers, MA, United States), and  $\beta$ -actin (Sigma) were diluted 1:1000 and incubated overnight at 4 °C. HRP-conjugated secondary antibodies (KPL, Gaithersburg, MD) were used at 0.02  $\mu$ g/mL and blots were developed using West Pico ECL reagent (Thermo, Rockford, IL, United States).

### TOPFlash assay

NCM460 cells (normal derived colon mucosa) were received by a cell licensing agreement with INCELL Corporation (San Antonio, TX, United States), and were propagated in M3:10 medium with addition of the conditioned medium (30%) from previously cultured NCM460 cells. Cells were transfected with a reporter construct containing TCF/luc to evaluate  $\beta$ -catenin transcription<sup>[28]</sup>. Transfected cells were pre-treated for 3 h with 5 mmol/L myo-inositol and overnight with 5 mmol/L myo-inositol or 10 ng/mL of TNF $\alpha$ <sup>[29,30]</sup>. Luciferase was detected with Luciferase Reagent (Promega, Madison, WI, United States).

### Human sample collection

Colon biopsies were obtained from adult patients undergoing diagnostic or surveillance colonoscopy or surgical resection at Northwestern University or the University of Chicago. For the NCI myo-inositol clinical trial, all patients were diagnosed as having colitis-induced low grade dysplasia (LGD). Informed consent was obtained from every patient and samples were coded. Collection of all material was approved by Northwestern University IRB and sample analysis was approved by the University of Kentucky IRB. Some biopsies were collected under the NCI Myo-Inositol Chemoprevention in Colitis-Associated Dysplasia trial, #NWU09-13-02.

### NCI clinical trial

Three patients with LGD completed the 90-d clinical trial; 1 was given placebo and 2 were given myo-inositol. Colon biopsies were collected at the start of the

study and again 90 d later. Participants were provided a 90 d supply of either maltodextrin (placebo) or myo-inositol in 9 g packets. Myo-inositol was manufactured by Tsuno Rice Fine Chemicals Co., Ltd (Wakayama, Japan) and filled into pouches by PharmOps, Inc (Phillipsburg, NJ, United States). Myo-inositol and the matching placebo were provided by NCI, DCP. For the first 14 d, participants dissolved one 9 g single-dose packet of study agent in 8 ounces of juice once daily. Following this initial period, participants escalated to 18 g/d taken in divided dose - one 9 g packet in the morning and one 9 g packet in the evening for the remaining 76 d. Patients were instructed to dose at approximately the same time each day, 2 h prior to eating. Pre- and post-study biopsies were assessed for general histological findings, IHC of p53 and Ki67, and patterns of nuclear  $\beta$ -cat.

### Digital analysis

Slides were scanned using an Aperio ScanScope XT<sup>TM</sup> slide scanner and visualized using ImageScope v11. A modified nuclear quantitation algorithm was used to identify DAB-positive and DAB-negative epithelial nuclei. Slides were assessed for  $\beta$ -cat staining using the following three criteria; (1) the number of crypts containing 2, 3, 4 or 5  $\beta$ -cat-positive cells; (2) the number of high powered fields of view containing 3 or more crypts, each containing 2 or more  $\beta$ -cat-positive cells (clustering or "high frequency" fields of view); and (3) the number of  $\beta$ -cat-positive cells per 100 IEC. For analysis of proliferation, numbers of Ki67-positive cells per 100 IEC were counted in high frequency fields of view as well as adjacent tissue.

### Statistical analysis

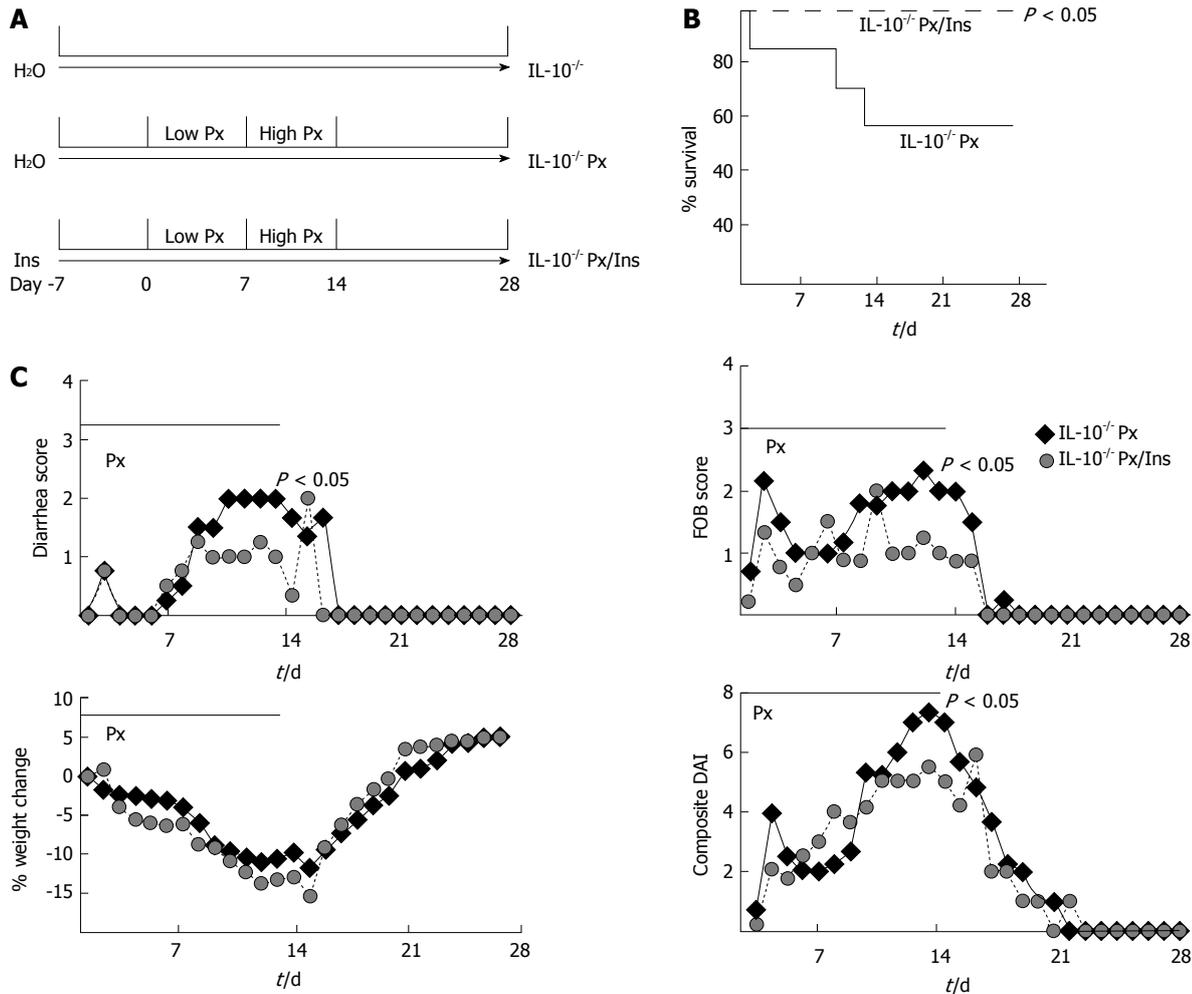
All pairwise comparisons were made using a Student's *t*-test; *P* < 0.05 was considered statistically significant. Comparisons among multiple groups were made using a one-way ANOVA with post-hoc Tukey's test.

## RESULTS

### Treatment with myo-inositol reduces $\beta$ -cat levels in murine colitis

Results from prior work indicate that myo-inositol inhibits PI3K and ERK signaling as evidenced by reduced p-Akt and p-ERK staining in dysplastic tissues<sup>[23]</sup>. Given our previous data linking PI3K signaling to  $\beta$ -catenin activation we considered the possibility that myo-inositol limits ISC activation in colitis<sup>[31]</sup>. The effects of myo-inositol on  $\beta$ -catenin activation were initially examined in the IL10<sup>-/-</sup> and DSS colitis models, which are known to progress from inflammation to CAC.

Initially, to establish the relationship between dietary intake and serum levels, myo-inositol levels was measured in untreated IL10<sup>-/-</sup> mice given 1% w/v myo-inositol in their drinking water. Baseline myo-inositol concentrations in IL10<sup>-/-</sup> mice were 2.0  $\pm$  0.2  $\mu$ mol/L,



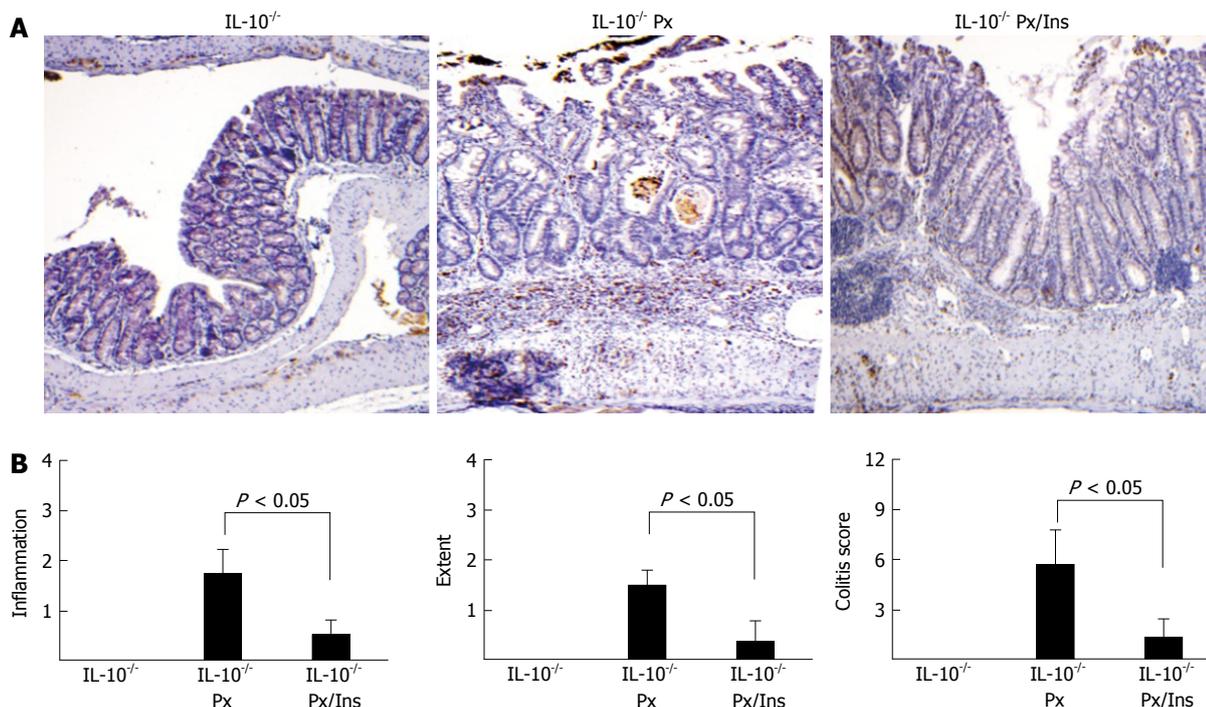
**Figure 1** Myo-inositol reduces mortality and disease activity indices in IL-10<sup>-/-</sup> Px mice. A: Schematic diagram of Px and myo-inositol treatment in IL-10<sup>-/-</sup> mice; B: A Kaplan-Meier survival curve shows decreased mortality in IL-10<sup>-/-</sup> Px/myo-inositol mice; C: Disease activity indices (DAIs) for IL-10<sup>-/-</sup> Px and IL-10<sup>-/-</sup> Px/Ins mice. For diarrhea and fecal occult blood (FOB), scores are based on a scale from 0-4, with 0 being normal and 4 being the most severe (Methods). Percent weight change was calculated as weight change relative to the baseline weight at day 1. The bar denotes Px treatment. Data points represent the mean,  $n = 4$  mice in each group.  $P < 0.05$  on day 14.

while provision of 1% myo-inositol in the drinking water raised serum myo-inositol concentrations 2.4-fold, to  $4.9 \pm 0.5 \mu\text{mol/L}$ ,  $P = 0.02$ . Although our experiments were conducted with 1% myo-inositol, we tested the effect of 2% myo-inositol as well, and serum levels increased to  $14.6 \pm 1.1 \mu\text{mol/L}$  ( $P = 0.01$  relative to 1% myo-inositol). These data document the correlation between dietary and serum levels of myo-inositol as used in these studies.

Piroxicam (Px) was used to induce colitis in IL-10<sup>-/-</sup> mice, and a cohort of these mice was treated with 1% myo-inositol in the drinking water (Figure 1A). While no IL-10<sup>-/-</sup> or IL-10<sup>-/-</sup> Px/myo-inositol mice died during the study, 42% of the IL-10<sup>-/-</sup> Px mice died during the second week of Px treatment (Figure 1B). Myo-inositol significantly reduced mean diarrhea scores in IL-10<sup>-/-</sup> Px mice from  $2.3 \pm 0.6$  to  $0.8 \pm 0.3$  ( $P = 0.02$ ), and occult fecal blood scores from  $2.0 \pm 0.4$  to  $0.9 \pm 0.3$  ( $P = 0.02$ ) (Figure 1C). There was no significant change in body weight between myo-inositol-treated and untreated mice, though myo-inositol may have

contributed to increased caloric intake. Composite DAI scores were reduced by myo-inositol treatment (Figure 1C). Given that myo-inositol has anti-inflammatory effects<sup>[32]</sup>, we next examined tissue histology in IL-10<sup>-/-</sup> Px and IL-10<sup>-/-</sup> Px/myo-inositol mice.

Tissue analysis revealed that myo-inositol significantly reduced inflammation in colonic tissue from IL-10<sup>-/-</sup> Px mice (Figure 2A and B). Images show that myo-inositol normalizes crypt distortion, transmural inflammation, and ulceration induced by Px in IL-10<sup>-/-</sup> mice. These changes correlate with increased myeloperoxidase (MPO) staining in IL-10<sup>-/-</sup> Px mice and greatly reduced MPO staining in IL-10<sup>-/-</sup> Px/myo-inositol mice (Figure 2A). This reduction was particularly evident near the junction of the proximal and distal colon, where IL-10<sup>-/-</sup> Px mice develop large lymphoid aggregates, ulcerated thickened mucosa, and crypt distortion. Histological grading of colitis (Methods) revealed that myo-inositol reduces inflammation scores, the extent of colitis, and composite colitis scores (Figure 2B). Inflammation scores were reduced



**Figure 2** Myo-inositol reduces inflammation in IL-10<sup>-/-</sup> Px mice. A: Representative MPO staining of the proximal-distal junction in IL-10<sup>-/-</sup> control, IL-10<sup>-/-</sup> Px and IL-10<sup>-/-</sup> Px/myo-inositol colon at day 28; B: Histological grading of colitis shows that myo-inositol reduces the severity of inflammation, the extent (thickness) of inflammation, and the combined colitis score. Untreated IL-10<sup>-/-</sup> mice did not exhibit inflammation at day 28. Values represent the mean  $\pm$  SE,  $n = 4$  mice in each group.

in myo-inositol-treated IL-10<sup>-/-</sup> Px mice from  $3.3 \pm 1.9$  to  $1.0 \pm 0.7$  ( $P < 0.05$ ) and myo-inositol completely abrogated crypt damage. Treatment with myo-inositol reduced the extent of colitis in IL-10<sup>-/-</sup> Px mice from  $2.5 \pm 1.2$  to  $0.5 \pm 0.5$  ( $P < 0.05$ ).

$\beta$ -cat staining can be used as a marker of activated stem and progenitor cells<sup>[25]</sup>. Thus, we examined the effect of myo-inositol on  $\beta$ -cat and pAkt levels in the IL-10<sup>-/-</sup> mice (Figure 3A and B). Results show that myo-inositol abrogates colitis-induced  $\beta$ -cat induction. Cell counting revealed that the number of  $\beta$ -cat-positive nuclei dropped by 63% in myo-inositol-treated IL-10<sup>-/-</sup> Px mice from  $0.3 \pm 0.02$  to  $1.9 \pm 0.02$  ( $P = 0.004$ ). Analysis of the crypt distribution of  $\beta$ -cat demonstrated that in IL-10<sup>-/-</sup> Px mice there were significantly more crypts with  $> 2$   $\beta$ -cat positive nuclei in mice with active colitis. By comparison, myo-inositol treatment normalized  $\beta$ -cat levels in IL-10<sup>-/-</sup>/Px mice (Figure 3B). Similar effects of myo-inositol were observed with pAkt staining, in which myo-inositol reduced pAkt nuclear staining from  $6.4 \pm 2.0$  to  $2.3 \pm 0.7$  ( $P < 0.05$ ) percent. Together these data suggest that myo-inositol impairs PI3K and  $\beta$ -catenin activation in colitis.

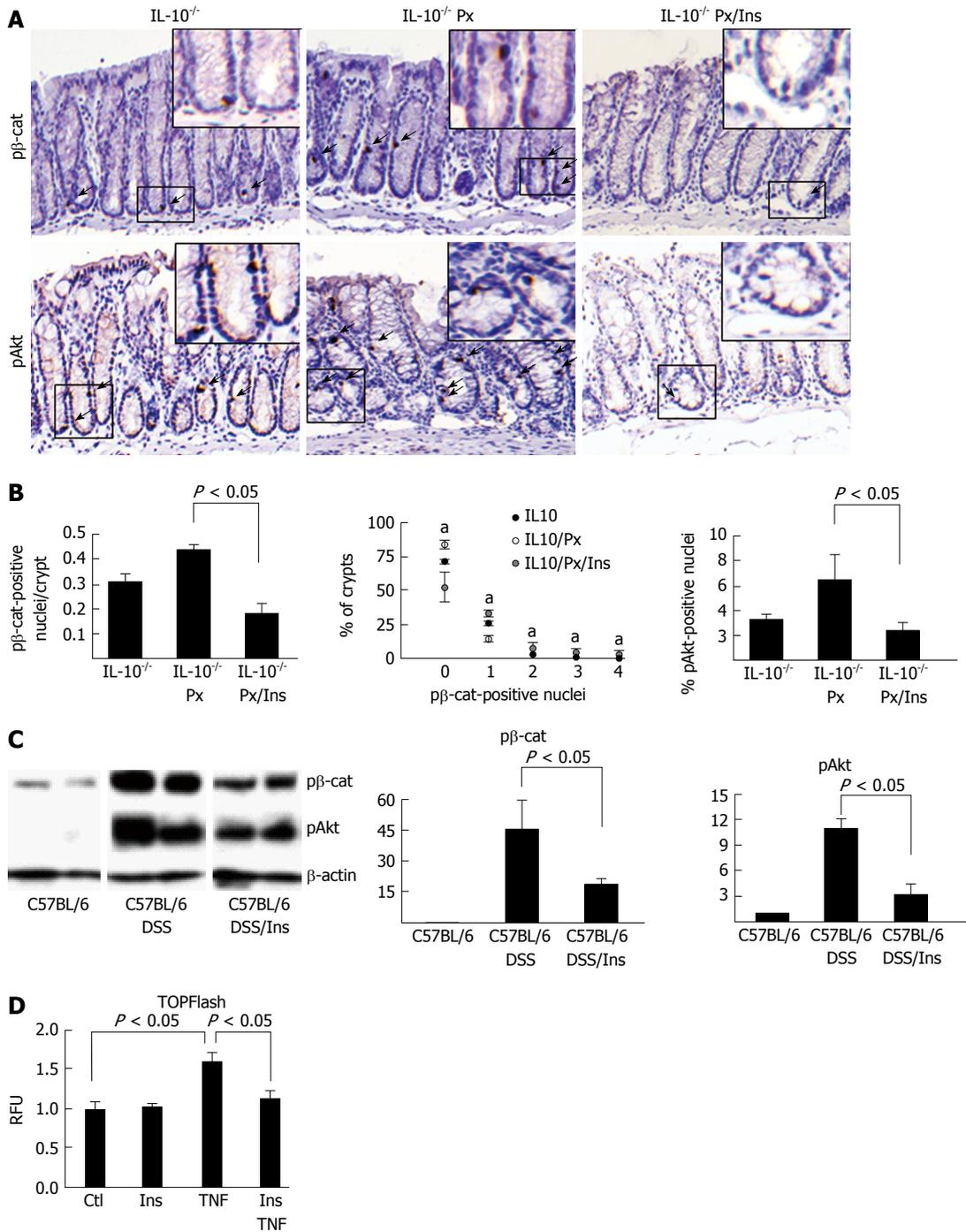
Previously we (GY) found that myo-inositol feeding reduced the incidence of cancer in a model of chronic DSS colitis<sup>[32]</sup>. To determine whether myo-inositol reduced levels of  $\beta$ -cat in these mice, we assessed protein levels of  $\beta$ -cat in isolated IEC from controls and mice treated with eight cycles of DSS with or without myo-inositol (Figure 3C). Levels of pAkt

were assessed to determine if myo-inositol reduces this potential activator of  $\beta$ -catenin signaling. WB data show that myo-inositol reduced  $\beta$ -cat levels by 59%, from  $45 \pm 14$ -fold to  $18 \pm 4$ -fold, relative to DSS-treated controls ( $P = 0.04$ ). Nuclear pAkt was reduced by 73%, from  $11 \pm 1$ -fold to  $3 \pm 1$ -fold ( $P = 0.006$ ). Together, data from two colitis models indicate that oral myo-inositol reduces both  $\beta$ -cat and PI3K signaling in colitis.

To determine if myo-inositol can reduce inflammation-induced  $\beta$ -catenin activation, we used the *in vitro* TOPFlash assay to assess  $\beta$ -catenin activity in response to treatment with TNF and myo-inositol. Myo-inositol treatment alone had no discernible effect on baseline  $\beta$ -catenin activation. NCM cells treated with 10 ng/mL TNF showed a 60% increase in  $\beta$ -catenin activation ( $1.00 \pm 0.08$  vs  $1.6 \pm 0.12$ ,  $P < 0.05$ ), which was abrogated by co-treatment with 5 mmol/L myo-inositol ( $1.6 \pm 0.12$  vs  $1.13 \pm 0.09$ ,  $P < 0.05$ ; Figure 3D). These findings provide evidence that myo-inositol directly impacts Wnt/ $\beta$ -cat signaling.

#### **Effect of myo-inositol on ISC activation in patients with a history of LGD**

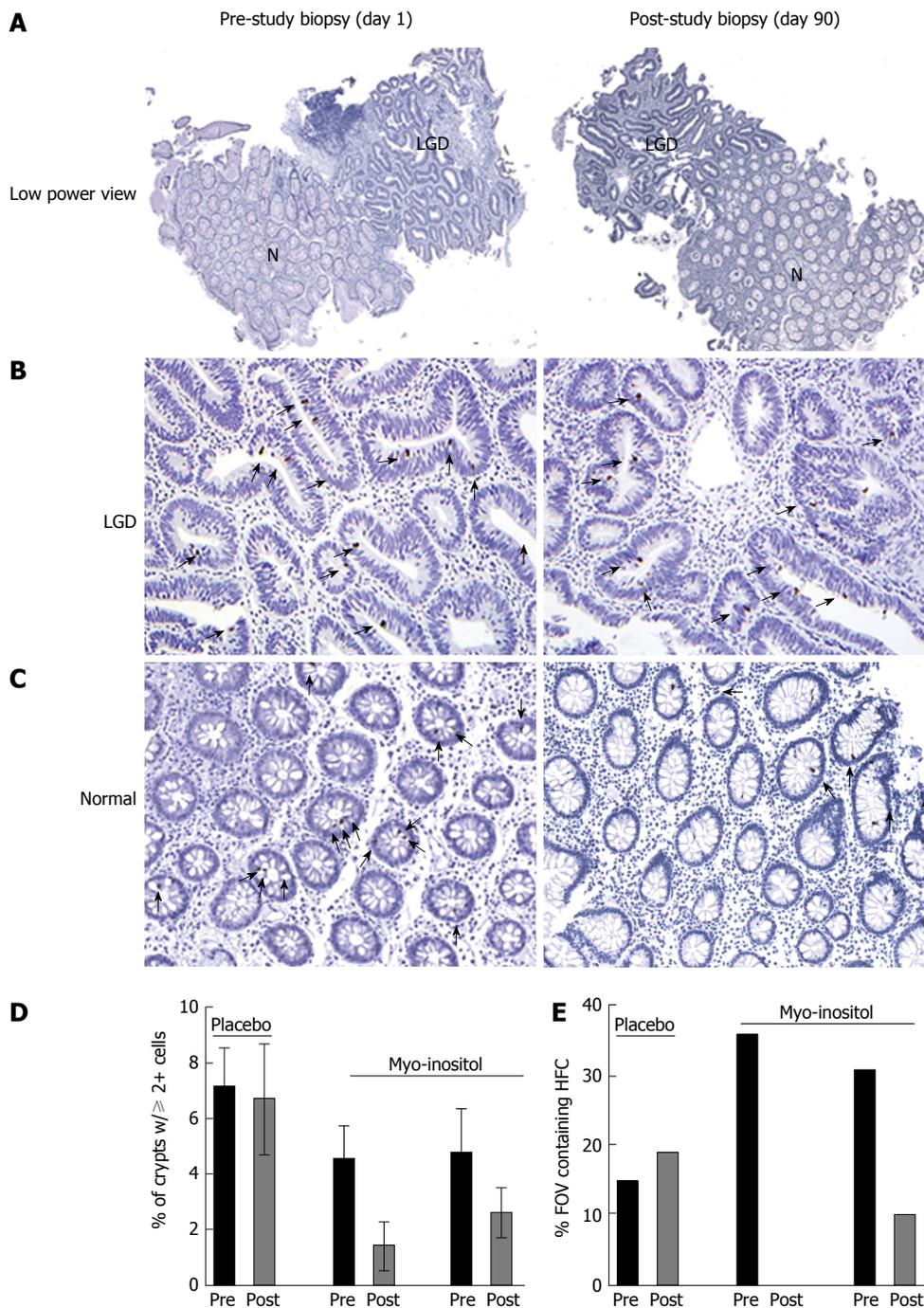
Several studies, in both human and mice, have shown the ability of myo-inositol to reduce dysplasia<sup>[23,32]</sup>. Given the chemopreventive effects of myo-inositol on CAC in mice<sup>[32]</sup> and the inhibition of  $\beta$ -catenin and PI3K signaling reported here, we postulated that myo-inositol may also reduce ISC activation and CAC in patients with longstanding colitis and a history



**Figure 3**  $\beta$ -cat and pAkt are reduced in colitis mice treated with myo-inositol. A: Representative images of p $\beta$ -cat and pAkt staining; B: Quantification of the number of p $\beta$ -cat- and pAkt-positive nuclei. Distribution of the percent of crypts containing 0-5 p $\beta$ -cat-positive nuclei. Values represent the mean  $\pm$  SE,  $n = 4$  mice in each group.  $^{\ast}P < 0.05$ ; C: Western blots of IEC showing levels of nuclear p $\beta$ -cat and pAkt in the 8 cycle model of DSS-induced colitis. Lanes were rearranged to be consistent with formatting. Densitometry was normalized to  $\beta$ -actin and presented as the fold change (mean  $\pm$  SE).  $n = 2$  control mice, 3 DSS-treated mice, and 3 DSS/myo-inositol-treated mice; D: TOPFlash reporter assay (shown in relative fluorescence units, RFU) for  $\beta$ -catenin activation in NCM460 cells treated with TNF or myo-inositol.  $n = 4$  separate experiments.

of recurrent dysplasia. To examine whether myo-inositol can be chemopreventive in CAC, we proposed a 3 mo trial in which myo-inositol was given to UC patients with LGD. We proposed that the frequency of p $\beta$ -cat-positive IEC would decrease in response to dietary myo-inositol treatment. Due to the challenges of finding colitis patients with current LGD at the time of consent, we were able to enter only five

patients into the trial. One patient dropped out due to adverse events (diarrhea, abdominal pain, flatulence, hemorrhoids and increased serum bilirubin), though it is not clear that these were study-related. One patient had the area of LGD removed with the initial biopsy, precluding further analysis at the completion of the study. Three patients completed the study; 1 treated with placebo and 2 treated with myo-inositol. Statistical

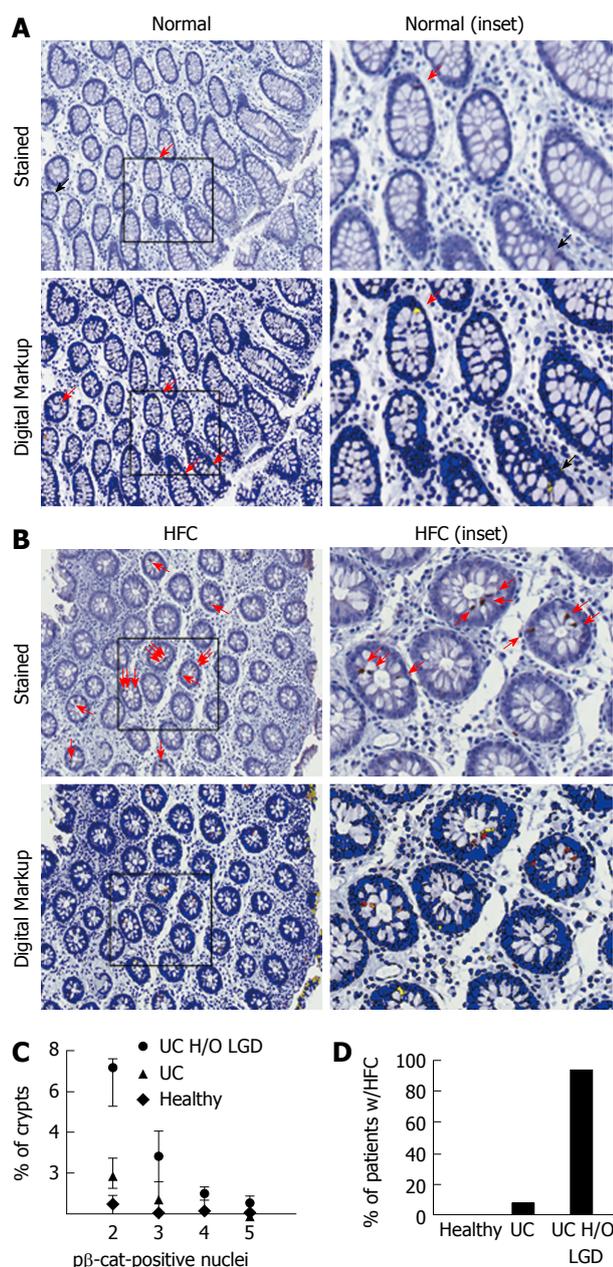


**Figure 4** Myo-inositol reduces the number of  $p\beta$ -cat-positive cells in ulcerative colitis patients. **A**: Representative low power images of biopsies stained for  $p\beta$ -cat from a patient before and after 90 d of myo-inositol treatment. **B**:  $p\beta$ -cat staining in an area of LGD; **C**:  $p\beta$ -cat staining in a non-dysplastic area adjacent to LGD. Note the HFC containing multiple  $p\beta$ -cat-positive nuclei; **D**: Graphic representation of the percent of crypts from each patient pre- and post-treatment containing 2 or more  $p\beta$ -cat-positive nuclei; **E**: The percent of all high powered fields of view (FOV) containing HFC in biopsies from each patient. N: Normal tissue; LGD: Low grade dysplasia.

analysis of this small study size is not possible. However, the results of the staining were used to identify a trend of myo-inositol-induced reductions in the number of crypts with high numbers of  $p\beta$ -cat-positive intestinal epithelial cells.

Data from the myo-inositol-treated patients who completed the study did not show reductions in established dysplasia (as in Figure 4A and B). However, in myo-inositol-treated patients there was a global

reduction in the percentage of crypts containing two or more  $p\beta$ -cat-positive cells in areas adjacent to LGD, which was not observed in the placebo-treated patient (Figure 4C). While analyzing the data from the 3 patients in the study, we observed “clusters” of crypts containing 2 or more  $p\beta$ -cat-positive cells in non-dysplastic areas where  $p\beta$ -cat staining was otherwise very sparse (Figure 4C). This aggregation of crypts with higher numbers of  $p\beta$ -cat-positive cells is suggestive



**Figure 5** Crypts with elevated p $\beta$ -cat staining identify ulcerative colitis patients at a high risk for low grade dysplasia. **A**: Representative images of p $\beta$ -cat staining and the digital markup in colon biopsies of UC patients with normal p $\beta$ -cat distribution (benign disease); and **B**: HFC. Red arrows indicate p $\beta$ -cat-positive nuclei identified by eye; black arrows indicate false positive or negative nuclei properly identified by digital analysis; **C**: Statistical distribution of p $\beta$ -cat-positive nuclei in normal biopsies showing that crypts with 2 more positive p $\beta$ -cat-positive nuclei are rarely detected in healthy patients (diamonds,  $n = 3$ ) or patients with benign, active UC (triangles,  $n = 12$ ) but are more frequent in UC patients with a previous history of LGD (circles,  $n = 16$ ). Data are shown as the percent of crypt cross-sections containing p $\beta$ -cat-positive nuclei; **D**: Biopsies from healthy controls, benign UC, and UC patients with a history of LGD were analyzed for the presence of HFC. UC: Ulcerative colitis; LGD: Low grade dysplasia; HFC: High frequency crypts.

of a field effect, in which foci of activated ISC are produced by expansion of mutated cells<sup>[14]</sup>. In order to quantify this effect, we counted the percentage of high power fields of view (20X) in which there were at least 3 crypts containing 2 or more p $\beta$ -cat-positive cells,

henceforth defined as high frequency crypts (HFC). In the placebo-treated patient, the percent of HFC at day 1 and day 90 was 15% and 19%, respectively. However, in the patients treated with myo-inositol, the percentages of HFC at day 1 were 36% and 31%, which were reduced to 0% and 10%, respectively, by day 90 (Figure 4D). Although these findings are far from conclusive, they support the notion that myo-inositol reduces the frequency of activated stem cells in patients at high risk for CAC.

#### Objective assessment of p $\beta$ -cat nuclear accumulation in colitis

We previously observed that numbers of p $\beta$ -cat-positive IEC are increased in patients within areas of colitis-induced LGD and cancer, and inflammation<sup>[25]</sup>. However, the observation of p $\beta$ -cat-positive cell clustering within non-dysplastic tissue adjacent to areas of colitis-induced dysplasia led us to speculate that patients with a history of recurrent LGD could be stratified based on the presence of HFC. To test this hypothesis, a digital algorithm was developed to objectively distinguish p $\beta$ -cat-positive and negative nuclei in human biopsies. This allowed us to more accurately compare staining of tissues that were processed and stained by a variety of institutions and laboratory personnel.

Digital analysis of sections improved the quality of data generated by discriminating true positive and true negative cells (Figure 5A and B). In some cases, digital imaging identified positive cells that were not detected by trained observers (Figure 5A, black arrow). In other sections, the computer algorithm correctly discriminated false positives. The ability to accurately identify positive and negative nuclei significantly increased the sensitivity of p $\beta$ -cat staining.

To test the hypothesis that HFC could be used to stratify patients with a history of LGD, biopsies from healthy control patients ( $n = 3$ ), patients with a diagnosis of active UC ( $n = 12$ ), and UC patients with a history of LGD ( $n = 16$ ) were stained for p $\beta$ -cat. In patients with a history of LGD, biopsies were taken from histologically normal areas of prior LGD, usually near mucosal tattoos marking resected LGD. In these tissues, we assessed the number of p $\beta$ -cat-positive cells within individual crypts and portrayed the relative distribution of crypts with distinct numbers of p $\beta$ -cat-positive cells/crypt (e.g., percentage of total crypts with 2,3,4 or 5 p $\beta$ -cat-positive cells/crypt) (Figure 5C). In order to stratify patients, histologically normal tissues from areas of previous dysplasia were evaluated for HFC (Figure 5D). When we calculated the percentage of patients that had at least one HFC, 8.3% (1 out of 12) patients with benign, active UC had HFC whereas 94% of patients with a history of LGD had at least one HFC (15 out of 16). These findings suggest that focal increases in p $\beta$ -cat staining identify patients at risk for dysplasia. If further validated in more extensive clinical trials, this biomarker may provide a

useful means for testing chemopreventive agents such as myo-inositol.

## DISCUSSION

The data presented in this study suggest that myo-inositol is a valuable chemopreventive agent that abrogates aberrant activation of ISC during inflammation and reduces the transition to dysplasia. Additionally, these data demonstrate that  $\beta$ -cat staining can be used to identify HFC within "pre-dysplastic" regions in patients with a previous history of colitis-induced dysplasia. A central hypothesis addressed in these studies was that myo-inositol reduces intestinal stem cell activation, detected by reduced nuclear  $\beta$ -cat and pAkt staining. Previous studies indicate that inositol compounds have both anti-inflammatory and chemopreventive properties<sup>[22,23,32-35]</sup>. Data here show that myo-inositol treatment reduces Akt activation in colitis. Additionally, we show that myo-inositol reduces  $\beta$ -catenin signaling, a key step in ISC activation. Prior data from our group demonstrated that Akt cooperates with Wnt to activate  $\beta$ -catenin<sup>[25]</sup>. In the models tested here, it is possible that PI3K inhibition by myo-inositol impaired Wnt signaling *via* reduced  $\beta$ -cat activation.

Because we were interested in early effects of myo-inositol during inflammation-induced neoplastic transformation, we analyzed data from IL-10<sup>-/-</sup> mice 14 d after the induction of colitis, 4 wk before the onset of dysplasia. The data presented here demonstrate that myo-inositol reduced inflammation, Akt activation, and  $\beta$ -cat nuclear localization. Additionally, in experiments with WT mice on 8 cycles of DSS, analysis of proteins from isolated IEC revealed that myo-inositol reduced nuclear  $\beta$ -cat and pAkt. In the same model, we reported that myo-inositol reduced tumor incidence, tumor multiplicity, and tumor volume<sup>[32]</sup>. Based on these results we propose that myo-inositol may reduce CAC by decreasing the numbers of activated ISC that may harbor pro-oncogenic genetic alterations.

5-ASA, a common therapeutic used in the treatment of UC, is a potent oxygen radical scavenger and has anti-proliferative and pro-apoptotic effects. 5-ASA reduces both pAkt and  $\beta$ -cat nuclear localization in human biopsies and in the IL-10<sup>-/-</sup> Px model of CAC<sup>[36,37]</sup>. However, in areas of established dysplasia, 5-ASA did not reduce proliferation or staining for  $\beta$ -cat and pAkt. We propose that like 5-ASA, myo-inositol reduces Akt/ $\beta$ -catenin signaling in the early stages of dysplastic transformation but not once it is established. We posit that 5-ASA and myo-inositol reduce CAC both by reducing inflammation-induced Wnt signaling in activated ISC at risk for acquiring founder mutations.

To address the question of whether a reduction in  $\beta$ -cat staining could be a biomarker of "fields" of pre-dysplastic cells in patients, we analyzed colonic biopsies from UC patients with benign disease or a history of LGD. We detected more regions with a high frequency of  $\beta$ -cat -positive nuclei (HFC) in tissue from patients

with a history of LGD compared to those with benign disease. Using the criterion that tissue sections must have high powered fields of view with three or more crypts containing two or more  $\beta$ -cat-positive IEC, we were able to distinguish sections from UC patients from those who had a history of LGD and those who did not. We hypothesize that these data reflect local expansions of activated ISC harboring mutations that affect Wnt/ $\beta$ -catenin signaling. Inflammation-induced carcinogenic genetic and epigenetic changes may transmit a survival advantage to select ISC populations. Thus, our observations suggest that higher numbers of activated ISC may be a biomarker for areas of tissue at higher risk for neoplastic transformation. What remains unclear is whether myo-inositol primarily affects ISC by reducing inflammation, pro-carcinogenic inflammatory insults and/or by altering critical ISC signaling pathways (*e.g.*, PI3K, Wnt/ $\beta$ -catenin) relevant to carcinogenesis. In unpublished studies, we found that enhanced nuclear staining of  $\beta$ -cat correlates highly with increased TCF4/LEF immunoprecipitation and increased expression of downstream TCF4 target genes. We propose that reductions of  $\beta$ -catenin activity in stem cells by myo-inositol are an essential mechanism for its chemopreventive properties. Because dysplasia typically arises from expansion of clonal stem cells that harbor mutations in key oncogenic processes<sup>[9,38,39]</sup>, reducing the numbers of activated stem cells in colitis would presumably lower the risk for generating neoplastic crypts. Additionally, myo-inositol is a potent anti-oxidant, and as such it may also reduce DNA damage caused by reactive oxygen species. We hypothesize that myo-inositol may exert chemopreventive properties through both of these mechanisms.

In order to test the hypothesis that myo-inositol can be used as a chemopreventive in CAC, we propose a revised clinical trial. In this trial, UC patients would be recruited who have history of recurrent LGD and evidence of elevated  $\beta$ -cat staining in areas adjacent to prior dysplasia. Myo-inositol would be given long-term (1 year or more) and the primary endpoints would include (1) a reduction in the recurrence of LGD; and (2) normalized or reduced  $\beta$ -cat staining in areas proximal to LGD. The primary goal would not be the reversal of LGD, but rather the prevention of stem cell activation in "high risk" areas of pre-dysplastic fields resulting in reduced emergence of new LGD. The goal would be to abort the progression to recurrent dysplasia in patients who have a history of recurrent disease. The clear benefit would be the provision of a safe, low cost agent for patients who are found to have recurrent LGD during surveillance colonoscopy for colitis.

## COMMENTS

### Background

Patients with inflammatory bowel disease (IBD) have an increased risk

of dysplasia and colon cancer due to chronic inflammation. Developing a biomarker and identifying areas of the colon that harbor the potential for neoplastic transformation prior to the onset of histologically-identifiable dysplasia is critical for early treatment and cancer prevention. Additionally, there is a need for inexpensive, accessible pharmaceuticals to prevent recurrent dysplasia and the progression to cancer in IBD patients.

### Research frontiers

Colitis-associated cancer (CAC) is a significant concern for patients with chronic inflammatory diseases, such as IBD. Although largely preventable by stringent surveillance colonoscopy and endoscopic or surgical removal of dysplastic tissue, many patients with limited access to health care resources remain at high risk for developing cancer. Myo-inositol is a safe, affordable, accessible sugar alcohol with limited side-effects and an established history in many clinical trials. We posit that myo-inositol is an attractive treatment option for patients at high risk for recurrent colonic dysplasia.

### Innovations and breakthroughs

This study demonstrated that nuclear staining patterns of Akt-phosphorylated  $\beta$ -catenin ( $p\beta$ -catenin<sup>S552</sup>) positively correlate with levels of inflammation and proliferation in both mouse and human models of intestinal inflammation and IBD. Additionally, nuclear staining patterns of  $p\beta$ -catenin<sup>S552</sup> can be used to stratify patients with benign colitis and those with a history of dysplasia. In mouse models of colitis, oral myo-inositol significantly reduced inflammation and numbers of activated stem cells. In a limited clinical trial, myo-inositol treatment did not reduce dysplasia, but successfully altered the pattern of  $p\beta$ -catenin<sup>S552</sup> staining in areas adjacent to dysplasia, suggesting a role for oral myo-inositol in limiting stem cell activation during colitis.

### Applications

In order to further test the hypothesis that myo-inositol can be used as a chemopreventive in CAC, the authors propose a revised clinical trial. In this trial, ulcerative colitis patients would be recruited who have history of recurrent low grade dysplasia (LGD) and evidence of elevated  $p\beta$ -cat staining in areas adjacent to prior dysplasia. Myo-inositol would be given long-term and primary endpoints would include a reduction in the recurrence of LGD and normalization of  $p\beta$ -cat staining in areas proximal to LGD. The primary goal would be the prevention of stem cell activation in "high risk" areas of pre-dysplastic fields resulting in reduced emergence of new LGD. The clear benefit would be the provision of a safe, low cost agent for patients who are found to have recurrent LGD during surveillance colonoscopy for colitis.

### Terminology

LGD: Mild architectural abnormalities characterized by basally-oriented nuclei, mild nuclear enlargement, and nuclear crowding.

### Peer-review

This paper describe some important things about:  $p\beta$ -catenin<sup>S552</sup> staining faithfully reported the effects of myo-inositol in reducing inflammation and intestinal stem cell activation in mice.

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P- Reviewer: Luo HS S- Editor: Ma YJ L- Editor: A  
E- Editor: Zhang FF





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ISSN 1007-9327

