Sex disparity in colonic adenomagenesis involves promotion by male hormones, not protection by female hormones

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It recently has been recognized that men develop colonic adenomas and carcinomas at an earlier age and at a higher rate than women. In the $Apc^{Pirc/+}$ (Pirc) rat model of early colonic cancer, this sex susceptibility was recapitulated, with male Pirc rats developing twice as many adenomas as females. Analysis of large datasets revealed that the $Apc^{Min/+}$ mouse also shows enhanced male susceptibility to adenomagenesis, but only in the colon. In addition, WT mice treated with injections of the carcinogen azoxymethane (AOM) showed increased numbers of colonic adenomas in males. The mechanism underlying these observations was investigated by manipulation of hormonal status. The preponderance of colonic adenomas in the Pirc rat model allowed a statistically significant investigation in vivo of the mechanism of sex hormone action on the development of colonic adenomas. Females depleted of endogenous hormones by ovariectomy did not exhibit a change in prevalence of adenomas, nor was any effect observed with replacement of one or a combination of female hormones. In contrast, depletion of male hormones by orchidectomy (castration) markedly protected the Pirc rat from adenoma development, whereas supplementation with testosterone reversed that effect. These observations were recapitulated in the AOM mouse model. Androgen receptor was undetectable in the colon or adenomas, making it likely that testosterone acts indirectly on the tumor lineage. Our findings suggest that indirect tumor-promoting effects of testosterone likely explain the disparity between the sexes in the development of colonic adenomas.

colon cancer | animal models | androgens | estrogens | intestinal regionality

Epidemiologic studies have identified a number of factors that influence the risk of sporadic adenomas and colorectal cancer (CRC). Age, familial predisposition, racial background, diet, physical activity, obesity and the metabolic syndrome, smoking, and heavy alcohol use are all established risk factors for the development of CRC. In addition, the risk of CRC also shows sexual dimorphism, with a lower incidence and delayed onset in women (1, 2). Colonoscopic screening of asymptomatic individuals has corroborated male sex as a risk factor for the development of both adenomas and CRC in all age groups (3, 4); however, whether this disparity depends on protective factors in women, tumor-promoting factors in males, or both is unknown.

A protective role of female hormones against the development of frank CRC is suggested by data from the Women's Health Initiative (WHI). In the WHI, two large randomized controlled trials examined the effects of hormonal replacement therapy on postmenopausal women over a 5-y period, using CRC development as one of the endpoints. The first study showed that combined treatment with both equine estrogen (E2) and medroxyprogesterone acetate (MPA) substantially reduced the risk of colorectal cancer compared with placebo (odds ratio, 0.63) after a 5-y follow-up (5). However, protection was not found in a second randomized controlled trial among women who had previously undergone hysterectomy and were treated solely with equine estrogen (odds ratio, 1.08) (6). Although treatment with a combination of female hormones may be protective against a 5-y incidence of CRC in postmenopausal women, whether this effect involves the same mechanism as that in the differences between the sexes in adenomagenesis and CRC is unknown.

Animal models of colonic neoplasia permit an experimental approach to examining the molecular basis of the disparity between males and females. The most frequently used mouse model of intestinal adenoma development is the $Apc^{Min/+}$ (Min) mouse. These mice carry a truncating mutation in the *Apc* gatekeeper tumor-suppressor gene, which is also mutated in the majority of adenomas and CRCs in humans (7, 8). However, Min and most other mouse genetic models of intestinal neoplasia develop tumors primarily in the small intestine. Thus, studies of risk factors for lesions in the colon of the Min mouse would require a major effort to achieve statistically significant results.

Significance

The age-adjusted incidence of colonic adenomas and colorectal cancer is higher in men than in women. In a careful analysis of two established animal models, we found that castration reduced, and testosterone supplementation restored, the number of adenomas in the male rat and mouse colon, whereas ovariectomy and replacement of female hormones had no measureable effect on colonic adenomagenesis. In Min mice, in which most of the tumors arise in the small intestine, this testosterone-dependent sexual dimorphism in mice was specific to the colon. Our results support a paradigm shift: Testosterone promotes early adenomagenesis through an indirect mechanism, explaining the enhanced susceptibility of males to colonic adenomagenesis in the human, rat, and mouse.

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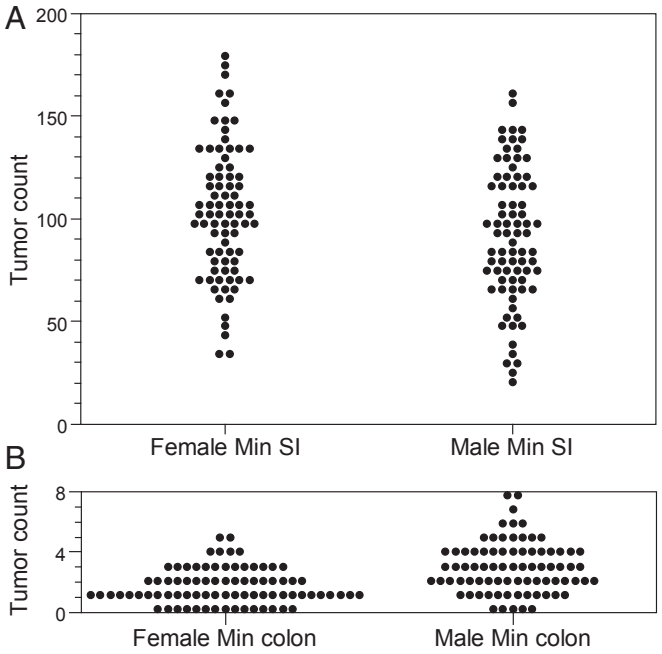

Fig. 1. Differential sex effect on the C57BL/6JD-*ApcMin* mouse phenotype. Male and female Min offspring from 57 litters were scored at sacrifice (90–120 d of age) for adenomas of the colon and small intestine. To minimize the effect of any variation among litters, the litters were selected as containing at least one Min animal of each sex. Tumor scoring was carried out as described previously (35), primarily by a single experienced observer (A.S.). Scatterplots were created for each sex for the small intestine (*A*) and colon (*B*). SI, small intestine.

We have previously reported a rat mutant model of adenoma development in which a nonsense mutation in the *Apc* gene of the rat, *Polyposis in the rat colon* ($Apc^{Pirc/+}$), leads to multiple adenomas, primarily in the colon. Similar to humans, Pirc males develop an increased adenoma burden with earlier onset than Pirc females (9). In agreement with this genetic model, male rats were found to be more susceptible to the chemical induction of colorectal tumors with the carcinogen dimethylhydrazine (10). Thus, it appears that sexual dimorphism in the incidence of colonic neoplasia results from intrinsic biological differences rather than from environmental differences, such as exposure to carcinogens. Given the Pirc rat's high multiplicity of colonic adenomas, we used this model to elucidate the underlying biological cause of this sex effect. We were able to substantiate these findings in a complementary model in which mice were treated with repeated injections of the carcinogen azoxymethane (AOM), in which a clear difference in colonic adenomagenesis owing to promotion by testosterone was observed as well.

Methods

Animal Experiments. All experiments were performed in accordance with guidelines of the Experimental Animal Committees of the Universities of Wisconsin and Amsterdam. Co-isogenic Pirc rats and WT controls were bred at the McArdle Laboratory for Cancer Research as described previously (9). For the AOM mouse model of adenomagenesis, C57BL/6JOlaHsd mice (Harlan) received six weekly injections of AOM 10 mg/kg following the protocol described by Neufert et al. (11) and were killed at the indicated time points.

Breeding of the C57BL/6JD-Apc^{Min} Colony. The congenic mouse strain carrying the *Min* allele of *Apc* on the C57BL/6J genetic background is subject to genetic modifiers arising by spontaneous mutation, either in our laboratory or in the production colony at the Jackson Laboratory (12). To maintain the classical Min phenotype, we established a "closed B6-Min colony" at the McArdle Laboratory for Cancer Research in which animals are maintained as breeders only if their Min progeny exhibit total intestinal tumor multiplicities of 100 ± 30. Following nomenclature protocol, this colony is designated C57BL/6JD-Apc^{Min}, where "D" stands for Dove.

Hormonal Manipulations. Animals were randomized within litters and subjected to OVX, ORX, or sham operation. For female hormone replacement in the rat, slow-release pellets of MPA, E2, a combination of MPA and E2, or vehicle only were implanted s.c. For male hormone replacement in the rat, DHT or vehicle-only pellets were used. For rat experiments, all hormones (Innovative Research of America) were added to the pellets, which were implanted s.c. in the nape of the neck at the time of OVX or ORX. Pellets with female hormones were fabricated as 90-d slow-release formulations and contained a total of 25 mg of MPA (~1 mg/kg/d) or 0.1 mg of E2 (~4 μg/kg/d). Pellets with DHT were fabricated as 90-d slow-release pellets containing 10 mg of DHT per pellet (~0.5 mg/kg/d) for Pirc animals. Vehicle-only (placebo) pellets were of the same size and composition as pellets containing the designated steroid hormone, but contained no functional substance. For the experiments in Pirc rats, surgery was performed at 30–40 d of age, and a new pellet was implanted 90 d later.

To supplement ORX mice with male hormones, we administered i.m. injections of testosterone enanthate 0.5 mg per mouse in a 50-μL volume or vehicle every 2 wk, as described by Zielinski and Vandenbergh (13). Hormone supplementation was initiated on the day of surgery.

Tissue Processing and Counting of Lesions. For scoring of Min, Pirc, and AOM adenomas, tissue was fixed in 10% formalin overnight at room temperature and then transferred to 70% ethanol. Only tumors >1 mm in maximum diameter were counted. Counting of lesions under a dissection microscope was performed blinded for sex and treatment.

Immunohistochemistry for *Ar*. Immunohistochemistry analyses in the mouse were performed using a rabbit polyclonal anti-*Ar* antibody (*N*-20, Sc-816; Santa Cruz Biotechnology). For this, 4-μm-thick sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol for 30 min. For antigen retrieval, slides were boiled at 100 °C for 20 min in 0.01 mol/L sodium citrate (pH 6), then blocked in PBS with 0.1% Triton X-100 and 1% BSA for 30 min, followed by incubation overnight at 4 °C with the primary antibody in PBS with 0.1% Triton X-100 and 1% BSA. Antibody binding was visualized with Powervision HRP-labeled secondary antibodies (Immunologic) and diaminobenzidine for substrate development. For the rat, immunohistochemistry analyses for *Ar* were performed by IDEXX BioResearch using polyclonal rabbit antibody RG-21 (Upstate Biotechnology).

qRT-PCR for *Ar* RNA in the Mouse. To examine *Ar* expression levels, we isolated RNA from homogenates of mouse testes, brain, liver, small intestine, and colon and from organoids of primary mouse small intestinal epithelium. Organoids were grown as described previously (14). To separate small intestinal and colonic epithelial cells from the rest of the intestinal mucosa, small pieces of intestine were incubated in ice-cold 5 mM EDTA containing 10 μM Rock inhibitor (Y27632; Sigma-Aldrich) for 20 min and then centrifuged at 800 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended and incubated for a further 20 min in ice-cold 5 mM EDTA containing 10 μM Rock inhibitor. After 5 min of centrifugation at 800 × *g*, the supernatant was again discarded, and the pellet was resuspended in PBS containing 2% FCS and 10 μM Rock inhibitor. Cells were then incubated with anti-EpCAM G8.8 FITC (1:50, SC-53532; tebu-bio) and anti-Cd45 PE-30 F11 (12–0451-82; Affymetrix eBioscience), and then sorted into an epithelial

Table 1. Effect of female hormones on body weight

Operation + hormone replacement	*n*	Weight, g, mean ± SD	*P* value* compared with OVX + placebo
Sham + placebo	7	261.9 ± 14.4	0.003
OVX + placebo	8	300.8 ± 16.1	NA
OVX + MPA	10	300.7 ± 20.6	0.96
OVX + MPA + E2	9	270.9 ± 20.6	0.007
OVX + E2	10	272.9 ± 30.2	0.03

Female Pirc rats were subjected either to OVX or to sham operation. The OVX females were then supplemented with MPA, MPA and E2, or E2 alone. E2 supplementation was sufficient to return the body weight of OVX females to that of sham-operated females ($P = 0.19$). NA, not applicable.
*Wilcoxon rank-sum test, two-sided.

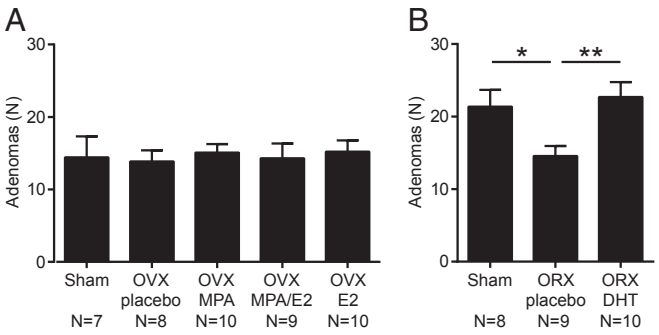

Fig. 2. Adenoma development in the Pirc rat is promoted by DHT and not affected by female hormones. (*A*) Numbers of adenomas in female Pirc rats are not affected by either OVX or OVX plus supplementation of female hormones. (*B*) Compared with sham-operated Pirc male rats, ORX reduces the numbers of adenomas to those observed in female rats. Treatment of ORX male Pirc rats with DHT causes an increase in the numbers of adenomas to levels seen in non-ORX males. Data are mean ± SEM. $*P < 0.05$; $**P < 0.01$.

fraction ($EpCAM^+$; Cd45) and a nonepithelial fraction ($EpCAM^-$) using fluorescence-activated cell sorting. Tissue, sorted cells, and organoids were lysed in RLT buffer with 1% β-mercaptoethanol. RNA was extracted with the RNEasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA synthesis was performed with RevertAid reverse transcriptase (Thermo Scientific) according to the manufacturer's protocol. Quantitative RT-PCR was performed with the Sensifast Sybr No-Rox Kit (GC Biotech) with Hot-Start Taq polymerase. The primers were as follows: mouse *Ar*: forward, GGTCTTCTGGGGTGGAAAGT; reverse, GGGACCTTGGATGGAGAACT; mouse *GAPDH*: forward, TGTGTCCGTCGTGGATCTGA; reverse, TTGCTGTTGAAGTCGCAGGAG. Methods for the RT-PCR experiments in the Pirc rat are described in Table S1, and primers for the rat are listed in Table S2.

Statistical Analysis of Tumor Count Data. All tumor count data are presented as mean ± SEM. Significance levels were calculated using the Student *t* test.

Results

To examine in detail the effect of sex on tumor multiplicity in the Min mouse, we assembled data on Min animals from the McArdle Laboratory's closed colony between 2006 and 2012 (*Methods*). Eighty females and 78 males from 57 litters were selected based on the following criteria: $Apc^{Min/+}$, 90–120 d old at death, no treatments, and at least one Min mouse of each sex in each litter. In the small intestine, Min females developed slightly more adenomas than males (females, 103 ± 4 vs. males, 92 ± 4; $P = 0.03$) (Fig. 1*A*). In contrast, a scatterplot of this large dataset demonstrates significantly enhanced adenoma multiplicity in the colon of Min males (males, 2.9 ± 0.2 vs. females, 1.6 ± 0.1; $P < 0.0001$) (Fig. 1*B*). In the past, this effect was obscured by the very low number of adenomas in the colon of the Min mouse and most other *Apc*-dependent mouse models of intestinal neoplasia. This differential sex bias can be easily detected and analyzed in the Pirc rat, with its preponderance of colonic tumors. The experiments were carried out on the coisogenic F344/NTac genetic strain, but the relative male susceptibility to colonic adenoma formation has also been observed in the ACI and BN congenic Pirc colonies at McArdle.

To test the effect of female hormones in our rat models, we performed ovariectomy (OVX) with and without hormone replacement. Controls were subjected to a sham operation, in which both ovaries were left in situ. During the operation, animals underwent s.c. implantation of pellets containing placebo, the progestin MPA, 17β-estradiol (E2), or a combination of the two steroids (Fig. S1). Body weight normally increases after OVX and decreases after the administration of E2 (15); thus, we followed body weight as a measure of steroid administration. Females that underwent OVX and received either E2 or the combination of E2 and MPA had reduced body weight and were much leaner than animals treated with MPA alone or placebo (Table 1).

Animals were killed at 210 d of age, when a significant adenoma load had developed. Despite the effect of the hormones on body weight (Table 1), no significant difference in colonic tumor numbers was observed between groups (Fig. 2*A*). Thus, our results indicate that female hormones do not influence the formation of adenomas in the Pirc rat colon.

We next analyzed the numbers of colonic lesions in male littermates of the females studied earlier. We corroborated our previously reported findings (9) that males develop more colonic adenomas than females [22.4 ± 3.9 ($n = 33$) vs. 12.1 ± 1.8 ($n = 44$); $P < 0.0001$]. Consequently, we tested a new hypothesis, that the observed disparity between sexes in colonic adenomagenesis is caused by a tumor-promoting effect of male hormones rather than a protective effect of female hormones.

Pirc males underwent sham operation or orchidectomy (ORX), followed by s.c. implantation of pellets containing either placebo or dihydrotestosterone (DHT) (Fig. S1). At ∼210 d of age, rats implanted with placebo-containing pellets had significantly fewer colonic adenomas than sham-operated males (14.6 ± 1.4 vs. 21.4 ± 2.3; $P = 0.02$), whereas ORX followed by DHT reversed this effect (22.7 ± 2.1 vs. ORX plus placebo; $P = 0.005$) (Fig. 2*B*). We note the tumor load in males that underwent ORX and were implanted with placebo-containing pellets was reduced to levels similar to those seen in females. Thus, it appears that the development of colonic adenomas is the direct or indirect result of testosterone, the principal hormone produced in male gonads.

To examine the tumor-promoting role of male hormones in a complementary model of adenomagenesis, we used a chemical model in which mice received six weekly injections with the carcinogen AOM (11) (Fig. S2). We compared the incidence of adenomas in males ($n = 10$) and females ($n = 9$) at 25 wk after the first AOM injection. As in humans and the Pirc rat, the incidence was higher in males (1.5 ± 0.2 vs. 0.9 ± 0.2; $P = 0.03$) (Fig. 3*A*). To test whether this result depended on male hormones, we performed a sham operation ($n = 13$) or ORX ($n = 13$) and initiated the AOM injections at 1 wk after surgery, then killed the animals at week 30 after the first AOM injection. We found that ORX substantially reduced the numbers of adenomas compared with the sham operation (2.4 ± 0.5 vs. 0.7 ± 0.2; $P = 0.005$) (Fig. 3*B*). In a third experiment, we performed ORX and then treated one group with i.m. vehicle injections every other week ($n = 10$) and a second group with i.m. testosterone enanthate 0.5 mg injections every other week ($n = 8$). At 25 wk after

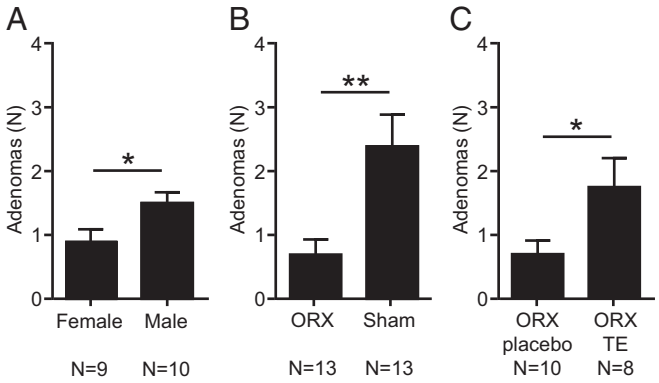

Fig. 3. Adenoma development is promoted by male hormones in the AOM mouse model. (*A*) Results of the male vs. female mouse experiment. In comparison with female mice, male mice have an increased adenoma burden. (*B*) Results of the ORX experiment. The numbers of adenomas in mice that underwent ORX are reduced compared with sham-operated mice. (*C*) Results of the testosterone supplementation experiment. Testosterone supplementation of male mice that had undergone ORX substantially increased the numbers of adenomas. Data are mean ± SEM. $*P < 0.05$; $**P < 0.01$.

Table 2. Expression of *Ar* in mouse tissues as determined by qRT-PCR

Tissue	Samples, *n*	Measurements per sample, *n*	AR fold mRNA expression,* mean ± SD
Testes	2	3	1.00 ± 0.14
Brain	2	3	0.37 ± 0.05
Liver	2	3	0.20 ± 0.05
Small intestine, proximal	2	1	0.02 ± 0.002
Small intestine, middle	2	1	0.01 ± 0.001
Small intestine, distal	2	1	0.02 ± 0.001
Colon	2	1	0.04 ± 0.01
Organoids	2	1	ND

Expression levels in small intestine and colon were very low compared with those in prostate, brain, and liver. No *Ar* expression was detected in organoid cultures of primary small intestinal epithelium. ND, not detectable.
*Relative to testes.

the first AOM injection, the mice receiving testosterone enanthate had substantially more adenomas than those receiving placebo (0.7 ± 0.2 vs. 1.8 ± 0.5; $P = 0.04$) (Fig. 3*C*). In these experiments, blood testosterone level measurements at 1 wk after the final injection revealed mean ± SEM values of 1.8 ± 0.4 nmol/L for intact males, 0.25 ± 0.05 nmol/L for orchidectomized males receiving vehicle-only injections, and 16.4 ± 2.3 nmol/L for orchidectomized males receiving testosterone enanthate. These results recapitulate those obtained in the Pirc rat and further support a tumor-promoting role of male hormones rather than a suppressive effect of female hormones.

To determine whether androgens act directly on intestinal epithelial cells to promote adenomagenesis, we examined the localization and expression of the androgen receptor gene (*Ar*) in the small intestine and colon of the mouse and rat. In the mouse, quantitative RT-PCR (qRT-PCR) on lysates of prostate, brain, liver, and various intestinal segments revealed ∼10- to 20-fold lower *Ar* expression in the small intestine and colon compared with the other organs (Table 2). In the rat, this difference in expression was similar (Table S1). Furthermore, no detectable *Ar* expression was observed in organoid cultures of pure mouse primary intestinal epithelial cells. We then isolated intestinal cells from fresh mouse small intestine and colon and sorted them into epithelial ($EpCAM^+$) and nonepithelial ($EpCAM^-$) cells, and confirmed that the *Ar* gene was expressed exclusively in the nonepithelial cells (Table 3).

These qRT-PCR findings were confirmed by immunohistochemical analysis of the androgen receptor. No *Ar*-positive cells were found in the epithelium of either small intestine or colon in the mouse or rat, using prostate and testis as positive controls (Fig. 4).

Discussion

In humans, the colonic adenoma-to-carcinoma sequence shows clear sexual dimorphism, with preferential male development of both adenomas and CRC (16). The reasons for this difference remain under investigation. Confounding factors in human studies include obesity, carcinogen exposure through diet or smoking, variations in physical activity, and comorbidities of other cancers. Thus, animal models may play an important role in elucidating this effect. Models carrying mutations in the *Apc* gene genetically parallel both familial and sporadic colon adenoma development in humans. The Min mouse and Pirc rat are such models.

The Pirc rat develops intestinal tumors preferentially in the colon, allowing ready analysis of male susceptibility to colonic adenomagenesis in this model. In female Pirc rats, adenoma development did not respond to OVX or hormone replacement. In contrast, abrogation of male hormone production by ORX resulted in reduction of tumor numbers to those seen in females (Fig. 2). Subsequent DHT replacement reversed this number to that seen in sham-operated littermates. These observations establish that in the Pirc rat, sex disparity in adenomagenesis depends directly or indirectly on a tumor-promoting effect of testosterone, rather than on a protective effect of female hormones.

A frequently used rodent model for the development of colonic adenomas involves repeated injections with the chemical carcinogen AOM. AOM treatment of the C57BL/6 mouse recapitulated the observations in the Pirc rat: preferential susceptibility of males and a reduced incidence of colonic tumors after ORX, with a return to a normal rate after testosterone replacement (Fig. 3). Thus, in this mouse model as in the Pirc rat, adenoma incidence is promoted by male hormones rather than suppressed by female hormones. Furthermore, because most mouse mutants are carried on a C57BL/6 background, this model can serve as a useful platform for molecular genetic analysis of the sexual dimorphism of colonic tumorigenesis.

Evidence for an indirect effect of testosterone has been found by analyzing the tissue distribution of *Ar*. In the intestinal epithelium of both mouse and rat, *Ar* expression levels were below detectable limits (Tables 2 and 3 and Table S1). We note that a promoting effect of testosterone on hepatocarcinogenesis extrinsic to the tumor lineage was previously demonstrated in an elegant use of mice mosaic for the *Tfm* mutation in the androgen receptor (17). One indirect mechanism for this effect is the increase in stress hormones, such as cortisol, affecting the tumor environment (18). Furthermore, studies of *Ar* KO mice have indicated that the immune system is regulated by androgens. For example, Chuang et al. (19) found reduced neutrophil counts in castrated males that could be restored to normal levels through androgen supplementation, implicating the innate immune system. Our evidence that testosterone acts indirectly to promote colonic adenomagenesis opens the possibility of testing these indirect mechanisms, using the power of the molecular genetics of the mouse and rat to ablate the *Ar* gene in somatic lineages that are candidates for the site of testosterone action (20).

Our findings should be compared with previous reports involving the Min mouse and the AOM rodent models, as well as with studies of hormonal replacement in human cohorts. The number of adenomas developing in the colon of Min mice is very low, limiting its usefulness in analyzing a sex effect on colonic adenomagenesis; a large number of animals is needed to

Table 3. *Ar* expression in mouse intestinal $EpCAM^+$, $EpCAM^-$ and unsorted cells

Tissue	No. of samples	No. of measurements per sample	*Ar*, relative mRNA expression × 10^{-4}, mean ± SD*		
			Unsorted	$EpCAM^-$	$EpCAM^+$
Small intestine	2	3	1.9 ± 1.0	1.6 ± 0.3	ND
Colon	2†	3	2.6 ± 0.7	7.4 ± 5.3	ND

Primers for mouse GAPDH: TGTGTCCGTCGTGGATCTGA and TTGCTGTTGAAGTCGCAGGAG. ND, not detectable.
*Relative to GAPDH.
†Colons from four animals pooled per sample.

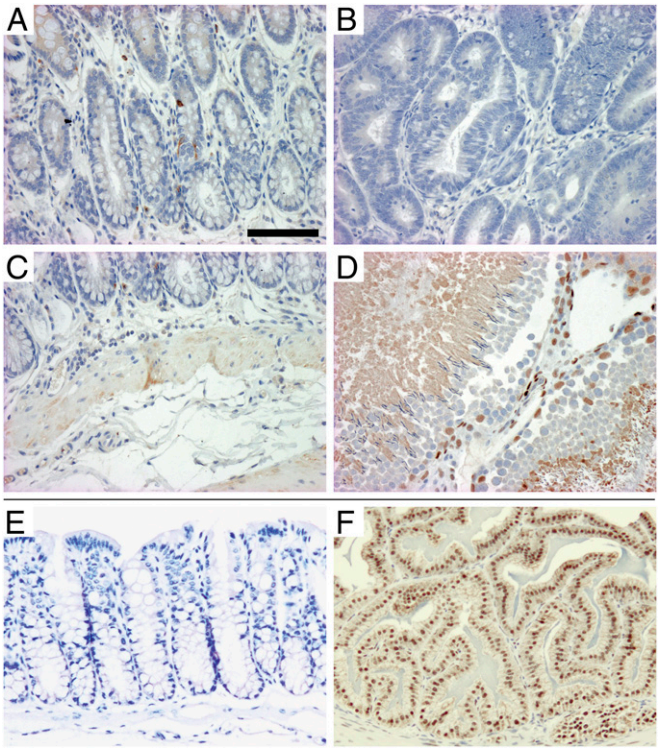

Fig. 4. IHC for androgen receptors in rat and mouse tissues. (*A*) Positive staining for the androgen receptor protein is seen in rare cells within the crypt and stroma of the mucosa. (*B*) Colonic adenomas show no staining for androgen receptor. (*C*) Diffuse staining is seen in the submucosa and muscularis in normal adjacent colon. (*D*) Positive control for androgen receptor staining in rat testis. (*E*) No positive staining for androgen receptor protein is seen in mouse colon. (*F*) Positive control for androgen receptor staining in mouse seminal vesicles.

confer statistical significance. Thus, only by analyzing a very large cohort of Min mice were we able to demonstrate the significantly higher (twofold) male susceptibility to colonic adenomagenesis (Fig. 1). In contrast, in the small intestine of the Min mouse, OVX reportedly increases adenomagenesis, perhaps owing to the loss of a suppressive estrogen effect (21, 22). However, our analysis of the large dataset of Min mice from the McArdle Laboratory provided only marginally significant evidence for a 1.1-fold increased female susceptibility to adenoma development in the small intestine (Fig. 1). How this subtle female susceptibility is related to the reported enhancement by OVX is not clear. Further investigation of the female susceptibility of the small intestine in the Min mouse is hindered by the small size of the effect.

A complication to understanding the published studies on the effects of OVX on colonic adenomas in the Min mouse is a lack of reproducibility, perhaps related to the low numbers of colonic tumors. One report described a significant reduction in colonic adenomas after OVX, with a reversal to normal numbers by supplementation with 17β-estradiol (22). This tumor-promoting role for estrogens is consistent with the strong estrogen enhancement effect on colonic tumorigenesis associated with inflammation in the mouse (23). This estrogen effect was not reproduced in a subsequent report by the same group, however (24). In a later analysis of the effects of estrogen on colonic adenomagenesis using *Er* receptor mutant mice (25), OVX plus hormone replacement was not performed, and male and female mice were not differentiated. Studies in mice with intact endogenous hormone production leave open the possibility that genetically inactivating only one of the two *Er* receptors leads to off-target hormone action. A well-known example of this effect is the increased plasma estrogen levels seen in *ERα* mutant mice (26). In this concept, changes in adenomagenesis could be caused by aberrant hormone action rather than by the absence of normal estrogen function.

Additional apparent contradictions to our observations are found in published studies of AOM-induced colonic adenomas in both rat and mouse. For example, one study found that AOM-induced tumor formation was dependent on male hormones in F344 rats (27); however, a second report by the same group found no effect of ORX in Sprague–Dawley rats (28). Another study reported female susceptibility to AOM-induced colonic adenomagenesis in the highly susceptible A/J mouse strain (29), in contrast to the male susceptibility in the C57BL/6 mouse strain reported herein. These differences are strain-dependent, perhaps owing to genetic variation in carcinogen metabolism trumping the biological variation of sex. AOM-induced colonic adenomagenesis in the mouse has been shown to be controlled by polymorphisms at numerous loci (30).

Finally, it is important to understand the distinction between our studies of adenomagenesis and studies of hormonal replacement in human populations. Two unique large interventional studies (the WHI) have clearly established that combination therapy with estradiol and MPA (but not with estradiol alone) protects against frank CRC formation in postmenopausal women (5). At first glance, these studies appear to contradict our findings; in rats, OVX and female hormone replacement had no effect on the incidence of colonic adenomas. However, in the Pirc rat model, our endpoint was adenoma formation, not CRC as in the WHI. The differences seen at the adenoma stage suggest that male hormones act at an early stage in the sequence leading from adenoma to carcinoma (31–33). Thus, the molecular basis for the 5-y protective effect of female hormone supplementation against CRC in postmenopausal women may differ from the molecular basis of the sex-based difference in adenoma formation.

Although abrogation of female hormone production by OVX allows the study of the contribution of female hormones, it must be recognized that it does not recapitulate the events occurring during menopause. Of note, postmenopausal ovaries are not inert but remain hormonally active and are a significant source of testosterone (34, 35). Other factors may have contributed to the apparent discrepancy between our results and those of the postmenopausal WHI studies. In particular, hormones affect many biological pathways important in tumor progression, such as inflammation, which differ between animal models and humans. Although the incidence of CRC was reduced by female hormone treatment in the WHI study, this might not be represented in our animal experiments that specifically targeted adenoma formation driven by *Apc* mutations and the carcinogen AOM. Recent reports of invasive colonic adenocarcinomas in both mouse and rat models (36, 37) may present an opportunity to explore this dichotomy. More fully delineating the roles of male and female hormones in the development of adenomas and CRC will be important to gaining a deeper understanding of sexual dimorphism in the colorectal adenoma-to-carcinoma sequence and in hormone replacement therapy in postmenopausal women.

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