Between cancer and therapy: Studies of the colon
Wielenga, M.C.B.

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CHAPTER 3

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


*McArdle Laboratory for Cancer Research, Department of Oncology, University of Wisconsin–Madison, Madison, WI, USA 53706
Laboratory of Genetics, University of Wisconsin–Madison, Madison, WI, USA 53706
Tytgat institute for Liver and Intestinal Research and Department of Gastroenterology and Hepatology, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands
Merck, Sharpe and Dohme, Women’s Health Department, Molenstraat 110, 5342 CC Oss, the Netherlands
Center for Inflammatory Bowel Diseases, University of California Los Angeles, 10945 Le Conte Avenue, Los Angeles, CA, USA 90095.

M.C.B.W., J.M.A.-L. and J.H. contributed equally to this work.

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ABSTRACT

It has recently become recognized that men develop colonic adenomas and carcinomas at an earlier age and at a higher incidence than women. In the ApcPirc/+ (Pirc) rat model of early colonic cancer this sex susceptibility is recapitulated with male Pirc rats developing twice as many adenomas as females. Analysis of large datasets reveal that the ApcMin/+ mouse also shows enhanced male susceptibility to adenomagenesis, but only in the colon. In addition, we find that wildtype mice treated with injections of the carcinogen azoxymethane (AOM) show increased numbers of colonic adenomas in males compared to females. The mechanism underlying these observations can be investigated by manipulation of hormonal status. The preponderance of colonic adenomas in the Pirc rat model has enabled 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 do not exhibit a change in prevalence of adenomas, nor is any effect observed with replacement of one or a combination of female hormones. By contrast, depletion of male hormones by orchidectomy (castration) markedly protects the Pirc rat from adenoma development, while supplementation with testosterone reverses 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. In conclusion, indirect tumor-promoting effects of testosterone likely explain the disparity between sexes in the development of colonic adenomas.

SIGNIFICANCE

The age-adjusted incidence of colonic adenomas and colorectal cancer is higher in men than women. In 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, while ovariectomy and replacement of female hormones had no measureable effect on colonic adenomagenesis. In Min mice, where 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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INTRODUCTION

Epidemiological 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 examples of 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. Colonoscopic screening of asymptomatic individuals has corroborated male sex as a risk factor in development of both adenomas and CRC among all age groups. However, it is not known if this disparity depends upon protective factors in women, tumor promoting factors in males, or both.

A protective role of female hormones against the development of frank CRC is suggested by data from the Women’s Health Initiative (WHI). Two very large randomized controlled trials examined the effects of hormonal replacement therapy on postmenopausal women over a five year interval, 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 to placebo (odds ratio = 0.63) after five years follow-up. However, protection was not found in a second randomized controlled trial among women who had previously undergone hysterectomy and were treated only with equine estrogen (odds ratio = 1.08). Although treatment with a combination of female hormones may be protective against a five-year incidence of CRC in postmenopausal women, it is unknown whether this effect involves the same mechanism as that in the differences between the sexes in adenomagenesis and CRC.

Animal models of colonic neoplasia permit an experimental approach to examine the molecular basis of the disparity between males and females. The most frequently used mouse model of intestinal adenoma development is the \textit{ApcMin/\textplus} (Min) mouse. These mice carry a truncating mutation in the \textit{Apc} gatekeeper tumor suppressor gene that is also mutated in the majority of adenomas and CRCs in the human. 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.

We have previously reported a rat mutant model of adenoma development: a nonsense mutation in the \textit{Apc} gene of the rat, \textit{Polyposis in the rat colon (ApcPirc/\textplus)}, leads to multiple adenomas primarily in the colon. Similar to humans, Pirc males develop an increased adenoma burden with earlier onset than Pirc females. In agreement with this genetic model, male rats have previously been shown to be more susceptible to the chemical induction of colorectal tumors with the carcinogen dimethylhydrazine. 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. Benefitting from its high multiplicity of colonic adenomas, we have employed the Pirc rat to elucidate the underlying biological cause of this sex effect. We were able to substantiate these findings with a complementary model in which mice are treated with repeated...
injections of the carcinogen azoxymethane; here a clear difference in colonic adenomagenesis owing to promotion by testosterone was also observed.

**METHODS**

**Animal experiments**

All experiments were performed according to the experimental animal committee guidelines from the Universities of Wisconsin and Amsterdam. Co-isogenic F344/NTac-ApcPirc/+ (Pirc) rats and wild-type controls were bred in the facility at the McArdle Laboratory for Cancer Research as described previously. For the AOM mouse model of adenomagenesis, C57BL/6JolaHsd mice (Harlan, Boxmeer, the Netherlands) were injected with 6 weekly injections of azoxymethane10 mg/kg according to the previously published protocol of Neufert et al. and sacrificed at the indicated time points.

**Breeding of the C57BL/6JD-ApcMin colony**

The congenic mouse strain carrying the *Multiple intestinal neoplasia* (*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. To maintain the classical Min phenotype, we have 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 give total intestinal tumor multiplicities of 100 ± 30. Following nomenclature protocol, this colony is designated C57BL/6JD-ApcMin, where D stands for Dove.

**Hormonal Manipulations**

Animals were randomized within litters and subjected to ovariectomies (OVX), orchidectomies (ORX) or sham operations. For female hormone replacement in the rat, slow release pellets with medroxyprogesterone acetate (MPA), 17β-estradiol (E2), or a combination of MPA and E2, or vehicle only were implanted. For male hormone replacement in the rat, dihydrotestosterone (DHT), or vehicle-only pellets were used. For rat experiments all hormones (Innovative Research of America, Sarasota, FL, USA) were added to pellets, which were implanted subcutaneously in the nape of the neck at the time of OVX or ORX. Pellets with female hormones were fabricated as 90-day slow release formulations and contained a total of 25 mg MPA (approximately 1 mg/kg/day) or 0.1 mg E2 (approximately 4 µg/kg/day). Pellets with DHT were fabricated as 90-day slow release pellets that contained 10 mg DHT per pellet (approximately 0.5 mg/kg/day) 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 experiments in Pirc rats, surgery was performed at 30-40 days of age and a new pellet was implanted 90 days later.

To supplement ORX mice with male hormone we used intramuscular injections of testosterone enanthate 0.5 mg per mouse in a 50 µl volume or vehicle every two weeks as previously described by Zielinski and Vandenbergh. Hormone supplementation was initiated on the day of the surgery.
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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 transferred to 70% ethanol. Only tumors greater than 1 mm in maximum diameter were counted. Counting of lesions under a dissection microscope was performed blinded for sex and treatment.

Immunohistochemistry for AR.

For immunohistochemistry in the mouse a rabbit polyclonal anti-Ar antibody was used (N-20, Sc-816, Santa Cruz). Sections of 4 µm were deparaffinized in xylene and rehydrated. Endogenous peroxidase was blocked using 0.3% H2O2 in methanol for 30 minutes. For antigen retrieval, slides were boiled at 100°C for 20 minutes in 0.01 mol/L sodium citrate (pH 6). Slides were then blocked in PBS with 0.1% Triton X-100 and 1% bovine serum albumin for 30 minutes, followed by incubation overnight at 4°C with the primary antibody in PBS with 0.1% Triton X-100 and 1% bovine serum albumin. Antibody binding was visualized with Powervision HRP labeled secondary antibodies from Immunologic (Duiven, the Netherlands) and diaminobenzidine for substrate development. For the rat, immunohistochemistry for the AR was performed by IDEXX Bioresearch (Columbia, MO), using the polyclonal rabbit antibody RG-21 (Upstate).

Quantitative RT-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 previously described14. To separate small intestinal and colonic epithelial cells from the rest of the intestinal mucosa, small pieces of intestine were incubated in ice cold 5mM EDTA containing 10µM Rock inhibitor (Sigma #Y27632) for 20 min and centrifuged at 800g for 5 min. The supernatant was discarded, and the pellet was resuspended and incubated for a further 20 min in ice cold 5mM EDTA containing 10µM Rock inhibitor. After 5 min centrifugation at 800g the supernatant was again discarded and the pellet was resuspended in PBS containing 2% fetal calf serum and 10µM Rock inhibitor. Cells were then incubated with Anti-Epcam G8.8 FITC (1:50, Tebu Bio #SC-53532) and Anti-Cd45 PE-30 F11 (Bioscience #12-0451-82) and subsequently sorted into an epithelial fraction (Epcam+, Cd45) and a non-epithelial fraction (Epcam-) using fluorescence-activated cell sorting. Tissue, sorted cells and organoids were lysed in RLT buffer with 1% 8-mercaptoethanol. RNA was extracted with the RNeasy (Qiagen) minikit according to the manufacturer’s protocol. cDNA synthesis was performed with revertaid transcriptase (Thermoscientific) 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. Primers: mouse androgen receptor-forward GGTCTTCTGGGTGAAGAT, mouse androgen receptor-reverse GGACCTTGGGATAGAAGACT, mouse gapdh-forward TGTTCCGTCTGATCTTA, mouse gapdh-reverse TTGCTGTGAAGTCGAGGAG.

Statistical analysis of tumor count data

All tumor count data are presented as mean ± standard error of the mean. Significance levels were calculated by the Student’s t-test. For animal experiments, a Student t test or a 1-way analysis of
variance (ANOVA) test was used. All ANOVA tests were followed by Bonferroni’s post-test for multiple comparisons.

RESULTS
To examine in detail the effect of sex on tumor multiplicity in the Min mouse we have assembled data on Min animals from the McArdle Laboratory closed colony between 2006 and 2012 (see Methods). 80 females and 78 males from 57 litters were selected by the following criteria: ApcMin/+, 90-120 days old at death, no treatments, and at least one Min mouse of each sex in each litter. In the small intestine, Min females develop slightly more adenomas than males (female 103 ± 4 versus male 92 ± 4, p = 0.03; Figure 1A). By contrast, a scatterplot of this large data-set demonstrates a highly significant enhancement of adenoma multiplicity in the colon of Min males (male 2.9 ± 0.2 versus female 1.6 ± 0.1, p < 0.0001; Figure 1B). In the past, this effect was obscured by the very low number of adenomas in the colon shown by the Min mouse and most other Apc-dependent mouse models of intestinal neoplasia. By contrast, 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 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 received subcutaneous pellets containing either placebo, the progestin MPA, 17β-estradiol (E2) or a combination of both steroids (Supplemental Figure S1). Body weight normally increases after OVX and decreases upon the administration of E2\(^{15}\). Therefore, 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 observed to be much leaner than animals treated with MPA alone or placebo (Table 1).

Animals were killed at 210 days 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 (Figure 2A). 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 above. We corroborated our previous findings\(^4\) that males develop more colonic adenomas than females [22.4 ± 3.9 (N=33) vs. 12.1 ± 1.8 (N=44), P < 0.0001]. We therefore 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 surgeries or ORX followed by implantation with pellets containing either placebo or DHT (Supplemental Figure S1). At ~210 days of age, rats implanted with placebo-containing
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Figure 1. Differential sex effect on the C57BL/6J-D-ApcMin mouse phenotype. Male and female Min offspring from 57 litters were scored at sacrifice (90-120 days of age) for adenomas of the colon and small intestine. To minimize the effect of any variation between litters, the litters were selected as containing at least one Min animal of each sex. Tumor scoring was carried out as described (35), primarily by a single experienced observer (AS). Scatterplots were created for each sex for the small intestine (A) and the colon (B). SI, small intestine.

Table 1. Effect of female hormones on body weight. 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).

<table>
<thead>
<tr>
<th>Operation + hormone replacement</th>
<th>N</th>
<th>Weight (g), mean ± SD</th>
<th>p-value* compared to OVX + placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + placebo</td>
<td>7</td>
<td>261.9 ± 14.4</td>
<td>0.003</td>
</tr>
<tr>
<td>OVX + placebo</td>
<td>8</td>
<td>300.8 ± 16.1</td>
<td>NA</td>
</tr>
<tr>
<td>OVX + MPA</td>
<td>10</td>
<td>300.7 ± 20.6</td>
<td>0.96</td>
</tr>
<tr>
<td>OVX + MPA + E2</td>
<td>9</td>
<td>270.9 ± 20.6</td>
<td>0.007</td>
</tr>
<tr>
<td>OVX + E2</td>
<td>10</td>
<td>272.9 ± 30.2</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Wilcoxon rank sum test, two-sided.
NA, not applicable.
pellets had significantly fewer colonic adenomas than sham-operated males (14.6 ± 1.4 vs. 21.4 ± 2.3, 
P = 0.02) while ORX followed by DHT reversed this effect (22.7 ± 2.1 vs. ORX plus placebo, P = 0.005) 
(Figure 2B). We note the tumor load in males that underwent ORX and were implanted with placebo-
containing pellets was reduced to levels similar to that in females. Thus, it appears that development of 
colonic adenomas is the direct or indirect result of testosterone, the principal hormone made in male 
gonads.

Figure 2. Adenoma development in the Pirc rat is promoted by DHT and not affected by female 
hormones. (A) Adenoma numbers in female Pirc rats are not affected by either OVX or OVX plus 
supplementation of female hormones. (B) Compared to sham-operated Pirc male rats, ORX reduces 
adenoma numbers to those observed in female rats. Treatment of ORX male Pirc rats with DHT 
causes adenoma numbers to increase to levels found in non-ORX males. Data is depicted as mean ± 
s.e.m. * = P < 0.05; ** = P < 0.01.

To examine the tumor promoting role of male hormones in a complementary model of adenomagenesis 
we used a chemical model in which mice were given six weekly injections with the carcinogen 
azoxymethane (AOM) (11) (Supplemental Figure S2). We compared adenoma incidence between 
males (n=10) and females (n=9) at 25 weeks after the first AOM injection. As in the human and Pirc 
rat, we found a higher incidence in males than females (1.5 ± 0.2 vs 0.9 ± 0.2, P = 0.03) (Figure 3A). To 
test if this result depended on male hormones we performed a sham operation (n=13) or ORX (n=13) 
and initiated the AOM injections one week after surgery. Animals were sacrificed at week 30 after the 
first AOM injection and we found that ORX substantially reduced adenoma numbers compared to the 
sham procedure (2.4 ± 0.5 vs 0.7 ± 0.2, P = 0.005) (Figure 3B). In a third experiment we performed 
ORX and treated one group with vehicle injections i.m. every other week (n=10) and a second group 
(n=8) were treated with injections every other week of testosterone enanthate 0.5 mg/mouse i.m. At
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Figure 3. Adenoma development is promoted by male hormones in the AOM mouse model. (A) Results of the male versus female mouse experiment. In comparison to female mice, male mice have an increased adenoma burden. (B) Results of the ORX experiment. Adenoma number in mice that have undergone ORX is reduced compared to sham operated mice. (C) Results of the testosterone supplementation experiment. Supplementtion of male mice that had undergone ORX with testosterone substantially increased the adenoma number. Data is depicted as mean ± s.e.m. * = P < 0.05; ** = P < 0.01.

25 weeks after the first AOM injection those receiving testosterone enanthate had substantially more adenomas than those on placebo (0.7 ± 0.2 vs. 1.8 ± 0.5, P = 0.04) (Figure 3C). Blood testosterone levels in these experiments were measured 1 week after the final injection and found to be 1.8 ± 0.4 for intact males, 0.25 ± 0.05 for orchidectomized males receiving vehicle-only injections and 16.4 ± 2.3 for orchidectomized males receiving testosterone enanthate (nmol/L, mean ± s.e.m.). Thus these results recapitulated 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 the intestinal epithelial cells to promote adenomagenesis, we examined the localization and expression of the androgen receptor in the small intestine and colon of the mouse and rat. In the mouse, quantitative RT-PCR on lysates of prostate, brain, liver and different intestinal segments revealed Ar expression ∼10-20x lower in the small intestine and colon compared to the other organs (Table 2). In the rat this expression difference was similar (Supplemental Table S1). Further, in organoid cultures of pure mouse primary intestinal epithelial cells, no detectable Ar expression was observed. We then isolated intestinal cells from fresh mouse small intestine and colon and sorted them into epithelial (Epcam+) and non-epithelial (Epcam-) cells and confirmed that the Ar gene is exclusively expressed in the non-epithelial cells (Table 3).
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Table 2. Expression of Ar in mouse tissues as determined by qRT-PCR. Expression of Ar in different tissues of the mouse as determined by quantitative RTPCR. Expression levels in small intestine and colon are very low compared to prostate, brain and liver. No Ar expression is detectable in organoid cultures of primary small intestinal epithelium.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N of samples</th>
<th>N of measurements per sample</th>
<th>Androgen receptor, fold mRNA expression* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes</td>
<td>2</td>
<td>3</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>Brain</td>
<td>2</td>
<td>3</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>3</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>Small intestine, proximal</td>
<td>2</td>
<td>1</td>
<td>± 0.001</td>
</tr>
<tr>
<td>Small intestine, middle</td>
<td>2</td>
<td>1</td>
<td>± 0.001</td>
</tr>
<tr>
<td>Small intestine, distal</td>
<td>2</td>
<td>1</td>
<td>± 0.001</td>
</tr>
<tr>
<td>Colon</td>
<td>2</td>
<td>1</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Organoids</td>
<td>2</td>
<td>1</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

* Relative to testes

Table 3. Ar expression in mouse intestinal EpCAM+, EpCAM– and unsorted cells. Mouse intestinal cells were sorted into epithelial cells (Epcam+) and non-epithelial cells (Epcam-) showing that Ar is expressed in non-epithelial cells. Primers for mouse GAPDH: TGTGTCCGTCGTGGATCTGA and TTGCTGTTGAAGTCGCAGGAG.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N of samples</th>
<th>N of measurements per sample</th>
<th>Androgen receptor, relative mRNA expression x 10^4 (mean ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unsorted</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2</td>
<td>3</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td>Colon</td>
<td>2**</td>
<td>3</td>
<td>2.6 ± 0.7</td>
</tr>
</tbody>
</table>

ND, not detectable. *Relative to GAPDH. †Colons from four animals pooled per sample.

These observations by quantitative RT-PCR 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 (Figure 4).
Figure 4. IHC for androgen receptors in rat and mouse tissues. IHC for androgen receptor on 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.
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DISCUSSION

In humans the colonic adenoma-to-carcinoma sequence shows clear sexual dimorphism with preferential male development of both adenomas and CRC\(^\text{16}\). The reasons for this difference remain a matter under investigation. Confounding factors in human studies include obesity, carcinogen exposure through diet or smoking, physical activity, and comorbidities of other cancers. Thus, animal models may play an important role understanding 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. Thus, male susceptibility to colonic adenomagenesis can be analyzed easily in this model. In the female, we find that adenoma development does not respond to OVX or hormone replacement. By contrast, abrogation of male hormone production by ORX results in reduction of tumor numbers to those observed in females (Figure 2). Subsequent DHT replacement reverses the number to that found in sham-operated littersmates. 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 a protective effect of female hormones.

A frequently used rodent model for the development of colonic adenomas utilizes 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, a reduction in the colonic tumor incidence upon ORX with a return to normal numbers after testosterone replacement (Figure 3). Thus, in this mouse model as in the Pirc rat, adenoma incidence is promoted by male hormones rather than suppressed by female hormones. Further, since most mouse mutants are carried on a C57BL/6 background, this will be a useful platform for the 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 the androgen receptor (Ar). In the intestinal epithelium of both mouse and rat, Ar expression levels are below detectable limits (Tables 2, 3, and 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\(^\text{17}\). One indirect mechanism for this effect is the increase in stress hormones, such as cortisol, affecting the tumor environment\(^\text{18}\). Further, studies of Ar KO mice indicate that the immune system is regulated by androgens. For example, Chuang and colleagues found that neutrophil counts are reduced in castrated males but can be restored to normal levels through androgen supplementation, implicating the innate immune system\(^\text{19}\). Our evidence that testosterone acts indirectly to promote colonic adenomagenesis, opens the possibility to test of these indirect mechanisms, using the power of the molecular genetics of the mouse and the rat to ablate the androgen receptor gene in somatic lineages that are candidates for the site of testosterone action\(^\text{20}\).
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Our observations should be compared with other 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 for statistical significance. Thus, only by analyzing a very large cohort of Min mice were we able to demonstrate the significant two-fold male susceptibility to colonic adenomagenesis (Figure 1). By contrast, in the small intestine of the Min mouse, it has been reported that OVX increases adenomagenesis, perhaps owing to loss of a suppressive estrogen effect. Yet, our analysis of the large data set of Min mice from the McArdle lab provided marginally significant evidence for a 1.1-fold relative female susceptibility to adenoma development in the small intestine (Figure 1). It is not clear how this subtle female susceptibility is related to the reported enhancement by OVX. Further investigation of the female susceptibility of the small intestine in the Min mouse is made daunting by the small size of the effect.

A complication to understanding the published studies of the effects of OVX on colonic adenomas in the Min mouse is lack of reproducibility, perhaps owing to the low numbers of colonic tumors. One report described a significant reduction of colonic adenomas after OVX, with a reversal to normal by supplementation with 17β-estradiol. Such a tumorpromoting role for estrogens is consistent with the strong estrogen enhancement found in colonic tumorigenesis associated with inflammation in the mouse. However, this estrogen effect was not reproduced in a subsequent report by the same group. In a later analysis of estrogen effects on colonic adenomagenesis using Er receptor mutant mice no OVX plus hormone replacement was performed, and the studies did not differentiate between male and female mice. Studies in mice that have kept endogenous hormone production intact leave open the possibility that genetically inactivating only one of the two Er receptors leads to off-target hormone action. A well-known example is the increased plasma levels of estrogens that are present in Erα mutant mice. In this view, 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 the rat and mouse. For example, one report showed that AOM-induced tumor formation was dependent on male hormones in F344 rats, yet a second report by the same group found no effect of orchidectomy in Sprague-Dawley rats. A further study reported female susceptibility to AOM-induced colonic adenomagenesis in the highly susceptible A/J strain of mice, which is in contrast to the observations of male susceptibility reported here on the C57BL/6 mouse strain. These differences are strain dependent, perhaps owing to genetic variation in carcinogen metabolism trumping the biological variation of the sex of the animal. AOM-induced colonic adenomagenesis in the mouse has been shown to be controlled by polymorphisms at numerous loci.

Finally, it is important to understand the distinction between our studies of adenomagenesis and those of hormonal replacement in human populations. Two unique large interventional studies (WHI)
have clearly established that combination therapy with estradiol and MPA (but not estradiol alone) protects against frank colorectal cancer formation in postmenopausal women. At first glance these studies appear to contrast with our findings: in rats OVX and female hormone replacement had no effect on the incidence of colonic adenomas. However, in the Pirc rat our end point was adenoma formation and 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. Thus the molecular basis for the fiveyear protective effect of female hormone supplementation against CRC in postmenopausal women may be different from that of the difference between men and women in adenoma formation.

Although abrogation of female hormone production by OVX allows study of the contribution of female hormones, it must be recognized that it does not recapitulate the events that take place during menopause. Notably, postmenopausal ovaries are not inert but remain hormonally active and are a significant source of testosterone. Other factors may have contributed to the seeming 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 CRC incidence was reduced by treatment by female hormones in the WHI study, these may 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 may present an opportunity to explore this dichotomy. Delineating more fully the roles of male and female hormones on the adenoma and CRC development will be important for a deeper understanding of the sexual dimorphism in the colorectal adenoma-to-carcinoma sequence and in hormone replacement of postmenopausal women.

ACKNOWLEDGEMENTS

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Sex disparity in colonic adenomagenesis involves promotion by male hormones

REFERENCES


Chapter 3


Sex disparity in colonic adenomagenesis involves promotion by male hormones

SUPPLEMENTAL INFORMATION

Supplemental Table S1. Androgen receptor expression in colon of the Pirc rat. RNA was extracted using the Qiagen (Valencia, CA) AllPrep RNA/DNA mini kit following the manufacturer’s instructions including the supplemental Dnase treatment on the column during the RNA extraction. RNA was quantitated using optical density measurements on a Nanodrop 8000 (Thermo Scientific, Wilmington DE). RNA was reverse transcribed using Superscript VILO cDNA synthesis kit (Invitrogen/Life Technologies, Grand Island, NY) using 400 ng of RNA in a 20μl reaction using the extended 2 hour incubation at 42°C. cDNA was amplified and quantitated using a BioRad CFX96 or CFX384 using predesigned assays from IDT shown in Supplemental Table S2. Samples were heated to 95°C for 30 seconds followed by 55 cycles of 95°C denaturation for 5 seconds and 60°C annealing for 30 seconds with fluorescence detection after each cycle. Samples were all run in triplicate with all replicates ± 0.5 cycles. Relative expression levels were calculated using BioRad and qBase software packages.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N of samples</th>
<th>N of measurements per sample</th>
<th>Androgen receptor, fold mRNA expression* (DDCt)(mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT testes</td>
<td>2</td>
<td>3</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>Male ORX, placebo, NE</td>
<td>5</td>
<td>3</td>
<td>0.023± 0.03</td>
</tr>
<tr>
<td>Male ORX, placebo, CT</td>
<td>5</td>
<td>3</td>
<td>0.025 ± 0.009</td>
</tr>
<tr>
<td>Male ORX, DHT, NE</td>
<td>6</td>
<td>3</td>
<td>0.017 ± 0.013</td>
</tr>
<tr>
<td>Male ORX, DHT, CT</td>
<td>6</td>
<td>3</td>
<td>0.019 ± 0.012</td>
</tr>
<tr>
<td>Female, NE</td>
<td>5</td>
<td>3</td>
<td>0.022 ± 0.008</td>
</tr>
<tr>
<td>Female, CT</td>
<td>5</td>
<td>3</td>
<td>0.025 ± 0.01</td>
</tr>
</tbody>
</table>

* Relative to testes using HPRT as the reference control probe. NE, normal epithelium adjacent to paired tumor sample; CT, colon tumor; ORX, orchidectomized; WT, wild type non-Pirc animal.
### Supplemental Table S2

<table>
<thead>
<tr>
<th>Gene (IDT assay number)</th>
<th>Probe</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
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<tbody>
<tr>
<td>Androgen Receptor</td>
<td>5’-6-FAM/AGC TGC ATC /ZEN/AAT TCA CTT TGG ACC TGC /IABkFQ/-3’</td>
<td>AAT CCC ACA TCC TGC TCA AG</td>
<td>GAA AGT CCA CGC TCA CCA TA</td>
</tr>
<tr>
<td>HPRT</td>
<td>5’-/5HEX/TGG ATA CAG/ZEN/GCC AGA CTT TGT TGG ATT/IABkFQ/-3’</td>
<td>GCT TTT CCA CTT TCG CTG ATG</td>
<td>GGT GAA AAG GAC CTC TCG AAG</td>
</tr>
</tbody>
</table>