Original Article
Testosterone strongly enhances azoxymethane/dextran sulfate sodium-induced colorectal cancer development in C57BL/6 mice

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Abstract: Colorectal cancer (CRC) is known to occur more frequently in males than in females, with sex hormones reportedly influencing the development. The purpose of the study was to investigate whether orchiectomy in C57BL/6 male mice reduces colorectal tumorigenesis and whether testosterone administration increases tumorigenesis after orchiectomy in an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model. Clinical symptoms, including colitis and tumor incidence, were evaluated in the absence or presence of testosterone in AOM/DSS-treated male, as well as orchiectomized (ORX) male and female mice. The levels of serum testosterone and colonic myeloperoxidase, interleukin (IL)-1β, and IL-6 were measured by ELISA. Target mRNA expression was assessed by quantitative real-time PCR. Orchiectomy significantly diminished the AOM/DSS-induced colitis indices, including disease activity index, colon shortening, and histological severity at week 2, and decreased tumor numbers and incidence rates in the distal part of the colon increased following AOM/DSS administration at week 13; this reduction was reversed by testosterone supplementation. Furthermore, it was confirmed that the ELISA level (MPO and IL-1β) and the mRNA expression of the inflammatory mediators (COX-2 and iNOS) were maintained at high levels in the tumors of the testosterone-treated group compared with AOM/DSS groups. Interestingly, both endogenous and exogenous testosterone administrations were associated with tumor development (> 2 mm in size) and submucosal invasive cancer. Based on multivariate logistic regression analysis, testosterone was identified as a reasonable hazard factor for the progression of submucosal invasive cancer of the distal colon. In conclusion, endogenous and exogenous testosterone presented a stimulating effect on AOM/DSS-induced colitis and carcinogenicity.

Keywords: Colorectal cancer, colitis, sex difference, sex hormone, orchiectomy, testosterone propionate, AOM/DSS mouse model

Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in the United States, with an estimated 147,950 new cancer cases and 53,200 cancer-related deaths reported in 2020 [1]. Typically, CRC is known to occur more frequently in males and females across all ages [2, 3]. Recently, female patients with right-sided tumors have shown improved response to

adjuvant chemotherapy when compared with male patients [4]. In families with hereditary non-polyposis cancer syndrome, CRC risk is considerably lower in females than in males [5]. However, female patients with CRC over the age of 65 show a higher mortality rate and a lower 5-year survival rate when compared with age-matched male patients [6]. Furthermore, the CRC occurrence shows sex-specific differences worldwide [7]. Collectively, these clinical

results suggest that sex hormones are associated with CRC development and progression.

It has been reported that estrogen acts a protective role in colon tumorigenesis [8, 9], neurodegenerative diseases [10], and cardiovascular diseases [11] in both men and women. Numerous studies have suggested that elevated female hormone levels owing to pregnancy and the administration of exogenous hormones, including oral contraceptives and postmenopausal hormone therapy, can be correlated with a lower CRC risk in females [12-14]. Estrogen and progestin trials in the Women's Health Initiative reported a 40% lower risk of invasive CRC in the treatment group when compared to the placebo group [9, 15]. Unlike estrogen, there was no significant clinical correlation between testosterone concentrations and outcomes in CRC patients [16, 17] so far. However, some studies have suggested that testosterone, which is one of the major male hormones, may enhance colon adenoma development through unknown mechanisms, that may explain the high CRC susceptibility observed in males. In Pirc rat models, which spontaneously develop intestinal tumors, male rats are known to be more sensitive to chemical induction of colon tumors by the carcinogen dimethylhydrazine, than females [18]. Furthermore, according to a recent report by Amos-Landgraf et al., colonic adenomagenesis was facilitated by male hormones such as dihydrotestosterone and testosterone enanthate in Pirc rats and azoxymethane (AOM)-induced mouse colon cancer models [19]. However, evidence concerning the correlation between endogenous male hormone concentrations and CRC risk remains scarce.

Several animal models have been developed and are being utilized to elucidate the underlying pathogenic mechanisms of human disease [20]. The most widely employed animal model for molecular-based investigations of colitis and colitis-associated CRC [21-23] involves a mouse model induced by administering AOM plus sodium dextran sulfate (DSS) [24-26]. This AOM and DSS combination model is a powerful tool capable of simulating multi-stage tumor progression based on abnormal crypt foci-adenoma-carcinoma sequences, along with molecular changes evaluated at each stage of carcinogenesis [22]. In previous studies, we have applied the AOM/DSS mouse model system to

investigate sex differences in colitis-associated CRC development and reported that CRC induced by AOM/DSS occurs more severely in males than female counterparts [27]. Reportedly, administration of 17β-estradiol (E2) (10 mg/kg) inhibits the onset of CRC by modulating nuclear factor erythropoietin 2-related factor 2 (Nrf2)-mediated pathways [28]. Furthermore, the removal of endogenous estrogen via oophorectomy in AOM/DSS-treated females resulted in an increased tumor numbers in the proximal colon [29]. Additionally, estrogen changes the intestinal microbiome, particularly in males with AOM/DSS treatment, by reducing the *Firmicutes/Bacteroidetes* ratio and altering Shannon and Simpson indices; this indicates that estrogen reduces the CRC risk by causing alterations in the intestinal microbiome [30].

To date, we have predominantly evaluated the effect of estrogen as a sex differentiation factor on CRC incidence. Hence, in this study, we tried to investigate the role of testosterone on the CRC incidence. We hypothesized that in male AOM/DSS mouse models, orchiectomy would reduce the incidence of colon tumors, which would be increased by testosterone administration. To assess the hypothesis, we focused on the key role of testosterone according to the anatomical location of CRC occurrence. Furthermore, we studies the regulatory mechanisms for inflammation-mediated CRC occurrence at the molecular level.

Materials and methods

Chemicals

AOM (#A5486), DSS (#160110), and Testosterone propionate (TP) (#T0028) were purchased from Sigma-Aldrich, MP Biomedicals, and Tokyo Chemical Industry Co., Ltd., respectively. The chemicals were dissolved in different solvent; phosphate-buffered saline (PBS), water, and olive oil used for dissolving AOM, DSS, and TP, restectively.

Mice

Seven-week-aged male and female C57BL/6 mice were purchased from Koatech (Pyeongtaek, South Korea). Mice were maintained in specific pathogen-free facilities with a 12-h light/12-h dark cycle, and controlled temperature at 23°C. The facility of our preclinical ani-

mal room is maintained in a good environment and managed by professional personnel following Animals in Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (permit number: BA1705-223/043-01).

Establishment of the colitis and CRC mouse model

The scheme is showed in **Figure 1A**. After adaptation during one week, an orchiectomized (ORX) group in male mice performed surgical orchiectomy [31] at 8 weeks of age. TP supplementation began on the day of surgery. Male, ORX, and female mice were divided into three subgroups: control mice (Con), AOM/DSS-treated mice (AOM/DSS), and AOM/DSS-treated mice supplemented with TP (AOM/DSS+TP). The animal number was maintained between 6-12 in each. To establishment of the colitis and CRC, the AOM solution of 100 μl was injected with 10 mg/kg per mouse, which was considered "Day 0" [32]. At 7th day, 2% DSS solution was provided as a tap water system for one week. In the AOM/DSS+TP group, TP was dissolved in olive oil and supplied twice a week by intramuscular (i.m.) injections (0.5 mg/kg) in a 50 μl volume; olive oil was administered to all other groups. Mice were sacrificed by CO_2 asphyxiation at week 2 (11-weeks age) and 13 (22-weeks age) after the AOM injection (**Figure 1A**).

Assessment of disease activity index

The disease activity index (DAI) is used as an index to evaluate clinical symptoms. The method of calculating the disease activity index (DAI) was referred to in the previous reports [33, 34]. In brief, in a blinded manner, two researchers were scored the DAI by dividing the sum of weight reduction, diarrhea, and rectal bleeding scores by three.

Macroscopic measurement of polyps

In brief, the colon was opened vertically and the feces were discarded by PBS washing. And, the colon (cecum to rectum) length was calculated by ruler. Polyps were counted independently by two researchers who were blinded to the experimental protocol [33, 34].

Histopathology

Whole colons were seperated to proximal and distal parts as previously described [29]. Briefly, colonic tissues with any abnormal lesions were fixed with 4% paraformaldehyde solution. After pafaffin embedding, each section was stained with hematoxylin and eosin (H&E). For the 2-week samples, the colonic damage score was calculated by sum of crypt damage and infiltration depth of inflammatory cells as previously described [35]. For the 13-week samples, the identification of adenoma and cancer was analyzed by a specialized histopathologist in blinded to the experimental protocol [36].

Inflammatory mediators

Total protein lysates were prepared to measure the concentrations of myeloperoxidase (MPO), IL-6, and IL-1β in colonic tussues, and performed enzyme-linked immunosorbent assay (ELISA). A mouse MPO ELISA kit (#HK210) was purchased from Hycult Biotechnology. A mouse IL-6 (#M6000B) and IL-1β (#MLB00C) ELISA kits were purchased from R&D Systems Inc. In particular, the 13-week samples were divided into the tumor and non-tumor groups, followed by ELISA analysis. All assays were performed in triplicate.

Serum testosterone

Blood was collected from 2-week and 13-week mice, and serum was prepared as previously described [29]. Briefly, the blood in clot-activator tube was kept at 4°C for 30 min. After centrifugation (3,000 rpm for 15 min), the serum (upper layer) was stored at -80°C. To measure the testosterone concentration within the serum of experimental mice, we used the Testosterone ELISA kit (#ADI-901-065, Enzo Life Sciences, Inc., USA), according to the manufacturer's instructions. The sensitivity was 5.67 pg/mL for the testosterone assay. All assays were performed in two times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from 2-week and 13-week colon tissue samples using TRIzol reagent (#15596026, Invitrogen, USA). Especially, AOM/DSS and AOM/DSS+TP samples were prepared from colon tumor tissue, and
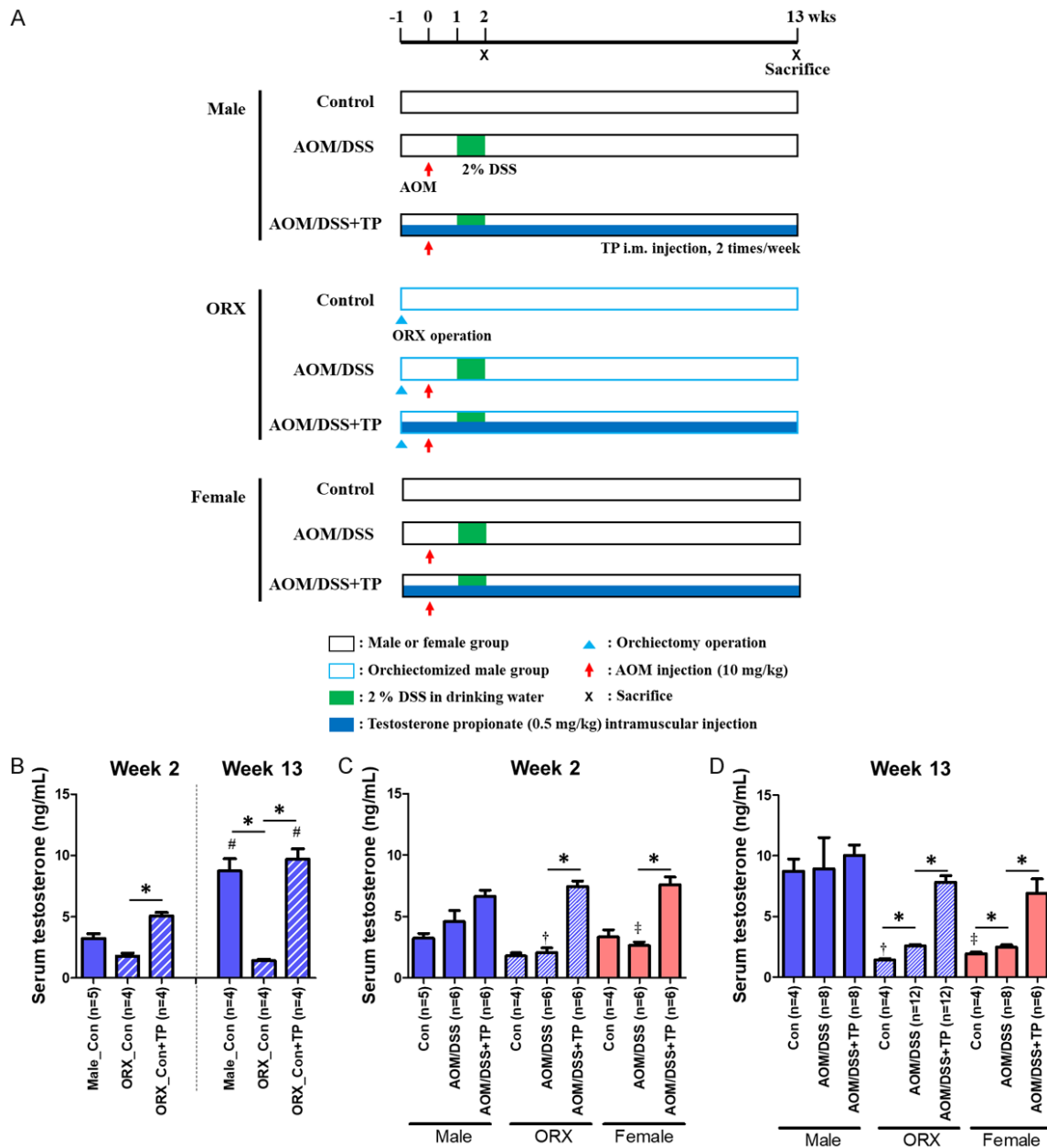
Figure 1. Serum testosterone levels are markedly increased following TP administration in AOM/DSS-treated ORX and female C57BL/6 mice. (A) Scheme to evaluate the role of TP on AOM/DSS-induced colitis (Week 2) and CRC (Week 13). One week after orchiectomy, animals were enrolled in the AOM/DSS protocol. The mice were injected with AOM on day 0 and provided DSS in drinking water (2%). TP administration was initiated on the day of surgery, administered twice a week by intramuscular (i.m.) injections. Mice were sacrificed at week 2 and 13. (B) The serum testosterone level, which was reduced following orchiectomy, is increased following TP supplementation similar to that of the male control group. *, P<0.05 for between indicated two groups; #, P<0.05 for week 2 *vs.* week 13. (C, D) Measurement of serum testosterone in male, ORX male, and female mice at week 2 (C) and 13 (D). *, P<0.05 for between indicated two groups; #, P<0.05 for week 2 *vs.* week 13; †, P<0.05 for male *vs.* ORX male; ‡, P<0.05 for male *vs.* female; §, P<0.05 for ORX male *vs.* female. Con, control; AOM, azoxymethane; DSS, dextran sulfate sodium; TP, testosterone propionate; ORX, orchiectomized.

control group samples were prepared from normal colon tissue. and then total RNA was extracted. Total RNA was used to synthesize cDNA using a reverse transcription kit (#436-

8814, Applied Biosystems, USA). Then, qRT-PCR was performed on a QuantStudio™ 7 Flex Real-Time PCR instrument using primer pairs for target gene (**Table 1**) and 2X PCR mixture

Table 1. List of primer sequences used for qRT-PCR

Gene	Primer sequence (5'→3')
COX-2	F: TGA GTA CCG CAA ACG CTT CTC R: TGG ACG AGG TTT TTC CAC CAG
iNOS	F: TGG TGG TGA CAA GCA CAT TT R: AAG GCC AAA CAC AGC ATA CC
Nrf2	F: ATG CCA GCC AGC TGA CCT CCT TA R: AGA CGG TGG CAG CAT GCC TTC
NQO1	F: GCG AGA AGA GCC CTG ATT GTA CTG R: TCT CAA ACC AGC CTT TCA GAA TGG
HO-1	F: CCT CAC TGG CAG GAA ATC ATC R: CCT CGT GGA GAC GCT TTA CAT A
GCLC	F: ACA TCT ACC ACG CAG TCA AGG ACC R: CTC AAG AAC ATC GCC TCC ATT CAG
GCLM	F: GCC ACC AGA TTT GAC TGC CTT TG R: TGC TCT TCA CGA TGA CCG AGT ACC
GAPDH	F: TTC ACC ACC ATG GAG AAG GC R: GGC ATG GAC TGT GGT CAT GA

COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; Nrf2, nuclear factor erythroid-derived 2-related factor 2; NQO1, NAD(P)H dehydrogenase (quinone) 1; HO-1, heme oxygenase 1; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

containing SYBR Green (#4367659, Applied Biosystems, UK). GAPDH was used as an internal control to normalize the expression level.

Statistical analysis

Statistical significance (*p*-value <0.05) was analyzed by the Mann-Whitney method using GraphPad Prism (ver. 5.01) and SPSS Statistics (ver. 18.0) programs. Statistical significance for tumor number and tumor incidence was analyzed by Fisher's exact test. The risk factors for CRC development were analyzed using multivariate and univariate analyses by employing the Firth logistic regression method. A bar graph expressed as the mean ± standard error of the mean (SEM) of the data was generated in the GraphPad Prism.

Results

Effect of testosterone propionate supplementation on serum testosterone levels in AOM/DSS-treated mice

To analyze the impact of testosterone on sex differences during colitis-associated CRC development, AOM/DSS male mice, with or with-

out orchiectomy, and AOM/DSS female mice were employed (**Figure 1A**). First, we measured serum testosterone levels to clarify the effects of orchiectomy and TP supplementation as a male hormone. Orchiectomy reduced serum testosterone levels (P=0.053 and P=0.020 for male *vs.* ORX male controls at weeks 2 and 13, respectively), which were significantly increased following TP supplementation (P=0.031 and P=0.020 for male control *vs.* control+TP at weeks 2 and 13, respectively), similar to those observed in male controls (**Figure 1B**). Furthermore, in male control mice, serum testosterone levels increased significantly with increasing age (**Figure 1B**, P=0.014 for week 2 *vs.* week 13), as in the previous report [37]. At week 2, immediately after DSS administration, serum testosterone levels in male mice were found to be enhanced following AOM/DSS treatment when compared with the control; levels were further increased with TP supplementation, but not by a significant extent. However, in the ORX male and female group, serum testosterone levels were unaltered following AOM/DSS treatment, significantly increased only by TP addition (**Figure 1C**, P=0.006 and P=0.022 for AOM/DSS *vs.* AOM/DSS+TP in ORX males and females, respectively). At week 13, basal testosterone levels were significantly lower in ORX male and female mice than those in males of the same age (**Figure 1D**, P=0.014 for male *vs.* ORX male control and P=0.025 for male *vs.* female control). Interestingly, serum testosterone levels in ORX male and female mice were significantly increased following AOM/DSS treatment when compared to controls (**Figure 1D**, P=0.003 for ORX male control *vs.* AOM/DSS and P=0.001 for female control *vs.* AOM/DSS), and further increased following TP supplementation, demonstrating levels similar to those observed in male mice (**Figure 1D**, P<0.001 and P=0.002 for AOM/DSS *vs.* AOM/DSS+TP in ORX males and females, respectively). The observed serum testosterone levels (**Figure 1B-D**) revealed that orchiectomy and TP supplementation were appropriately performed in the present study.

Aggravation of colon inflammation by TP administration during DSS treatment

Next, we assessed the effect of TP on colitis-associated symptoms, including the DAI score, colon shortening, and colonic epithelial damage, to assess the early impact of TP. AOM/
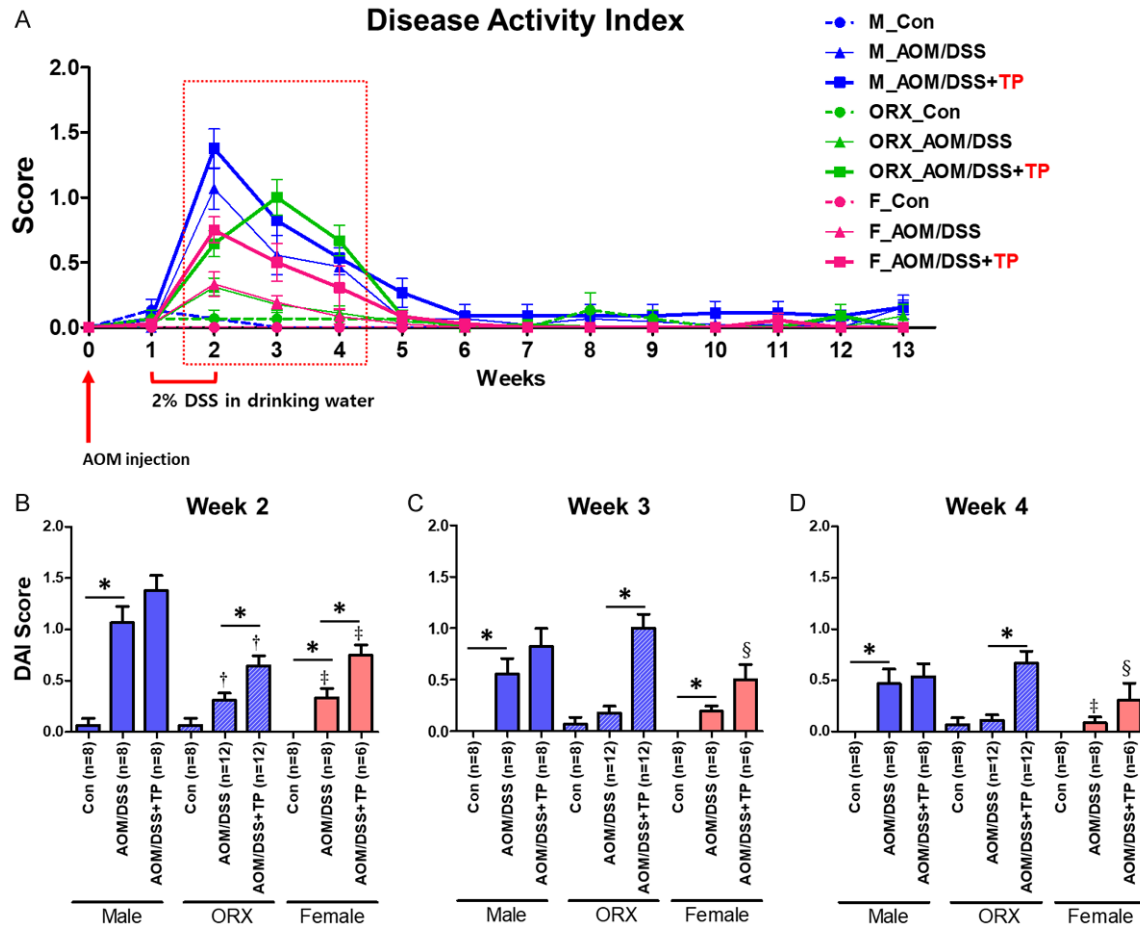
Figure 2. Effect of TP on DAI score. (A-D) DAI score for the entire duration of the experiment (A), at 2-week (B), at 3-week (C), and 4-week (D). *, P<0.05 for between indicated two groups; †, P<0.05 for male *vs.* ORX male; ‡, P<0.05 for male *vs.* female; §, P<0.05 for ORX male *vs.* female. DAI, disease activity index; Con, control; AOM, azoxymethane; DSS, dextran sulfate sodium; TP, testosterone propionate; ORX, orchiectomized.

DSS treatment increased the DAI score, which was further increased following TP supplementation (**Figure 2A**). At week 2, the increased DAI score, induced by AOM/DSS treatment, was significantly higher in the male mice than in the ORX males or females (**Figure 2B**, P<0.001 for male *vs.* ORX male AOM/DSS and P=0.001 for male *vs.* female AOM/DSS). TP administration further increased the DAI score, but statistical significance was observed only in the ORX and female groups (**Figure 2B**, P=0.012 for AOM/DSS *vs.* AOM/DSS+TP in both ORX males and females). The DAI scores elevated with AOM/DSS treatment, and the impact of TP supplementation on the DAI weakened at weeks 3 and 4 when compared with week 2 in the male and female groups (**Figure 2**). In the ORX male group, the effect of DSS on the DAI score was highest at week 2, while the effect of TP on the

DAI score was observed up to week 3, peaking at week 3 (**Figure 2**). In all AOM/DSS-treated groups, colons were significantly shortened by inflammation at week 2 when compared with the control group (**Figure 3A**, P=0.006, P= 0.048, and P=0.005 for control *vs.* AOM/DSS in males, ORX males, and females, respectively). TP supplementation further exacerbated colon shortening induced by AOM/DSS in males and ORX males, but not in females (**Figure 3A**, P=0.008 and P=0.028 for AOM/DSS *vs.* AOM/DSS+TP in male and ORX male, respectively). Furthermore, colonic epithelial damage scores (**Figure 3B**) and representative histopathological images (**Figure 3C**) revealed that crypt loss and inflammatory cell infiltration in colon tissues, induced by AOM/DSS, were aggravated following TP supplementation in all groups (**Figure 3B**, P=0.002, P=0.042, and P=0.039
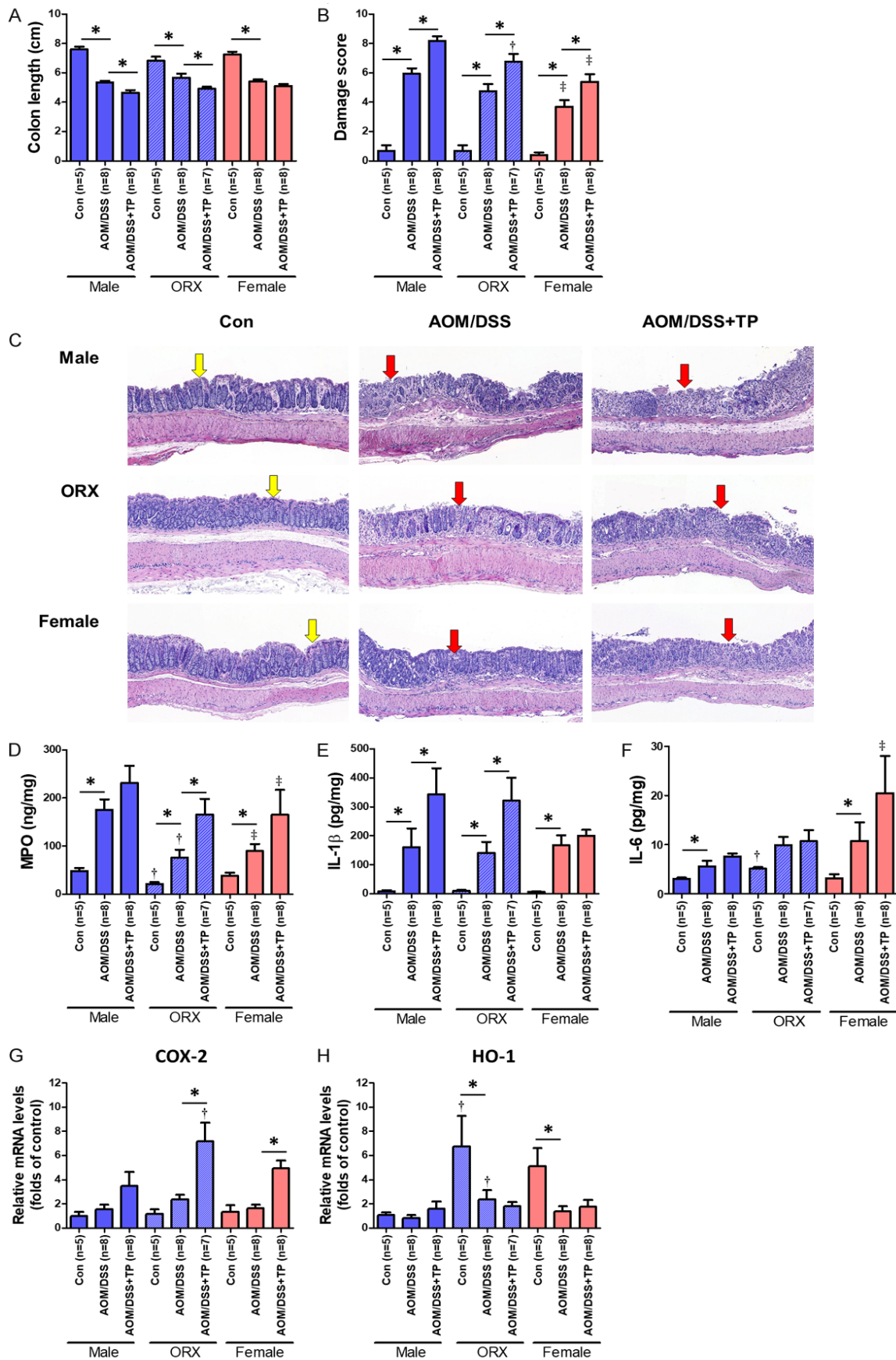
Figure 3. Promoting effects of TP on inflammatory indices in AOM/DSS-induced mouse colitis. (A) Colon length shortening owing to AOM/DSS treatment is more severe following TP supplementation in male and ORX male mice at week 2. (B, C) Colonic epithelial damage attributed to AOM/DSS treatment is more severe following TP supplementation in male, ORX male, and female mice. (B) Microscopic damage score at week 2. (C) Representative histopathology photos of colonic tissues at 2-week. Magnification, 100 ×. Normal crypt in control mice (yellow arrow). Crypt loss and marked inflammatory cell infiltration in all AOM/DSS-treated mice (red arrow). Histological damage caused by AOM/DSS is aggravated by TP supplementation (red arrow). (D-F) Effects of orchiectomy and TP on the concentration of proinflammatory mediators in colonic tissues at week 2. The concentration of MPO (D), IL-1β (E), and IL-6 (F) in colonic tissues at week 2. (G, H) The mRNA expression of COX-2 and HO-1 in colonic tissues at 2-week. *, P<0.05 for between indicated two groups; †, P<0.05 for male *vs.* ORX male; ‡, P<0.05 for male *vs.* female; §, P<0.05 for ORX male *vs.* female. Con, control; AOM, azoxymethane; DSS, dextran sulfate sodium; TP, testosterone propionate; ORX, orchiectomized; MPO, myeloperoxidase; IL-1β, interleukin 1 beta.

for AOM/DSS *vs.* AOM/DSS+TP in males, ORX males, and females). However, the microscopic damage score increased following AOM/DSS treatment and TP supplementation was significantly lower in female than in male mice (**Figure 3B**, P=0.006 and P=0.001 for males *vs.* females in the AOM/DSS and AOM/DSS+TP groups, respectively). Next, we performed an ELISA to measure the concentration of MPO, IL-1β, and IL-6, mediators associated with intestinal inflammation, in the colon tissue at week 2 and 13. At week 2, the MPO levels increased by AOM/DSS treatment was further increased by TP supplementation in all groups and was significant only in the ORX group (**Figure 3D**, P=0.047 for AOM/DSS vs. AOM/DSS+TP). The AOM/DSS-mediated MPO levels were significantly lower in ORX and females than in males (**Figure 3D**, P=0.002 for ORX AOM/DSS and P=0.004 for female AOM/DSS vs. male AOM/DSS). Interestingly, the IL-1β levels increased by AOM/DSS treatment were strongly elevated by TP supplementation in males and ORX, excluding females (**Figure 3E**, P=0.023 and P=0.017 for AOM/DSS vs. AOM/DSS+TP in males and ORX, respectively). Unlike MPO or IL-1β, IL-6 levels were not only less responsive by AOM/DSS treatment, but also showed no difference due to TP supplementation in C57BL/6 background mice at week 2 (**Figure 3F**). Next, we further conducted qRT-PCR analysis to analyze mRNA expression of pro-inflammatory mediator COX-2 and anti-oxidant enzyme HO-1 gene in 2-week colon tissue samples. The mRNA expression of COX-2 was strongly elevated by TP supplementation (**Figure 3G**, P=0.015 and P=0.002 for AOM/DSS vs. AOM/DSS+TP in ORX and females, respectively). Interestingly, ORX and female controls had higher levels of HO-1 mRNA expression than males, and those levels were significantly reduced by AOM/DSS treatment (**Figure 3H**,

P=0.042 and P=0.033 for control vs. AOM/DSS in ORX and females, respectively). No additional change was observed by the TP treatment compared with AOM/DSS group. Collectively, these data indicated that TP aggravates the severity of DSS-induced colitis, as revealed by the DAI score, colon length shortening, and histopathological damage, and inflammatory mediators.

Aggravation of colitis-associated tumorigenesis by TP treatment at week 13

To determine the effect of TP on colitis-associated tumorigenesis, we assessed tumor incidence by macroscopic estimation of tumors, assessing both size and location, at week 13. The incidence of tumor multiplicity and microscopic colon neoplasms is summarized in **Table 2**. In all AOM/DSS-treated groups, tumors were well-developed at week 13, mostly in the distal part of the colon (**Figure 4A** and **Table 2**), consistent with observations in a previous report [25]. In the distal colon, AOM/DSS-treated male mice presented a greater number of tumors than AOM/DSS-treated ORX males and females, with a significant increase only when the size exceeded 2 mm (**Figure 4A** and **Table 2**, P=0.018 for male *vs.* ORX male and P=0.007 for male *vs.* female in the AOM/DSS group, respectively). Furthermore, TP supplementation increased tumor incidence when compared with the AOM/DSS-treated group, and revealed a significant increase in the incidence of tumors exceeding 2 mm in size only in females (**Figure 4A** and **Table 2**, P=0.022 for AOM/DSS *vs.* AOM/DSS+TP in females). These results provide crucial evidence regarding the pro-tumorigenic effect of testosterone in colitis-related tumor development. A summary of the incidence and tumor multiplicity of microscopic neoplasms in the distal colon is provided in **Table 2**. Representative histopathological

Table 2. Incidence and multiplicity of adenoma and cancer at the distal part of the colon

	Low grade adenoma incidence	High grade adenoma incidence	Cancer with mucosa invasion	Cancer with submucosa invasion	Adenoma/ Cancer incidence	Adenoma/ Cancer multiplicity
Male						
Con (n=8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0
AOM/DSS (n=8)	0.0 (0/8)	0.0 (0/8)	62.5 (5/8)	0.0 (0/8)	62.5 (5/8)	4.9 ± 1.1
AOM/DSS+TP (n=8)	0.0 (0/8)	0.0 (0/8)	50.0 (4/8)	50.0 (4/8)	100.0 (8/8)	6.5 ± 1.0
p-value[a]	1.000	1.000	**0.026**	1.000	**0.026**	**0.001**
p-value[b]	1.000	1.000	1.000	0.077	0.200	0.125
ORX male						
Con (n=8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0
AOM/DSS (n=12)	0.0 (0/12)	0.0 (0/12)	25.0 (3/12)	8.3 (1/12)	33.3 (4/12)	3.1 ± 0.7
AOM/DSS+TP (n=12)	8.3 (1/12)	0.0 (0/12)	58.3 (7/12)	8.3 (1/12)	75 (9/12)	3.8 ± 0.6
p-value[a]	1.000	1.000	0.242	1.000	0.117	**0.002**
p-value[b]	1.000	1.000	0.214	1.000	0.100	0.503
Female						
Con (n=8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0
AOM/DSS (n=8)	12.5 (1/8)	12.5 (1/8)	37.5 (3/8)	0.0 (0/8)	62.5 (5/8)	2.0 ± 0.9
AOM/DSS+TP (n=6)	0.0 (0/6)	0.0 (0/6)	83.3 (5/6)	16.7 (1/6)	100.0 (6/6)	3.0 ± 0.8
p-value[a]	1.000	1.000	0.200	1.000	**0.026**	**0.010**
p-value[b]	1.000	1.000	0.138	0.429	0.209	0.145
Male vs. ORX						
p-value[c]	1.000	1.000	0.167	1.000	0.362	0.638
p-value[d]	1.000	1.000	1.000	0.109	0.242	0.087
Male vs. Female						
p-value[c]	1.000	1.000	0.619	1.000	1.000	0.115
p-value[d]	1.000	1.000	0.301	0.301	1.000	0.076
ORX vs. Female						
p-value[c]	0.400	0.400	0.642	1.000	0.362	0.305
p-value[d]	1.000	1.000	0.600	1.000	0.515	0.601

Values are expressed as the % (number/subtotal) for the adenoma/cancer incidence and number ± SEM for the tumor multiplicity. ORX, orchiectomized; Con, control; AOM, azoxymethane; DSS, dextran sodium sulfate; TP, Testosterone propionate. a, control vs. AOM/DSS; b, AOM/DSS vs. AOM/DSS+TP; c, AOM/DSS group comparison; d, AOM/DSS+TP group comparison (by Fisher's exact test for a 2 × 2 table); bold numbers, statistical significance (*P*<0.05).

H&E images are provided in **Figure 4B**. The occurrence of mucosal invasive adenocarcinoma was significant in the male AOM/DSS group, but not in ORX males and females (**Figure 4C** and **Table 2**, P=0.026 for control *vs.* AOM/DSS in males). Following TP supplementation, submucosal invasive adenocarcinomas were increased in males and females treated with AOM/DSS, presenting no statistical significance (**Figure 4C** and **Table 2**). To determine the risks associated with TP supplementation in AOM/DSS-induced CRC development, we performed multivariate and univariate logistic regression analyses using the Firth logistic regression method [38]. Multivariate analysis showed that TP supplementation significantly increased adenoma/cancer incidence when compared with the AOM/DSS-treated group (OR=7.59, P=0.008). In particular, TP supplementation influenced the development of cancer in the distal colon (OR=9.02, P=0.003), but not in the proximal colon (OR=2.32, P=0.247) (**Figure 5A**). Univariate analysis revealed that males (OR=43.48, P=0.029) showed a higher prevalence of CRC development induced by AOM/DSS treatment than orchiectomy (OR=9.01, P=0.185) and females (OR=27.03, P=0.054) (**Figure 5B**). Testosterone was confirmed to be a risk factor for the occurrence of submucosal invasive adenocarcinoma of the
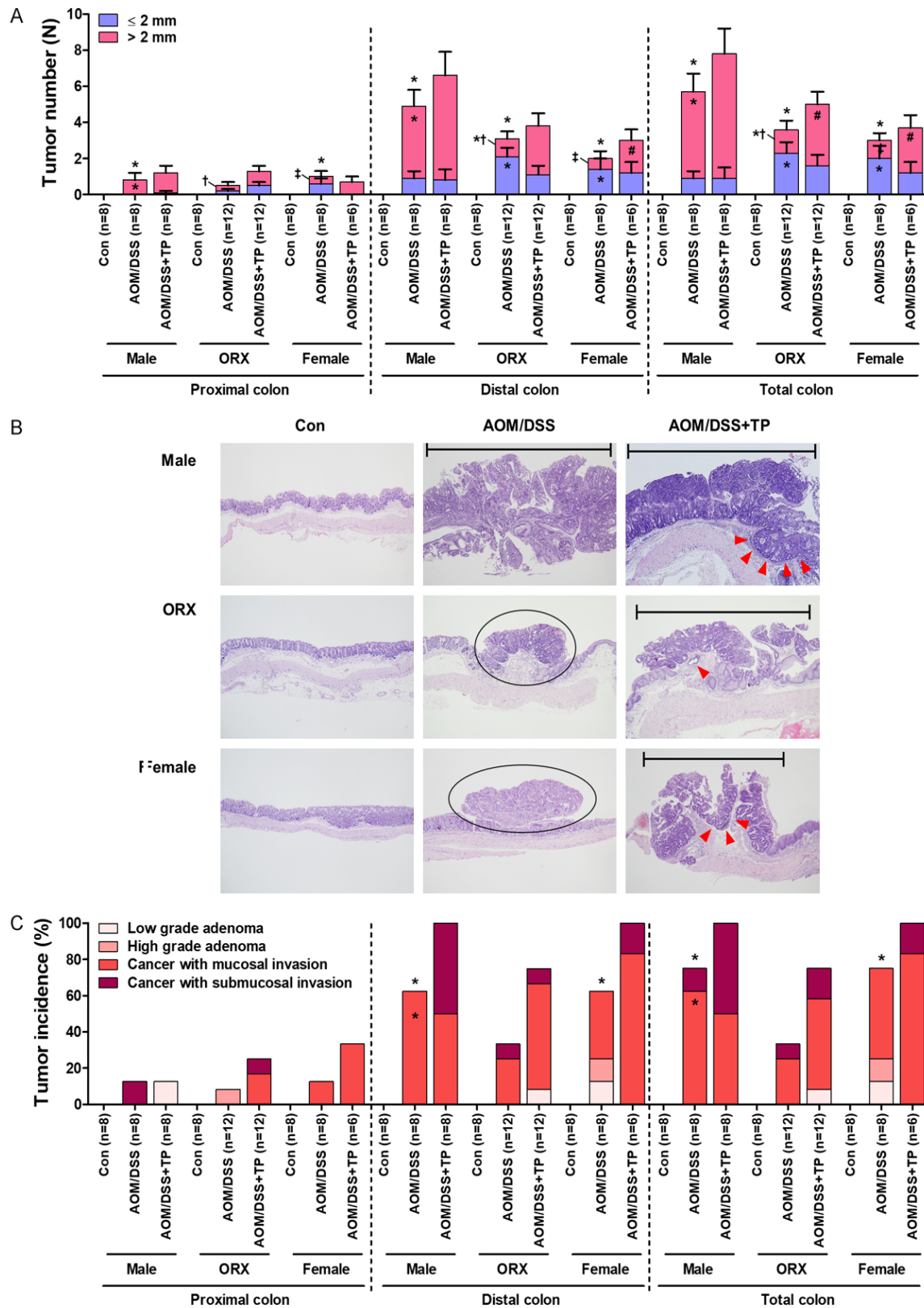
Figure 4. Effects of orchiectomy and TP on the colon tumor multiplicity at week 13. A. Average tumor numbers and size distribution in the proximal, distal, and whole colon in each group sacrificed at week 13 following AOM injection.

B. Representative histological images after H&E staining at week 13. Adenocarcinoma is indicated by a circle and a bar. Submucosal invasion is indicated by a red arrow. Magnification, 40 ×. C. Quantification of adenoma/adenocarcinoma incidence and invasion in each group by microscopic evaluation of colonic tissues at the carcinogenesis stage (at week 13). *, P<0.05 for Con *vs.* AOM/DSS; #, P<0.05 for AOM/DSS *vs.* AOM/DSS+TP; †, P<0.05 for male *vs.* ORX male; ‡, P<0.05 for male vs. female; §, P<0.05 for ORX male *vs.* female. The statistical significance of the sum is shown at the top of the graph. Con, control; AOM, azoxymethane; DSS, dextran sulfate sodium; TP, testosterone propionate; ORX, orchiectomized; H&E, hematoxylin-eosin.

distal colon in males (OR=17.00, P=0.095) and females (OR=4.54, P=0.404) (**Figure 5C**). These data suggest that both endogenous and exogenous testosterone can promote colitis-associated tumorigenesis, especially within the distal colon.

Promoting effects of TP on the pro-inflammatory mediators at week 13

Next, we performed an ELISA to measure the concentration of MPO, IL-1β, and IL-6, mediators associated with colitis-associated tumorigenesis, in the colon tumors at week 13. In case of 13-week samples were divided into tumor and non-tumor, and then performed ELISA. MPO and IL-1β levels increased by AOM/DSS in tumor tissues were strongly lower compared to that of 2-week in all groups (**Figure 6A**, **6B**). Interestingly, the levels of MPO and IL-1β increased by AOM/DSS treatment in tumor tissues were significantly increased by TP supplementation, similar to those in the AOM/DSS-treated 2-week group (**Figure 6A**, **6B**). As similar to week 2, IL-6 levels were not only less responsive by AOM/DSS treatment, but also showed no difference due to TP supplementation in C57BL/6 background mice at week 13 (**Figure 6C**). Next, to analyze expression of pro-inflammatory mediators and anti-oxidant enzyme genes in 13-week samples, AOM/DSS and AOM/DSS+TP group samples were prepared from colon tumor tissue, and control group samples were prepared from normal colon tissue. The mRNA expression of COX-2 (**Figure 6D**) and iNOS (**Figure 6E**) was also regulated by AOM/DSS and TP supplementation, similar to MPO and IL-1β. However, the mRNA expression of Nrf2 and its target genes (NQO1, HO-1, GCLC, and GCLM) was not consistently regulated by testosterone in male, ORX, and female groups (**Figure 6F-J**). These data suggest that endogenous testosterone supplementation has a stimulating effect on pro-inflammatory mediators and may contribute to aggressive tumor formation due to maintaining high levels of inflammatory factors in tumor tissues.

Discussion

In the present study, our results revealed that orchiectomy attenuated the colitis index scores induced by AOM/DSS, including DAI and colonic epithelial damage, at week 2, and decreasing the AOM/DSS-induced development and incidence rates of tumors in the colon, mainly developed in the distal part of the colon, at week 13; testosterone supplementation reversed these observed reductions to those observed at endogenous levels. Notably, in male mice, AOM/DSS-induced distal colon tumors, in particular, those over 2 mm in size significantly reduced following orchiectomy and enhanced following testosterone supplementation. Furthermore, the development of submucosal invasive cancer was considerably promoted following testosterone supplementation in males, ORX males, and females with AOM/DSS treatment. The present results confirm that testosterone plays an important role in promoting colon tumorigenesis, especially impacting severity such as larger tumors and depth of invasion.

CRC is one of the leading causes of cancer-related deaths worldwide [3, 39]. The CRC risk is marginally higher in males than in females [5], and sex differences in incidence and mechanism have been previously reported [7]. Emerging evidence suggests that female hormones, especially estrogen, act a role in preventing colitis and the progression of CRC [40], while male hormone androgens could cause CRC [41]. In this study, serum testosterone concentrations were measured to determine 1) whether the orchiectomy was successfully performed and 2) whether intramuscular TP injections restored levels to endogenous testosterone levels. Moreover, we aimed to determine 3) the relationship between serum testosterone levels and pathomechanisms of inflammation-mediated CRC. As expected, ORX control mice
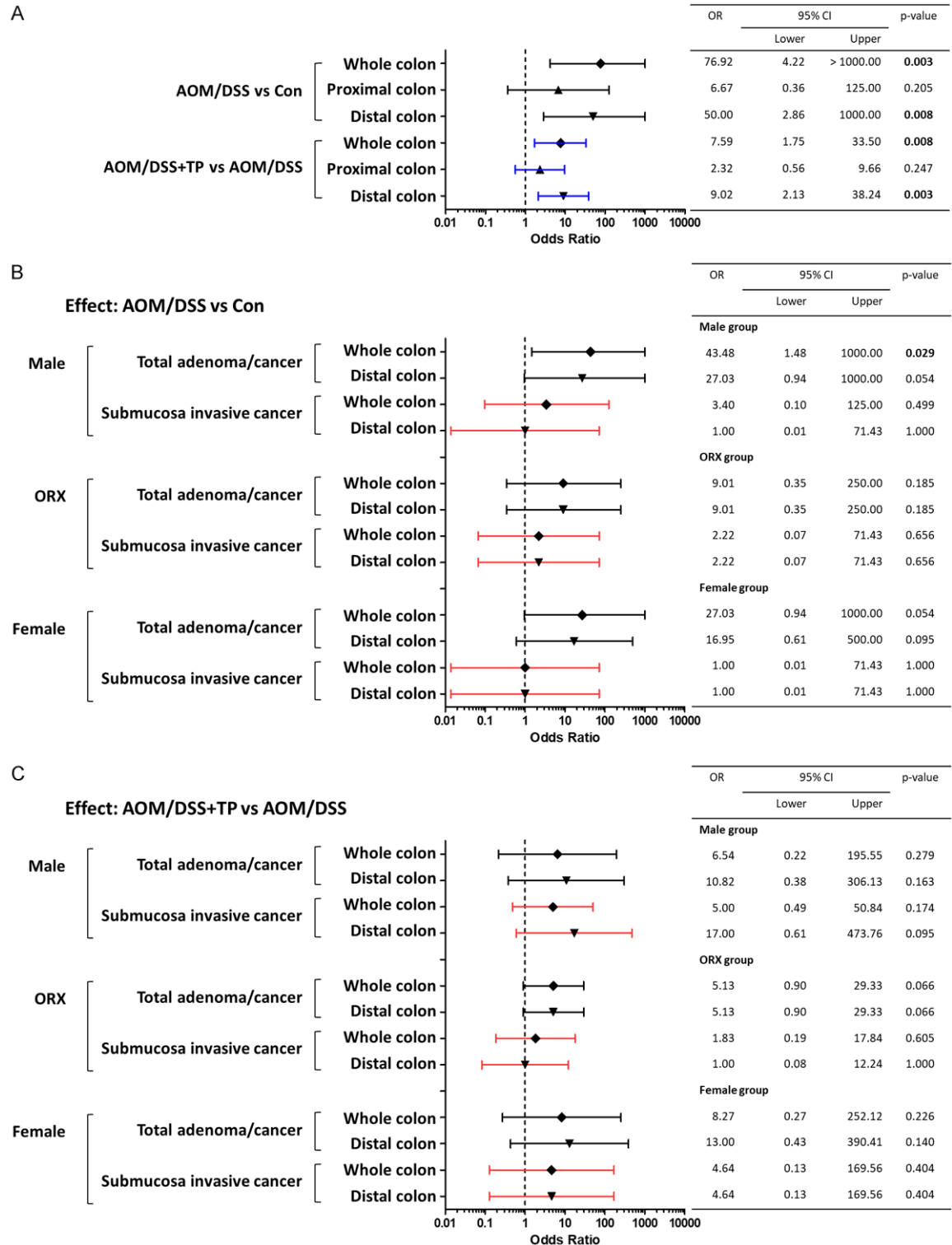
Figure 5. Forest plots of odds ratios of AOM/DSS-induced CRC associated with TP supplementation. A. Multivariate analysis using the Firth logistic regression method. AOM/DSS or AOM/DSS+TP effect according to tumor location in CRC mouse model. B. Univariate analysis using the Firth logistic regression method. AOM/DSS effect according to sex, tumor location, and submucosal invasive cancer in the CRC mouse model. C. Univariate analysis using the Firth logistic regression method. AOM/DSS+TP effect according to sex, tumor location, and submucosal invasive cancer in the CRC mouse model. Horizontal lines represent 95% confidence intervals. The dashed vertical line is at the null value (OR=1.0). Bold numbers, statistical significance (P<0.05). OR, odds ratio; CI, confidence interval; Con, control; AOM, azoxymethane; DSS, dextran sulfate sodium; TP, testosterone propionate; ORX, orchiectomized; CRC, colorectal cancer.
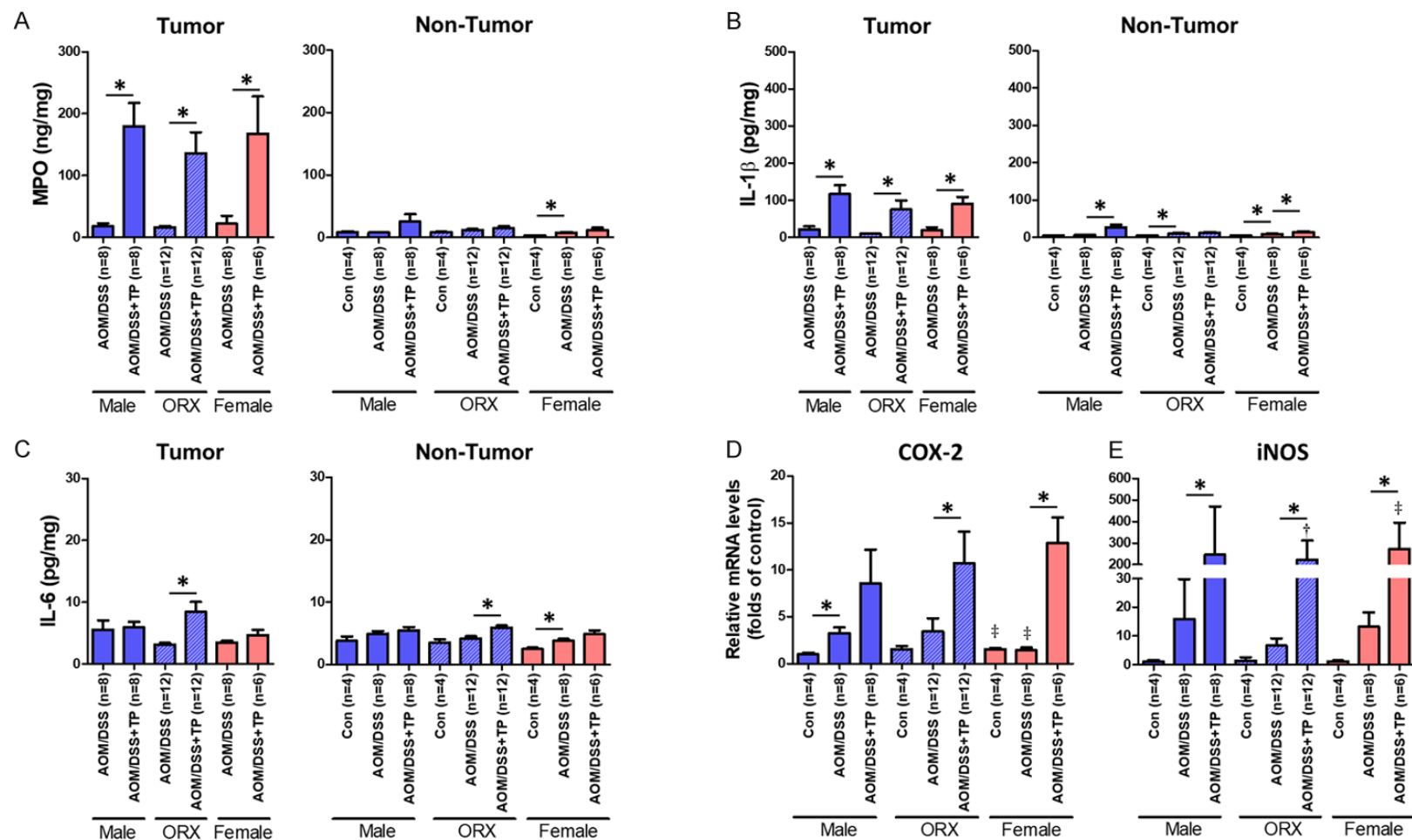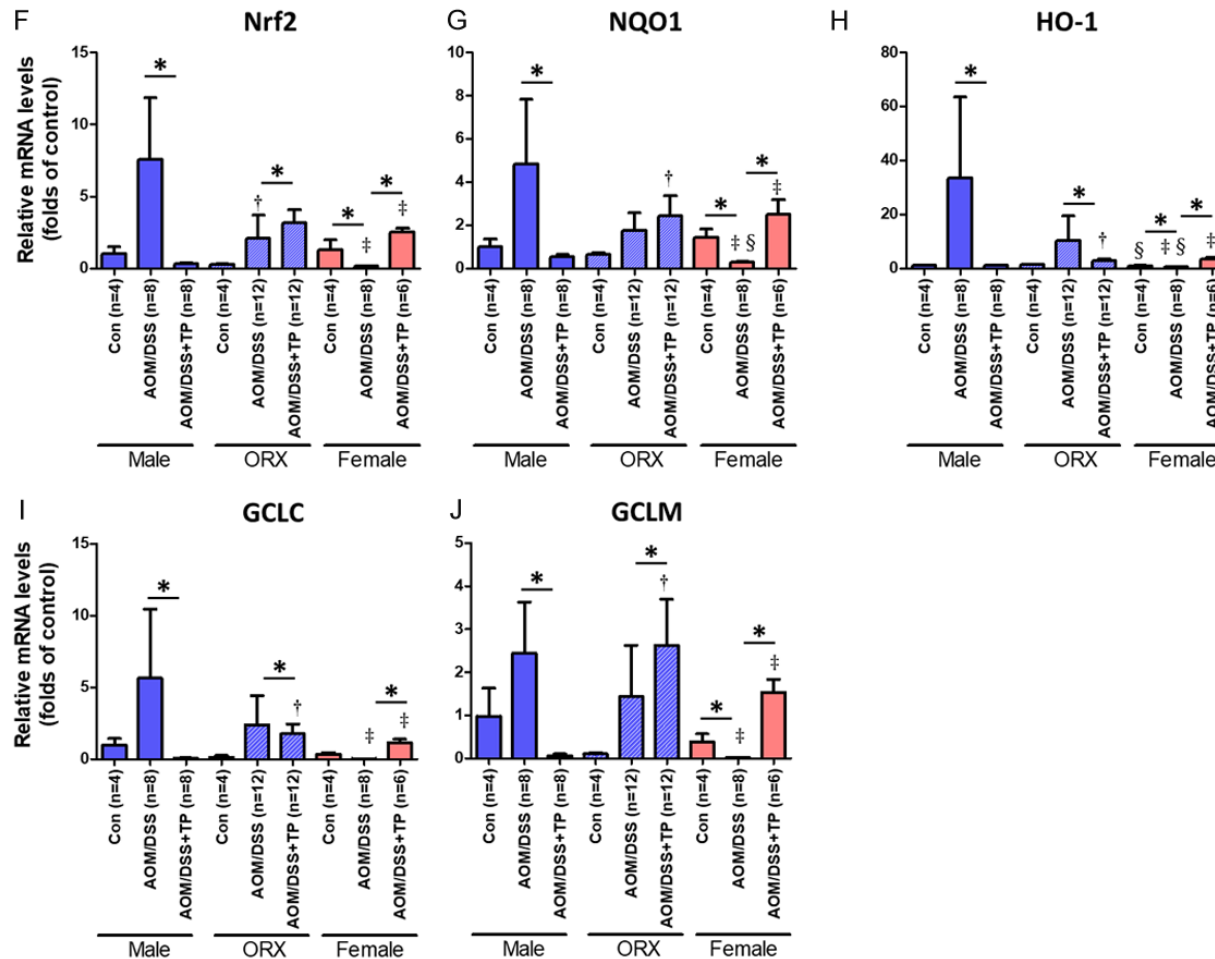
Figure 6. Effects of orchiectomy and TP on the concentration and the expression levels of proinflammatory mediators and anti-oxidant enzyme genes in colon tumors at week 13. (A-C) The concentration of MPO (A), IL-1β (B), and IL-6 (C) in the tumor and non-tumor tissues of the colon at week 13. (D-J) At week 13, AOM/DSS and AOM/DSS+TP group samples were prepared from colon tumor tissue, and control samples were prepared from normal colon tissue to analyze mRNA expression of COX-2 (D), iNOS (E), Nrf2 (F), NQO1 (G), HO-1 (H), GCLC (I), and GCLM (J) by qRT-PCR analysis. *, P<0.05 for between indicated two groups; †, P<0.05 for male vs. ORX male; ‡, P<0.05 for male vs. female; §, P<0.05 for ORX male vs. female. Con, control; AOM, azoxymethane; DSS, dextran sulfate sodium; TP, testosterone propionate; ORX, orchiectomized; MPO, myeloperoxidase; IL-1β, interleukin 1 beta; COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H dehydrogenase (quinone) 1; HO-1, Heme oxygenase 1; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit.

showed lower serum testosterone concentrations than intact male controls, with serum levels restored following exogenous administration. In male control mice, serum testosterone levels were significantly higher at week 13 (22 weeks of age) than at week 2 (11 weeks of age), revealing an age-dependent increase, as revealed in a previous report [37]. In ORX male and female mice, serum testosterone levels were unaltered following AOM/DSS treatment compared to controls in the inflammation stage (week 2); but, the levels were strongly enhanced in the AOM/DSS-treated mice when compared to the controls in the carcinogenesis stage (week 13). Numerous reports have revealed that elevated testosterone concentrations within serum are positively associated with the diseases risk including liver cancer [42], prostate cancer, lung cancer [17], and breast cancer recurrence [43]. Based on previous findings [17, 42, 43], our data support that circulating testosterone could be useful as a predictive marker of CRC progression in ORX and female mice after AOM/DSS treatment, but not in untreated males.

After tumor development (week 13), we observed interesting differences between the male AOM/DSS group and ORX male or female AOM/DSS group, and between the AOM/DSS and AOM/DSS+TP groups. Most colon tumors, as in previous reports [25], developed distal. In the distal colon, AOM/DSS-treated male mice presented a higher tumor numbers than AOM/DSS-treated ORX males and females, especially tumors > 2 mm in size. Moreover, TP administration increased the tumor numbers compared to the AOM/DSS group, and only females significantly increased the tumor numbers (> 2 mm). Interestingly, TP administration increased the development of submucosal invasive adenocarcinoma in males and females treated with AOM/DSS. Furthermore, endogenous and exogenous testosterone levels were identified as reasonable risk factors for developing distal colon submucosal invasive adenocarcinoma, based on multivariate and univariate logistic regression analyses. According to a recent report, the molecular, pathological, and clinical characteristics of colon cancer and survival rates in colon cancer differ depending on the anatomical location [44]. Furthermore, there is a sex difference in the CRC location. Generally, female CRC is more prevalent in the proximal colon, but in males, CRC is mainly located in the distal colon [7]. Furthermore, the depletion of endogenous estrogen by oophorectomy significantly increases tumor numbers and CRC incidence only in the proximal colon after AOM/DSS treatment compared to females treated with AOM/DSS, and these increments were strongly decreased following administration of exogenous E2 [29]. But, oophorectomy did not affect the occurrence of CRC in the distal colon [29]. These findings suggest a relationship between sex hormones and the anatomical location of CRC. The colon is anatomically divided into proximal (right side) and distal (left side) part. Proximal colon cancer is frequently mucinous and has higher microsatellite instability (MSI), with major carcinogenic pathways such as mutant BRAF[V600E] mutated more often, regardless of the histological type [44]. Distal colon cancer is more frequently accompanied by chromosomal instability and epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) amplification, often overexpressing epiregulin [44]. These present results strongly support the key role of testosterone in colitis and colon tumorigenesis, especially in the tumorigenesis of the distal part of the colon, as shown in ORX mice and female mice. However, no key pathogenic mechanism has clarified why testosterone causes this specific CRC. In-depth mechanistic studies are needed to investigate the severity of colitis and CRC progression based on the anatomical distribution of the colon in ORX male mice, and we are plan to undertake this research challenge.

Another limitation is that the animals were sacrificed at week 13 instead of week 16 after the AOM injection. In our previous studies, the colitis-associated CRC mouse model was induced using AOM (10 mg/kg) and 2.5% (w/v) DSS. In the current study, several male mice died following TP supplementation under the same conditions; hence, animals could not be harvested at week 16 after AOM injection, similar to previous studies. Hence, we lowered the DSS concentration from 2.5% to 2% and altered the timepoint for harvesting tissues from week 16 to week 13 after the AOM injection. Accordingly, the number and severity of tumors in AOM/DSS-treated males in the present study were slightly lower than those observed in similarly treated male mice in previous studies.

In conclusion, our results show that testosterone supplementation further increased inflammation and carcinogenesis in males, ORX males, and females in a chemically induced colitis-associated CRC mouse model. Our data revealed that both endogenous and exogenous testosterone are associated with CRC progression, which is associated with larger tumors and greater depth of invasion. A comprehensive description of the role of sex hormones in colitis and colon carcinogenesis is important for a deeper understanding of sex-specific differences in CRC progression. These findings may provide useful therapeutic strategies depending on sex, sex hormone levels, and disease state.

Acknowledgements

Disclosure of conflict of interest

None.

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