Activation of intestinal human pregnane X receptor protects against azoxymethane/dextran sulfate sodium-induced colon cancer

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ABBREVIATIONS: AOM, azoxymethane; BrdU, bromodeoxyuridine; COX, cyclooxygenase; CDK, cyclin-dependent kinase; CRC, colorectal cancer; DSS, dextran sulfate sodium; FITC, fluorescein isothiocyanate; IBS, irritable bowel syndrome; IKKβ, inhibitor of kB kinase beta; IL, interleukin; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI, propidium iodide; PXR, pregnane X receptor; qPCR, quantitative polymerase chain reaction; RXR, retinoid X receptor; TNFα, tumor necrosis factor α.

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ABSTRACT

The role of intestinal human pregnane X receptor (PXR) in colon cancer was determined through investigation of the chemopreventive role of rifaximin, a specific agonist of intestinal human PXR, towards azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colon cancer. Rifaximin treatment significantly decreased the numbers of colon tumors induced by AOM/DSS treatment in *PXR*-humanized mice but not wild-type and *Pxr*-null mice. Additionally, rifaximin treatment markedly increased the survival rate of *PXR*-humanized mice but not wild-type and *Pxr*-null mice. These data indicated a human PXR-dependent therapeutic chemoprevention of rifaximin towards AOM/DSS-induced colon cancer. NF-κB-mediated inflammatory signaling was up-regulated in AOM/DSS-treated mice, and inhibited by rifaximin in *PXR*-humanized mice. Cell proliferation and apoptosis were also modulated by rifaximin treatment in the AOM/DSS model. *In vitro* cell-based assays further revealed that rifaximin regulated cell apoptosis and cell cycle in a human PXR-dependent manner. These results suggested that specific activation of intestinal human PXR exhibited a chemopreventive role towards AOM/DSS-induced colon cancer through mediating anti-inflammation, anti-proliferation, and pro-apoptotic events.

Introduction

Colorectal cancer (CRC) is one of the most common forms of fatal cancer in the world, however, the underlying molecular pathogenesis and effective strategies for prevention and treatment of spontaneous CRC are not fully understood (le Clercq and Sanduleanu, 2014). Chronic inflammation is a known risk factor for carcinogenesis, and accumulated data indicate that up to 15% of human cancer incidence is associated with inflammation (Drexler and Yazdi, 2013). Inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, dramatically increase the risk of colorectal cancer. Pro-inflammatory pathways and mediators including eicosanoids catalyzed by cyclooxygenases (COXs) or 5-lipoxygenase and cytokines/chemokines promote tumorigenesis. Anti-inflammatory strategies such as inhibition of COXs by non-steroidal anti-inflammatory drugs are among the most promising approaches for the chemoprevention of colon cancer (Stolfi et al., 2013). In addition, the importance of inflammation is further highlighted by the dependence of tumor type and progression on activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Mladenova et al., 2013). The NF-κB pathway activation observed during inflammatory bowel diseases may contribute to tumor formation by providing anti-apoptotic survival signals to the colonic epithelial cells (Onizawa et al., 2009). Indeed, in mice harboring a mutation in intestinal inhibitor of κB kinase β (IKKβ) that leads to suppression of the NF-κB pathway, colon tumorigenesis was significantly attenuated (Greten et al., 2004).

Rifaximin (Xifaxan®), a non-synthetic antibiotic that has low gastrointestinal absorption while retaining antibacterial activity within the intestine, was approved in 2004 for therapy of traveler’s diarrhea and in 2010 for therapy of hepatic encephalopathy

due to its antibiotic-based inhibition of ammonia-producing enteric bacteria that reduces circulating gut-derived ammonia in patients with cirrhosis (Mullen et al., 2013). In addition, numerous clinical trials revealed that rifaximin has efficacy toward irritable bowel syndrome (IBS), presumably as a result of alteration of intestinal microbiota (Xu et al., 2014). Thus, rifaximin has therapeutic applications beyond its antibiotic activity and is especially attractive due to its minimal systemic absorption and safety profile. A previous study indicated that rifaximin is the gut-specific agonist of human pregnane X receptor (PXR, NR1I2) (Ma et al., 2007) and is also a potential drug candidate for acute colitis through its anti-inflammatory activity, due in part to inhibition of the NF-κB signaling cascade through specific activation of intestinal PXR (Cheng et al., 2010). Therefore, the chemopreventive role of rifaximin towards colitis-associated colon cancer was examined in the present study.

The experimental colitis-mediated mouse colon cancer model was created through the injection of azoxymethane (AOM), a mutagenic agent, and dextran sodium sulfate (DSS), a pro-inflammatory chemical (Kohno et al., 2005). This model was used in the present study to determine the role of specific activation of intestinal human PXR in the suppression of AOM/DSS-induced colon cancer. The therapeutic role of rifaximin towards AOM/DSS-induced colon cancer in *PXR*-humanized, wild-type, and *Pxr*-null mice was evaluated. Furthermore, the effect of rifaximin on pro-inflammatory cytokines and apoptosis during development of colitis and colon carcinogenesis was also investigated. The results suggested that specific activation of intestinal PXR by rifaximin might exert chemopreventive role on AOM/DSS-induced colon cancer through inhibition of the NF-κB pathway and induction of cellular apoptosis.

Materials and Methods

Animals and Chemicals. Male *PXR*-humanized, wild-type, and *Pxr*-null mice were housed in temperature- and light-controlled rooms and were given water and pelleted chow *ad libitum*. All animal experiments were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee. Rifaximin (Xifaxan®**)** was provided by Salix Pharmaceuticals, Inc. (Morrisville, NC.). AOM was purchased from Sigma and DSS (Mr 36,000 - 50,000 Da) was from MP Biochemicals (Solon, OH).

AOM/DSS-induced Colon Cancer Model and Rifaximin Treatment. Before starting the experiments, mice were acclimatized for 1 week. Two- to three-month-old *PXR*-humanized, wild-type, and *Pxr*-null male mice were divided into control group, AOM/DSS group, and AOM/DSS+rifaximin group (n=20 for each group). To induce colon cancer, *PXR*-humanized, wild-type, and *Pxr*-null mice were given an intraperitoneal injection with 10 mg/kg AOM in 0.1 mL of phosphate-buffered saline (PBS) followed by three cycles of 2% DSS in drinking water for 1 week and normal drinking water for 2 weeks. The AOM/DSS+rifaximin group was administered rifaximin in the 10 mg/kg rifaximin-containing AIN93G diet (Dyets Inc., Bethlehem, PA), while mice in the AOM/DSS group and control group received the AIN93G diet without rifaximin. This dose of rifaximin is equivalent to oral administration of 1 mg/kg/day for each mouse. All mice in the AOM/DSS+rifaximin group received rifaximin-containing diet for 1 week before the AOM/DSS treatment until the end of the experiment. Body weights, food and water consumption were monitored once per week throughout the

experiment. Clinical assessment of all DSS/AOM-treated animals for body weight, stool consistency, rectal bleeding and general appearance was performed weekly. At day 180 of the experiment, all mice were killed, and the whole colorectal tissues collected for biochemical and pathological analyses.

Macroscopic and Histopathological Evaluation Analysis. After killing the mice, the colons were excised from the ileocecal junction to the anal verge and flushed with PBS. Blood was collected for the preparation of serum. Colons were weighed and colon length (from the colocecal junction to the rectum) was measured for assessment of morphologic changes. The colon was opened longitudinally. Gross examination was performed to measure the pattern of tumor development, including quantity, size, and the location of each tumor within the large bowel. The tumor incidence was defined as number of mice with tumors/total mice in the group. For histological analysis, the colon tissues were fixed in 10% formalin for 24 h, followed by paraffin-embedding. The incidence of tumors with diameter > 4mm was also assessed because most large tumors with diameter > 4mm examined contained carcinoma components.

Short-term Anti-proliferation Effect of Rifaximin. To observe the short-term anti-proliferation role of rifaximin, *PXR*-humanized (n=9), wild-type (n=9), and *Pxr*-null (n=9) mice were given an intraperitoneal injection with 10 mg/kg AOM dissolved in 0.1 mL of PBS followed by one cycle of 2% DSS in drinking water for 1 week. Among them, four mice were used for bromodeoxyuridine (BrdU) staining experiments. Mice were injected with 200 μmol/kg body weight of BrdU. After 1 h, the mice were killed and the colons

taken for BrdU staining as detailed in a previous study (Wang et al., 2008). The BrdU labeling index was calculated using the ratio of BrdU staining-positive cell number versus the total cell number. The colons taken from the other five mice were subjected to quantitative polymerase chain reaction (qPCR) analysis for specific mRNAs.

qPCR Analysis. Total RNA was extracted from colon tissues of each mouse using TRIzol reagent (Invitrogen, Carlsbad, CA) and qPCR performed using cDNA generated from 1 μg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Primers for qPCR were designed using the Primer Express software (Applied Biosystems, Foster City, CA) and sequences are available upon request. qPCR reactions were carried out using SYBR Green PCR master mix (SuperArray, Frederick, MD) by using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Values were quantitated using the comparative cycle threshold method, and results were normalized to mouse β-actin.

Cell Apoptosis Assays by Flow Cytometry. Human colonic epithelial cells HT-29 were grown at 37°C with 5% CO_2 in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA) and 1% penicillin-streptomycin (Invitrogen). HT-29 cells were seeded at density of 5×10^4 cells/well in 24-well plates. Expression vectors for human PXR and retinoid X receptor α (RXRα) constructs were transfected into cells using Fugene transfection reagent (Roche, Indianapolis, IN). Twenty-four hours post-transfection, the cells were incubated with DMSO (vehicle) or rifaximin for an additional 24 h, followed by cell apoptosis analysis as follows. Cells

were treated with fluorescein isothiocyanate (FITC)-conjugated annexin V in conjunction with propidium iodide (PI) that stains necrotic cells (Southern Biotech) to identify apoptotic cells measured by flow cytometry (BD Biosciences). Cells were differentiated in early apoptosis (Annexin V+, PI−) from those in late apoptosis (Annexin V+, PI+) stage. To confirm expression of the human PXR in the transfected HT-29 cells, nuclear extracts of cells 24 hours after transfection were prepared by use of the NE-PER kit (Pierce). Nuclear extracts (20 μg) were loaded on an SDS-polyacrylamide gel and western blotting carried out using an Human PXR Common/NR112 mAb (Clone H4417) (R&D Systems) and Histone H3 (D2B12) XR rabbit mAb (Cell Signaling Technology) diluted 1:1000 in 5% skim milk. Nuclear extracts of liver tissues from *PXR*-humanized and *Pxr*-null mice were also loaded as a positive and negative control, respectively. The human PXR mAb recognizes human PXR1 and 2. The immunogen was amino acids 1-40 of human PXR. Primers used for qPCR are shown in the Supplemental Fig. 5 legend.

Statistics. Experimental values are expressed as mean ± standard deviation (SD). Statistical analysis was performed with two-tailed Student's t tests and a *P* value of <0.05 were considered to be statistically significant.

Results

Rifaximin Prevents AOM/DSS-induced Colon Carcinogenesis in a Human PXR-dependent Manner. *PXR*-humanized mice with AOM/DSS+rifaximin treatment had the highest survival rate among *PXR*-humanized, wild-type and *Pxr*-null mice treated with AOM/DSS+rifaximin (Fig. 1A). However, there were no significant differences in survival rates among the three mouse lines treated with AOM/DSS only (Supplemental Fig. 1). The AOM/DSS-induced colon cancer produced similar total tumor numbers per colon in *PXR*-humanized, wild-type and *Pxr*-null mice fed a control diet (Fig. 1B). However, rifaximin treatment led to reduced tumor numbers only in *PXR*-humanized mice in comparison with wild-type and *Pxr*-null mice (Fig. 1C). Total tumor incidence in the *PXR*-humanized mice treated AOM/DSS+rifaximin was 42% compared to 100% in the AOM/DSS group, and 100% in the wild-type and *Pxr*-null mice treated with AOM/DSS and AOM/DSS+rifaximin (Fig. 2A). Among the tumors found in the AOM/DSS group in the *PXR*-humanized mice, 24% were > 4 mm (Fig. 2B) with histological characteristics of adenocarcinomas (Supplemental Fig. 2). All of the tumors in the *PXR*-humanized mice treated with AOM/DSS+rifaximin were smaller, < 4 mm (Fig. 2C). Wild-type and *Pxr*-null mice treated with AOM/DSS had 10% and 25% > 4 mm tumors, respectively, and when treated with AOM/DSS+rifaximin, had 4% and 18% with > 4 mm tumors, respectively (Fig. 2B). In a second cohort of *PXR*-humanized mice treated with AOM/DSS+rifaximin, there was a 15% tumor incidence compared to 100% in the AOM/DSS-treated *PXR*-humanized group, and 100% for the wild-type and *Pxr*-null mice treated with AOM/DSS with or without rifaximin (Supplemental Fig. 3A). In the *PXR*-humanized mice treated with AOM/DSS, 38% of the tumors were < 4 mm

(Supplemental Fig. 3B) while all of the tumors in the *PXR*-humanized mice treated with AOM/DSS+rifaximin were < 4 mm (Supplemental Fig. 3C). Interestingly, in this cohort, the wild-type mice treated with AOM/DSS+rifaximin had mostly (75%) > 4 mm tumors with AOM/DSS+rifaximin treatment (Supplementary Fig. 3B). The reason for this difference between the first cohort of mice (Fig. 2) is not known. Tumor incidence and % of small and large tumors were similar in both cohorts of the *Pxr*-null mice treated with AOM/DSS with and without rifaximin (Fig. 2 and Supplemental Fig 3). Both cohorts revealed that rifaximin inhibited AOM/DSS-induced tumorigenesis, notably, the production of large > 4 mm tumors.

Rifaximin Inhibits the NF-κB Pathway via Human PXR Activation in Colitis-associated Colorectal Carcinogenesis. To investigate the effects of rifaximin-enriched diet on the expression of mRNAs encoding pro-inflammatory cytokines, qPCR was used to quantify mRNAs in tumor-adjacent tissue of the colon. Expression of tumor necrosis factor alpha (*Tnfa*) and interleukin 1 beta (*Il-1b*) mRNAs significantly increased after AOM/DSS treatment ($P<0.01$); rifaximin treatment suppressed the increased expression induced by AOM/DSS in *PXR*-humanized mice (Fig. 3). No suppression of rifaximin towards the AOM/DSS-induced elevated expression of *Tnfa* and *Il-1b* mRNAs was found in wild-type and *Pxr*-null mice. In *PXR*-humanized mice, compared with AOM/DSS group, expression of inducible nitric oxide synthase (*iNos),* interleukin 6 *(Il-6) and Il-10* mRNAs were significantly reduced ($P<0.05$) in the DSS/AOM+rifaximin group. However, this effect was not observed for wild-type and *Pxr*-null mice (Fig. 3). These results indicated that NF-κB signaling pathway was induced in AOM/DSS-induced colon

cancer model in *PXR*-humanized, wild-type and *Pxr*-null mice, and that NF-κB pathway was suppressed only in *PXR*-humanized mice upon rifaximin treatment. Therefore, inhibition of the NF-κB signaling pathway might contribute to the prevention of AOM/DSS-induced colon cancer in *PXR*-humanized mice via human PXR activation by rifaximin.

Effect of Rifaximin on Cell Proliferation and Apoptosis. To explore the effect of rifaximin treatment on proliferation and apoptosis in inflammation-related colon cancer mouse model, the expression of several target genes were investigated. The results revealed that *p21* mRNA was significantly increased in AOM/DSS+rifaximin group in comparison with the AOM/DSS group in *PXR*-humanized mice while not in wild-type and *Pxr*-null mice (Fig. 4). The expression of mRNAs encoded by genes involved in apoptosis, *Bcl-2* and *Bclx* was significantly decreased and significantly increased, respectively, in the AOM/DSS+rifaximin group when compared with AOM/DSS group in *PXR*-humanized mice, but not in wild-type and *Pxr*-null mice (Fig. 4). The expression of mRNA encoding *C*-myc, an oncoprotein expressed in proliferating and transformed cells, was also examined (Qu et al., 2014). Suppression of *C-myc* mRNA was only observed in of AOM/DSS-treated *PXR*-humanized mice, and not in wild-type and *Pxr*-null mice upon rifaximin treatment, indicating that rifaximin efficiently decreased cell proliferation via human PXR (Fig. 4). Cyclin-dependent kinases (CDKs) are a family of protein kinases having important roles in regulating the cell cycle through binding to regulatory proteins called cyclins (Lim and Kaldis, 2013). The treatment of rifaximin prevented the increased expression of many *Cdk* and *Cyclin* mRNAs induced by

AOM/DSS treatment in *PXR*-humanized mice but not in wild-type and *Pxr*-null mice, including *Cdk2, Cdk4, Cdk6, and Ccnd1, Ccnd2, Ccna2, Ccne1,* and *Ccne2* (Fig. 5). To confirm the inhibitory action of rifaximin towards cell proliferation in *PXR*-humanized mice, BrdU staining was performed. Treatment of *PXR*-humanized mice with rifaximin significantly decreased incorporation of BrdU into colon epithelial cells induced by AOM/DSS treatment when compared with rifaximin-treated wild-type and *Pxr*-null mice (Fig. 6A and B). Short-term treatment with rifaximin can activate human intestinal PXR, but not mouse intestinal PXR (Ma et al., 2007) (Supplemental Fig. 4), and the expression of the corresponding *Cdk* and *Cyclin* mRNAs were also significantly decreased in PXR-humanized mice in comparison with wild-type and *Pxr*-null mice (Fig. 7).

***In vitro* Transfection of Human PXR and Rifaximin Treatment Increased Apoptosis and Decreased Proliferation in HT-29 cells.** To analyze whether rifaximin can modulate apoptosis and proliferation in a human PXR-dependent manner, induction of apoptosis was evaluated in cells transfected with or without human PXR. Expression of human PXR and RXRα from the expression vector plasmids was demonstrated by analysis of human PXR mRNA and protein using qPCR and western blotting, and RXRα mRNA by qPCR (Supplemental Fig. 5). Cells with either RXRα or PXR/RXRα were simultaneously stained with propidium iodide (PI) and Annexin V, and monitored by flow cytometry. Cells were treated with DMSO (control), low-dose rifaximin (1 μM, L) and high-dose rifaximin (100 μM, H). Cells transfected with the RXRα expression plasmid remained viable throughout the experiment, indicating that the treatment did not induce apoptosis without human PXR expression (Supplemental Fig. 6). A low-dose of

rifaximin produced negligible influence on apoptosis and proliferation of HT-29 cells transfected with the human PXR expression vector. However, a high dose of rifaximin significantly increased the numbers of total, early and late apoptosis, with a slight although not significant elevation of cell numbers in the G0/G1 phase (Fig. 8).

Discussion

Inflammatory bowel disease leads to increased risk to develop colon cancer. The risk of colon cancer development is much higher in patients with inflammatory bowel disease than in the general population (Derikx et al., 2014). The tumor-promoting effect of inflammation is widely recognized to be related to the induction of gene mutations or epigenetic alterations, increasing expression of factors involved in carcinogenesis (such as NF-κB and COX-2), production of reactive oxygen species, and inhibition of apoptosis and stimulation of cell proliferation (Savari et al., 2014). It is also widely accepted that chronic inflammation promotes carcinogenesis by inducing production of a variety of cytokines and chemokines that produce a localized inflammatory response by activating NF-κB, which is followed by increased expression of iNOS and pro-inflammatory cytokines including TNFα and IL-6. These findings have important implications for the development of colon cancer chemoprevention strategies.

PXR regulates genes involved in the metabolism and disposition of various xenobiotics and endobiotics (Cheng et al., 2011). However, several studies reported potentially novel physiological functions of PXR, including regulation of inflammatory pathways (Xie and Tian, 2006). By this mechanism, the role of PXR in the therapy of

acute colitis and colon cancer has been investigated. PXR suppressed the proliferation of colon tumor cells in culture by modulating the cell cycle at G0/G1 cell phase by an unknown mechanism (Ouyang et al., 2010). Forced expression of human PXR in colon cancer cell lines inhibited proliferation and increased apoptosis, and the tumor size was significantly suppressed in a xenograft model (Xie et al., 2009; Ouyang et al., 2010). However, a contradictory role of PXR in colon cancer also exists. Treatment of a PXR-expressing colon cancer cell line, LS180, with the PXR agonist rifampicin inhibited apoptosis. Activation of PXR up-regulated the anti-apoptotic signaling molecules BAG3, BIRC2, and MCL-1, and down-regulated the pro-apoptotic factors BAK1 and p53 (Zhou et al., 2008; Habano et al., 2011). Others showed that activation of PXR induced tumor aggressiveness in mice and humans in a fibroblast growth factor 19-dependent manner (Wang et al., 2011). Based on these studies, the role of PXR in colon cancer remains unclear, thus the need for further investigation.

Rifaximin is a gut-specific agonist of human PXR, and thus can be used to investigate the role of intestinal PXR in colon cancer. However, this can only be accomplished using *PXR*-humanized mice since rifaximin does not activate the mouse PXR. With this agonist, the role of human intestinal PXR in acute colitis therapy has been was uncovered (Cheng et al., 2010). The preventive and therapeutic role of rifaximin in experimental models of colitis was demonstrated in the *PXR*-humanized mouse model where rifaximin not only prevented colitis before an inflammatory insult, as revealed by use of the DSS and 2,4,6-trinitrobenzene sulfonic acid-induced murine acute colitis models, but also decreased the symptoms after the onset of colitis (Cheng et al., 2010). These data indicated that the treatment with rifaximin might have value in the

long-term prevention of colon inflammation, and that activation of intestinal PXR might also be employed to prevent inflammation-driven colon cancer. The present study focused on the efficient therapy of rifaximin towards ulcerative colitis-associated colorectal cancer using the AOM/DSS-induced colon cancer model. The results revealed decreased tumor numbers and total tumor incidence upon rifaximin treatment only in *PXR*-humanized mice, but not in wild-type and *Pxr*-null mice. The percentage of tumors with diameter > 4 mm was reduced only in *PXR*-humanized mice and not in wild-type and *Pxr*-null mice. In addition, survival rates were markedly increased in *PXR*-humanized mice compared to wild-type and *Pxr*-null mice when rifaximin was administered concomitant with AOM/DSS. These results suggest that rifaximin prevents AOM/DSS-induced colon carcinogenesis in a human PXR-dependent manner. Furthermore, this study showed that the expression of cytokines significantly increased in response to DSS/AOM treatment; these are involved in the 'inflammation–carcinoma sequence' (Okayasu, 2012). Treatment with rifaximin decreased cytokine expression, which was in line with data showing that oral feeding with rifaximin modulates and attenuates the colonic inflammation in experimental animal colitis (Cheng et al., 2010). These results suggested that rifaximin exerts anti-inflammatory and growth inhibitory effects on colon cancer development by reducing cytokine expression. These results demonstrate that rifaximin has anti-tumor activity in *PXR*-humanized mice. This effect is correlated with decreased pro-inflammatory cytokines, attenuated colorectal inflammation and a lack of NF-κB target gene expression in the colonic tissue at the end of the experimental model.

The effects of rifaximin on cell proliferation and apoptosis were also evaluated. Rifaximin treatment induced apoptosis and reduced proliferation activity in colon tissues of mice treated with AOM/DSS. The proliferation-inhibiting properties of rifaximin can also be demonstrated in the short-term AOM/DSS treatment model. Moreover, rifaximin displayed the ability to induce apoptosis and control proliferation in G0/G1 phase in sporadic colon carcinoma cell line transfected with a human PXR expression vector. These results revealed the potential effects of rifaximin towards colon cancer via regulating cell proliferation and apoptosis.

In summary, the ability of rifaximin to prevent the development of colon carcinogenesis was associated with attenuated colorectal inflammation and lower tumor incidence only in AOM/DSS-treated *PXR*-humanized mice, thus indicating a chemopreventive role for specific activation of intestinal human PXR towards colon cancer. The mechanism is due in part to anti-inflammation, induction of apoptosis, and inhibition of cell proliferation.

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Zhou J, Liu M, Zhai Y and Xie W (2008) The antiapoptotic role of pregnane X receptor in human colon cancer cells. *Mol Endocrinol* **22**:868-880.

Figure legends

Fig. 1. Treatment with rifaximin prevents AOM/DSS-induced colon carcinogenesis in *PXR*-humanized mice. A. Survival rate of *PXR*-humanized (hPXR), wild-type (WT), and *Pxr*-null mice with rifaximin treatment in the AOM/DSS-induced colon carcinogenesis model (n=11 per group). ***P*<0.01, N.S., not significant. B. Tumor numbers in hPXR, WT, and *Pxr*-null mice without rifaximin treatment in AOM/DSS-induced colon carcinogenesis. C. Tumor number in hPXR, WT, and *Pxr*-null mice with rifaximin treatment in AOM/DSS-induced colon cancer. ***P*<0.01; N.S., not significant.

Fig. 2. Tumor incidence in cohort 1 of *PXR*-humanized (hPXR), wild-type (WT), and *Pxr*-null mice with or without rifaximin treatment in AOM/DSS-induced colon carcinogenesis (n=10 per group). A. Total tumor incidence in hPXR, WT, and *Pxr*-null mice with or without rifaximin treatment in AOM/DSS-induced colon cancer. B. Percentage of tumors with diameter > 4 mm in hPXR, WT, and *Pxr*-null mice with or without rifaximin treatment. C. Percentage of tumors with diameter < 4 mm in hPXR, WT, and *Pxr*-null mice with or without rifaximin treatment.

Fig. 3. Expression of human PXR and NF-κB target genes in tumor-adjacent tissue of colon in *PXR*-humanized (hPXR), wild-type (WT), and *Pxr*-null mice with or without rifaximin treatment in AOM/DSS-induced colon carcinogenesis (n=5 for AOM/DSS and AOM/DSS+rifaximin group, n=4 for the normal control group). #*P*<0.05, ##*P*<0.01

compared with control (normal tissue); **P*<0.05, ***P*<0.01 compared with AOM/DSS-treated group.

Fig. 4. Expression of *p21*, *Bcl-2*, *Bclx*, and *c-Myc* mRNAs in tumor-adjacent tissue of colon in *PXR*-humanized (hPXR), wild-type (WT), and *Pxr*-null mice with or without rifaximin treatment in AOM/DSS-induced colon carcinogenesis (n=5 for AOM/DSS and AOM/DSS-Rifaximin group, n=4 for normal control group). #*P*<0.05, compared with control (normal tissue); **P*<0.05 compared with AOM/DSS-treated group.

Fig. 5. Expressions of mRNAs encoded by cyclin-dependent kinases and cyclin genes in tumor-adjacent tissue of colon in *PXR*-humanized (hPXR), wild-type (WT), and *Pxr*-null mice with or without rifaximin treatment in AOM/DSS-induced colon carcinogenesis mice (n=5 for AOM/DSS and AOM/DSS+rifaximin group, n=4 for normal control group). #*P*<0.05, compared with control (normal tissue); **P*<0.05, ***P*<0.01 compared with AOM/DSS-treated group.

Fig. 6. Colon epithelial cells exhibited lower proliferation in *PXR*-humanized (hPXR) mice than wild-type (WT) and *Pxr*-null mice upon short-term treatment with rifaximin and AOM/DSS. (A) Representative BrdU staining in colon tissues obtained from hPXR, wild-type, and *Pxr*-null mice treated with rifaximin and AOM/DSS. Arrows indicate BrdU-positive nuclei. (B) BrdU labeling index comparison in hPXR, WT, *Pxr*-null mice treated with rifaximin and AOM/DSS. ***P*<0.01; ****P*<0.001.

Fig. 7. Gene expression of cyclin-dependent kinases and cyclin genes in colon tissues in *PXR*-humanized (hPXR), wild-type (WT), and *Pxr*-null mice with short-term treatment with AOM/DSS+rifaximin (n=5 for each groups). *P<0.05.

Fig. 8. Apoptosis and cell cycle in HT-29 cell transfected with human PXR with or without rifaximin treatment, including total apoptosis, early apoptosis, late apoptosis, and cell cycle. Human PXR and RXRα expression vectors were transfected to HT29 cells. These transfected cells were treated with DMSO or two different concentrations of rifaximin. Cont: cell treated with DMSO, L: cell treated with low dosage (1 μM) of rifaximin, H: cell treated with high dosage (100 μM) of rifaximin. Cells were treated in two batches (n=3 per batch), therefore, cont1, L1, H1, cont2, L2, and H2 were labeled as listed.

Figure 1

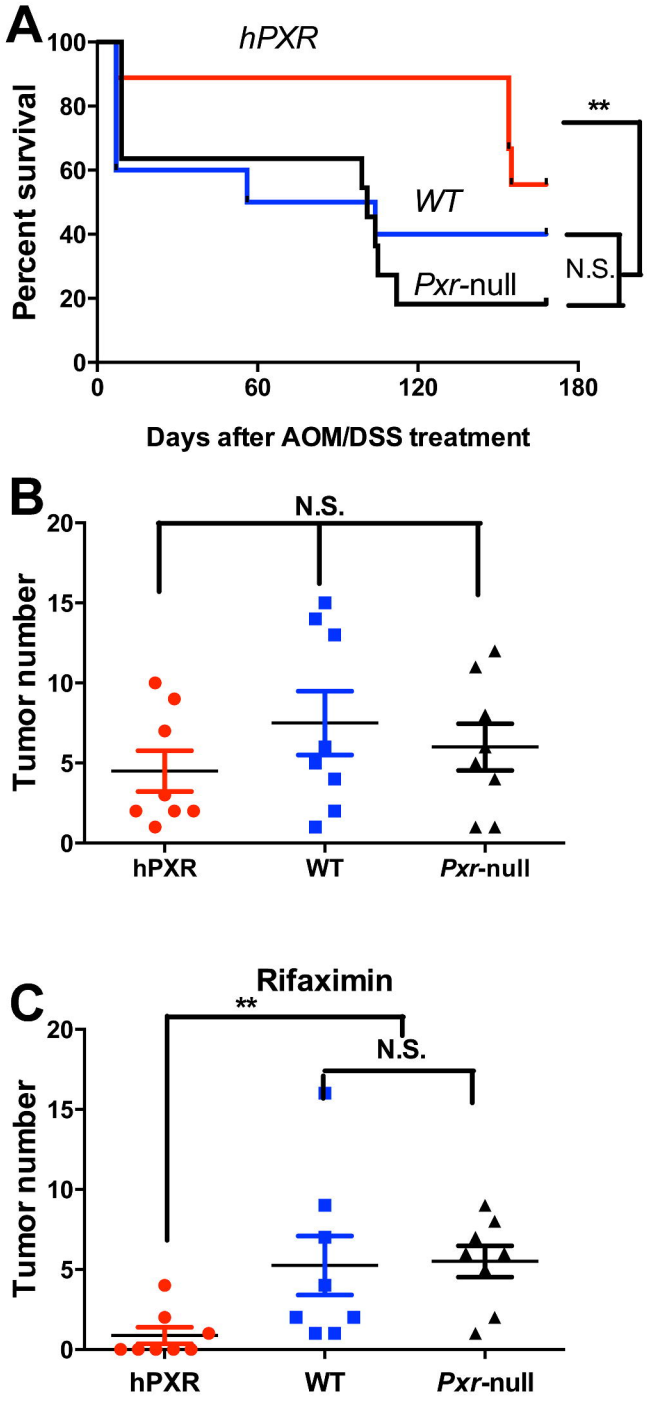

A
hPXR
WT
Pxr-null
**
N.S.
Percent survival
100
80
60
40
20
0
0
60
120
180
Days after AOM/DSS treatment
B
N.S.
Tumor number
20
15
10
5
0
hPXR
WT
Pxr-null
C
Rifaximin
**
N.S.
Tumor number
20
15
10
5
0
hPXR
WT
Pxr-null

Figure 2

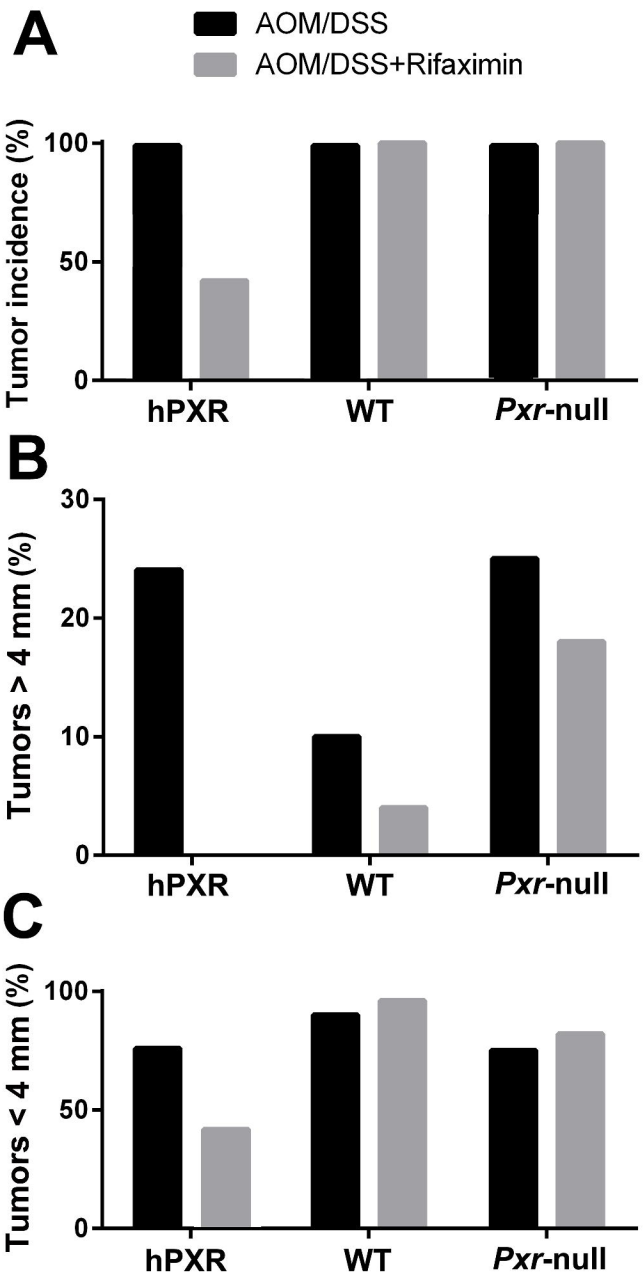

A
AOM/DSS
AOM/DSS+Rifaximin
Tumor incidence (%)
100
50
0
hPXR
WT
Pxr-null
B
Tumors > 4 mm (%)
30
20
10
0
hPXR
WT
Pxr-null
C
Tumors < 4 mm (%)
100
50
0
hPXR
WT
Pxr-null

Figure 3

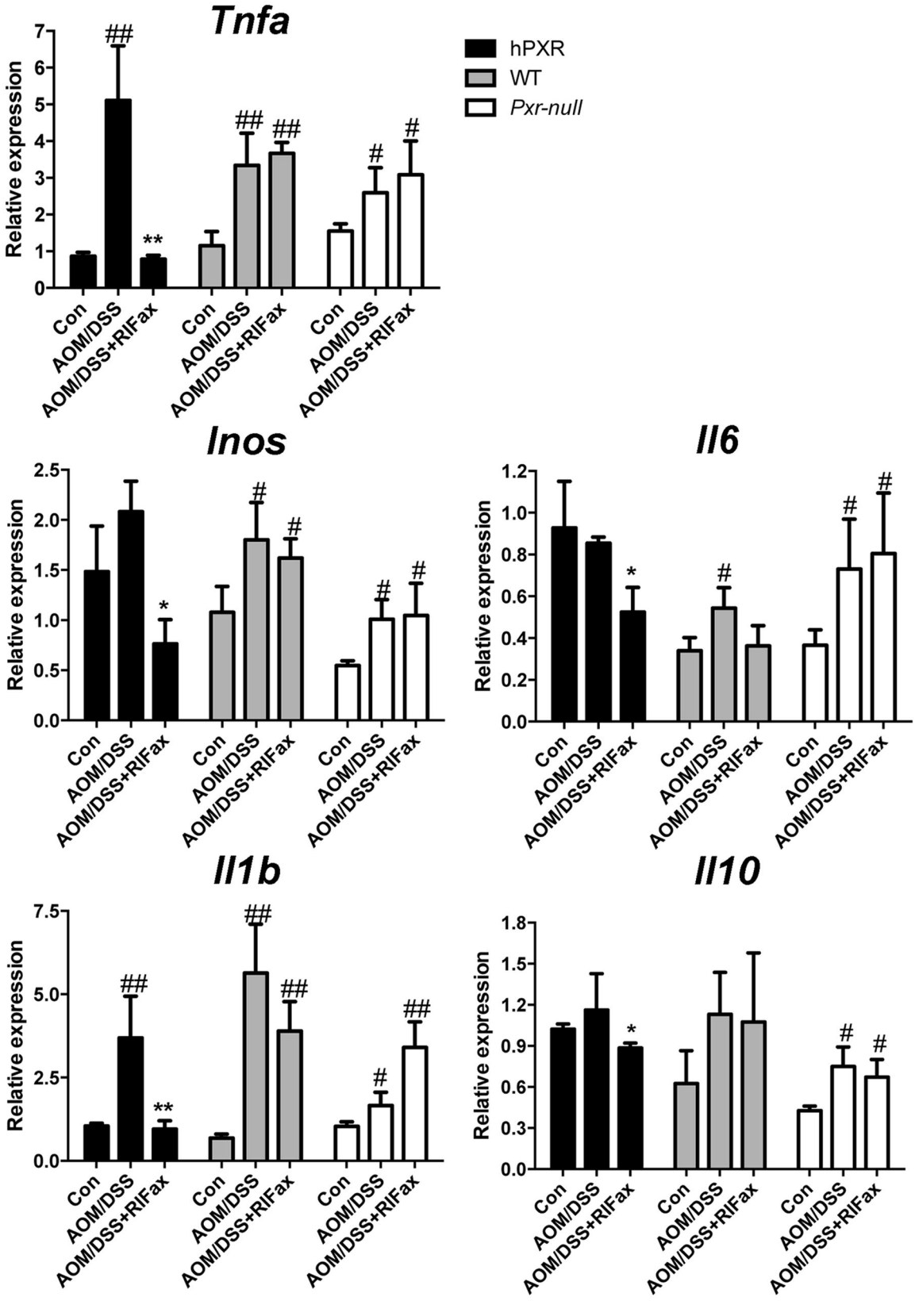

Tnfa
hPXR
WT
Pxr-null
Relative expression
Con
AOM/DSS
AOM/DSS+RIFax
Inos
Il6
Il1b
Il10

Figure 4

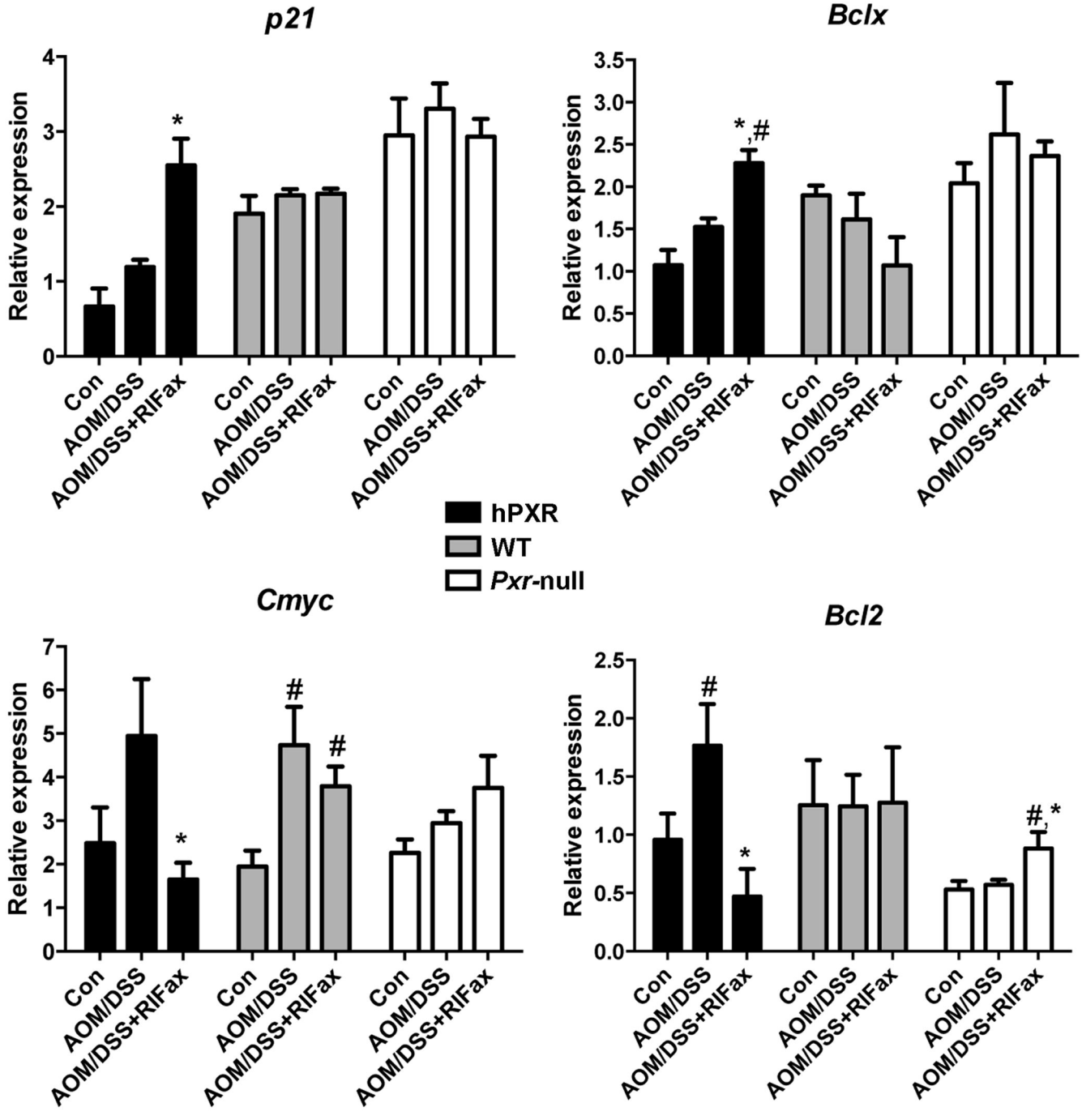

p21
Bclx
Cmyc
Bcl2
Relative expression
Con
AOM/DSS
AOM/DSS+RIFax
hPXR
WT
Pxr-null

Figure 5

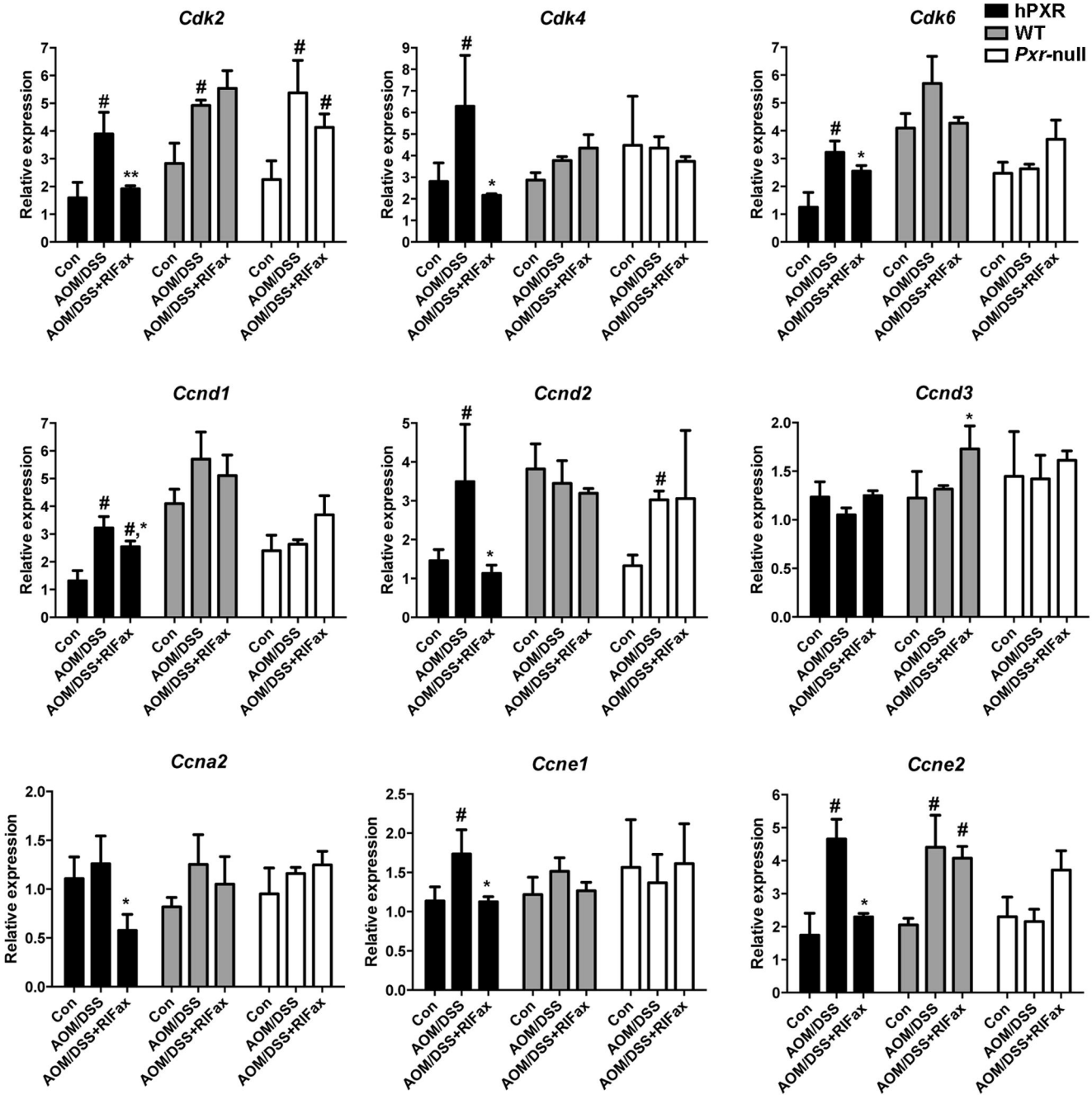
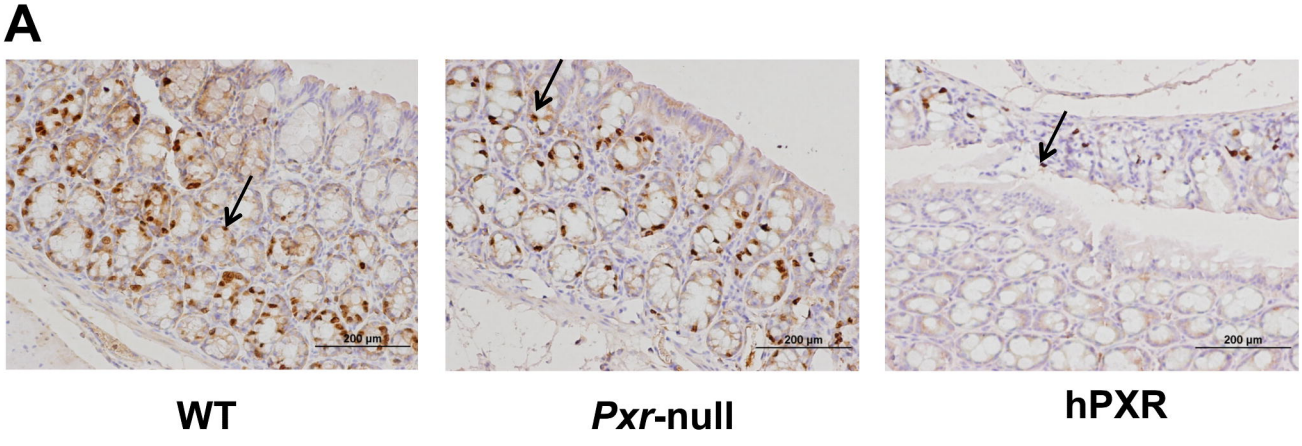

Cdk2
Cdk4
Cdk6
hPXR
WT
Pxr-null
Ccnd1
Ccnd2
Ccnd3
Ccna2
Ccne1
Ccne2
Relative expression
Con
AOM/DSS
AOM/DSS+RIFax

Figure 6


A
200 µm
200 µm
200 µm
WT
Pxr-null
hPXR

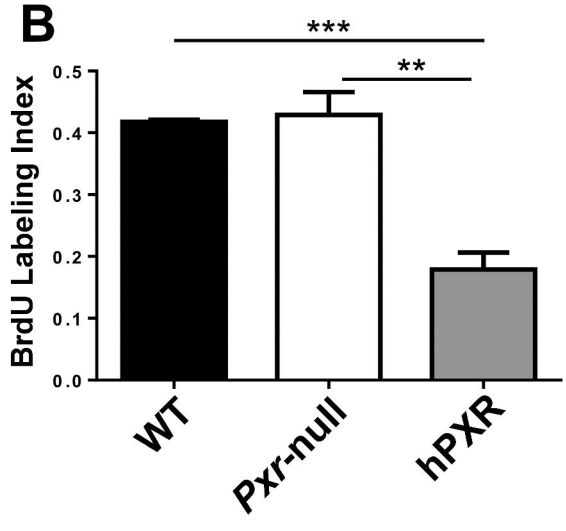

B
***
**
BrdU Labeling Index
0.5
0.4
0.3
0.2
0.1
0.0
WT
Pxr-null
hPXR

Figure 7

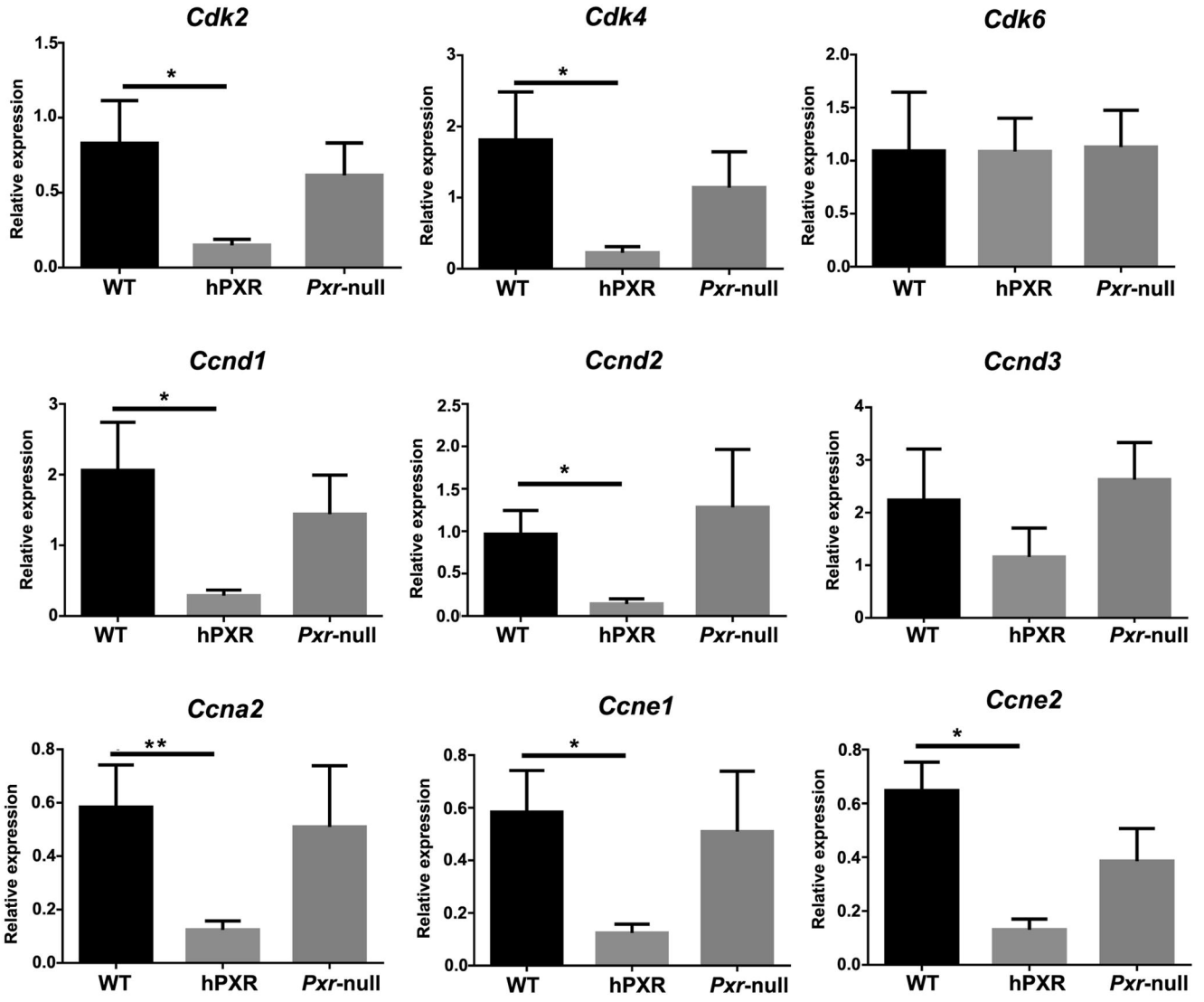
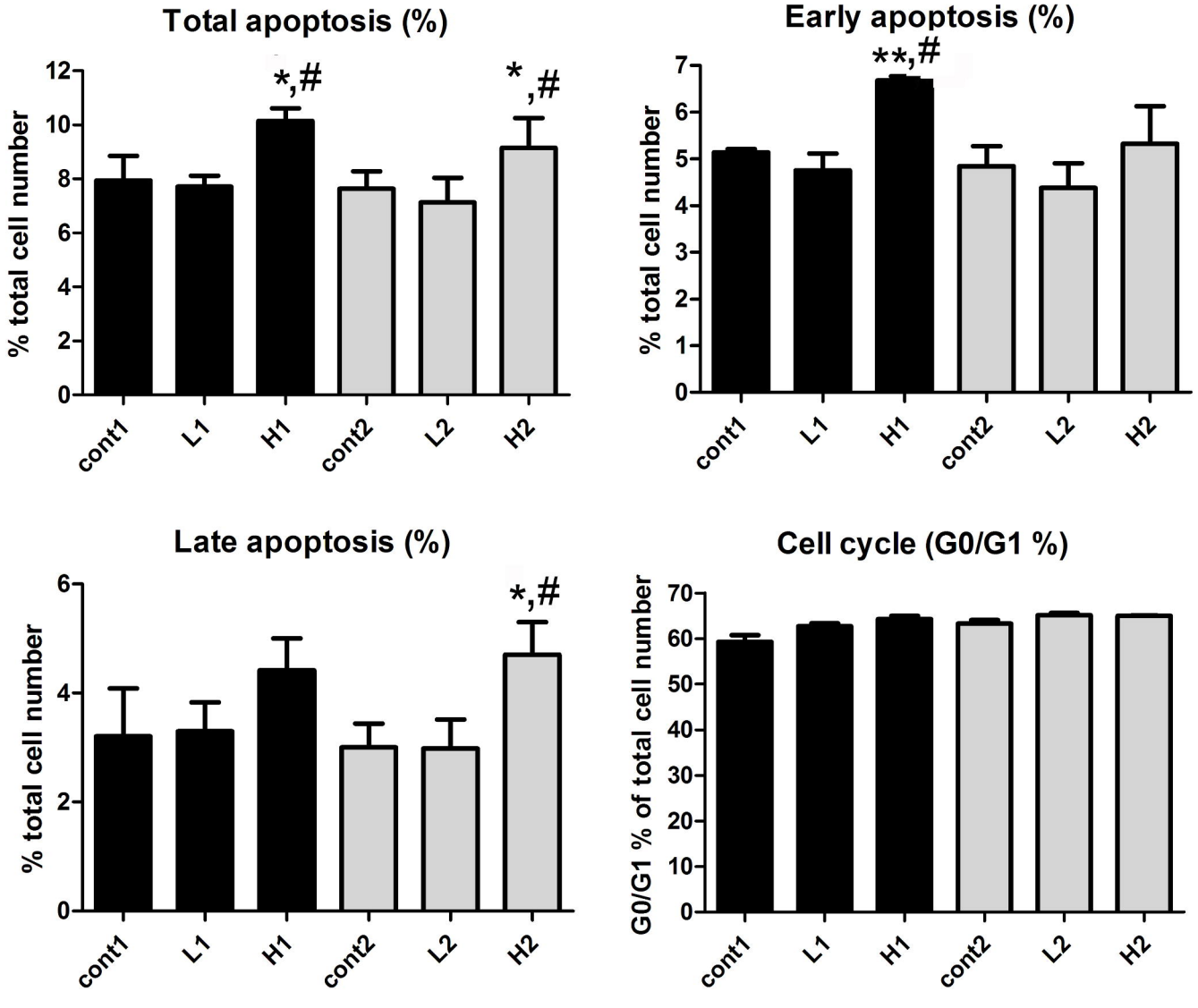

Cdk2
Cdk4
Cdk6
Ccnd1
Ccnd2
Ccnd3
Ccna2
Ccne1
Ccne2
Relative expression
WT
hPXR
Pxr-null
*
**

Figure 8


Total apoptosis (%)
% total cell number
12
10
8
6
4
2
0
*,#
*,#
cont1
L1
H1
cont2
L2
H2
Early apoptosis (%)
% total cell number
7
6
5
4
3
2
1
0
**,#
cont1
L1
H1
cont2
L2
H2
Late apoptosis (%)
% total cell number
6
4
2
0
*,#
cont1
L1
H1
cont2
L2
H2
Cell cycle (G0/G1 %)
G0/G1 % of total cell number
70
60
50
40
30
20
10
0
cont1
L1
H1
cont2
L2
H2