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Targeted *Cox2* gene deletion in intestinal epithelial cells decreases tumorigenesis in female, but not male, *Apc*$^{Min/+}$ mice

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ABSTRACT

Mice heterozygous for mutations in the adenomatous polyposis coli gene (*Apc*$^{+/-}$ mice) develop intestinal neoplasia. *Apc*$^{+/-}$ tumor formation is thought to be dependent on cyclooxygenase 2 (COX2) expression; both pharmacologic COX2 inhibition and global *Cox2* gene deletion reduce the number of intestinal tumors in *Apc*$^{+/-}$ mice. COX2 expression is reported in epithelial cells, fibroblasts, macrophages and endothelial cells of *Apc*$^{+/-}$ mouse polyps. However, the cell type(s) in which COX2 expression is required for *Apc*$^{+/-}$ tumor induction is not known. To address this question, we developed *Apc*$^{Min/+}$ mice in which the *Cox2* gene is specifically deleted either in intestinal epithelial cells or in myeloid cells. There is no significant difference in intestinal polyp number between *Apc*$^{Min/+}$ mice with a targeted *Cox2* gene deletion in myeloid cells and their control littermate *Apc*$^{Min/+}$ mice. In contrast, *Apc*$^{Min/+}$ mice with a targeted *Cox2* deletion in intestinal epithelial cells have reduced intestinal tumorigenesis when compared to their littermate control *Apc*$^{Min/+}$ mice. However, two gender-specific effects are notable. First, female *Apc*$^{Min/+}$ mice developed more intestinal tumors than male *Apc*$^{Min/+}$ mice. Second, targeted intestinal epithelial cell *Cox2* deletion decreased tumorigenesis in female, but not in male, *Apc*$^{Min/+}$ mice. Considered in the light of pharmacologic studies and studies with global *Cox2* gene knockout mice, our data suggest that (i) intrinsic COX2 expression in intestinal epithelial cells plays a gender-specific role in tumor development in *Apc*$^{Min/+}$ mice, and (ii) COX2 expression in cell type(s) other than intestinal epithelial cells also modulates intestinal tumorigenesis in *Apc*$^{Min/+}$ mice, by a paracrine process.

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1. Introduction

Heterozygous inactivating adenomatous polyposis coli (*APC*) gene mutations cause familial adenomatous polyposis (FAP) in patients. Most sporadic human colorectal cancers also exhibit *APC* gene mutations. $Apc^{+/-}$ mutant mice have been widely studied, to better understand mechanisms of $Apc^{+/-}$ intestinal tumorigenesis (McCart et al., 2008). One of the most commonly used $Apc^{+/-}$ mouse models, the multiple intestinal neoplasia (*Min*) or $Apc^{Min/+}$ mouse (Moser et al., 1990), develops ~30–100 tumors, predominantly in the small intestine (McCart et al., 2008).

Cyclooxygenase-2 (COX2) is an inducible form of cyclooxygenase, the enzyme(s) that catalyze the first two steps in the conversion of arachidonic acid to prostaglandins. COX2 mRNA is often elevated in human colorectal adenomas and adenocarcinomas (Eberhart et al., 1994; Maekawa et al., 1998), suggesting that COX2 may play a role in the early stages of colorectal cancer. Markedly increased COX2 mRNA and protein levels are also observed in intestinal tumors of $Apc^{\Delta716/+}$ and $Apc^{Min/+}$ mice (Oshima et al., 1996; Williams et al., 1996; Backlund et al., 2005). *Cox2* gene disruption reduced intestinal tumorigenesis in both $Apc^{\Delta716/+}$ and $Apc^{Min/+}$ *Cox2* knockout mice (Oshima et al., 1996; Chulada et al., 2000); selective pharmacologic COX2 enzyme inhibition also decreased intestinal tumor frequency in $Apc^{\Delta716/+}$ and $Apc^{Min/+}$ mice (Jacoby et al., 2000; Oshima et al., 2001). These studies with $Apc^{+/-}$ mice provide direct evidence for a role of COX2-derived prostaglandins in $Apc^{+/-}$ intestinal tumorigenesis. Celecoxib (a COX2 selective inhibitor) treatment attenuated the number and size of polyps in FAP patients (Higuchi et al., 2003; Lynch et al., 2010) also suggesting a role for COX2 in hereditary *APC*-dependent tumor formation.

Despite genetic and pharmacologic evidence, how COX2 promotes intestinal tumor development in $Apc^{+/-}$ mice and FAP patients is still unclear. Epithelial cell COX2 staining was observed in all adenocarcinomas (Brosens et al., 2008) and in epithelial and stromal cells (Jungck et al., 2004) from FAP patients. Oshima et al. (1996) observed COX2 expression in the interstitial cells of polyps from $Apc^{\Delta716/+}$ mice, but not in the dysplastic epithelium. Williams et al. (1996) concurrently reported the presence of COX2 in the epithelial cells of polyps from $Apc^{Min/+}$ mice. Sonoshita et al. (2002) subsequently reported the presence of COX2 in fibroblasts and endothelial cells in polyps from both APC patients and $Apc^{\Delta716/+}$ mice. However, they did not observe COX2 expression in macrophages or lymphocytes. In contrast, Hull et al. (1999) reported the presence of COX2 immunostaining in the lamina propria and in macrophages of polyps from $Apc^{Min/+}$ mice.

The studies described above report, in $Apc^{+/-}$ mice and FAP patients, the presence of COX2 in intestinal tumor epithelial cells, stromal cells of the surrounding tumor microenvironment, and macrophages present in the tumors. In their *Nature Reviews* article discussing colorectal cancer and COX2, published over a decade ago, Gupta and DuBois (2001) comment "There is no consensus at present on what cell types within a tumor express COX2..." They pose the question "Does understanding the exact location of COX2 in colorectal tumors have any bearing on understanding how COX2 promotes colorectal cancer cell growth?" and end with the comment that "knowing the specific cell type that expresses COX2 would be essential for understanding how COX2 promotes colorectal cancer progression. The most direct experimental approach to address these issues could be through the use of mice with tissue specific genetic ablation of *Cox2*. In such experiments, it would be possible to find out whether removal of COX2 only from epithelial or macrophage lineages, for example, decreases polyp burden in $Apc^{Min/+}$ mice (Gupta and DuBois, 2001)."

The ambiguities regarding the role(s) of cell-type specific COX2 expression in driving tumor progression in $Apc^{+/-}$ tumors have not changed since Gupta and DuBois (Gupta and DuBois, 2001) wrote their review. To address these questions, we generated $Apc^{Min/+}$ mice with specific *Cox2* gene deletions in intestinal epithelial cells or in myeloid cells and examine here the effect of these targeted *Cox2* deletions on COX2 expression, intestinal polyp number, and polyp size in $Apc^{Min/+}$ mice.

2. Materials and methods

2.1. Mice

A conditional *Cox2* gene knockout mouse, $Cox2^{flox/flox}$, in which exons 4 and 5 of the *Cox2* gene are flanked by loxP sites was generated previously (Ishikawa and Herschman, 2006). $Apc^{Min/+}$ mice (C57BL/6J-Apc^{Min}/J), LysMCre knock-in mice (B6.129P2-$Lyz2^{tm1(cre)Ifo}$/J) and VillinCre transgenic mice (B6.SJL-Tg(Vil-cre)997Gum/J) were purchased from the Jackson Laboratory (Bar Harbor, MA). All animal experiments were conducted with the approval of the Animal Research Committee, University of California, Los Angeles.

$Apc^{Min/+}$ mice, $LysM^{Cre/+}$ knock-in mice, and VillinCre transgenic mice were crossed with $Cox2^{flox/+}$ or $Cox2^{flox/flox}$ mice to generate $Cox2^{flox/flox}$ $Apc^{Min/+}$ mice, $Cox2^{flox/flox}$ $LysM^{Cre/+}$ mice, and $Cox2^{flox/flox}$ $VillinCre^{Tg}$ mice. To generate $Apc^{Min/+}$ mice with homozygous *Cox2* deletions specifically in intestinal epithelial cells, genetic crosses between $Cox2^{flox/flox}$ $Apc^{Min/+}$ mice and $Cox2^{flox/flox}$ $VillinCre^{Tg}$ mice were performed. These crosses produced $Apc^{Min/+}$ mice homozygous for the *Cox2* floxed alleles and positive for the VillinCre transgene ($Cox2^{flox/flox}$ $Apc^{Min/+}$ $VillinCre^{Tg}$ mice) or negative for the $VillinCre^{Tg}$ allele ($Cox2^{flox/flox}$ $Apc^{Min/+}$ mice). Similarly, $Cox2^{flox/flox}$ $Apc^{Min/+}$ mice were crossed with $Cox2^{flox/flox}$ $LysM^{Cre/+}$ mice to generate $Apc^{Min/+}$ mice with myeloid cell-specific homozygous *Cox2* deletions ($Cox2^{flox/flox}$ $Apc^{Min/+}$ $LysM^{Cre/+}$ mice) and their littermate controls ($Cox2^{flox/flox}$ $Apc^{Min/+}$ mice).

$Cox2^{flox/flox}$ $Apc^{Min/+}$ $VillinCre^{Tg}$ mice, in which the *Cox2* gene is deleted in intestinal epithelial cells, will be referred to as $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice. $Cox2^{flox/flox}$ $Apc^{Min/+}$ $LysM^{Cre/+}$ mice, in which the *Cox2* gene is deleted in myeloid cells, will be referred to as $Apc^{Min/+}$ $Cox2^{\Delta M}$ mice. Littermate control mice for both $Apc^{Min/+}$ $Cox2^{\Delta E}$ and $Apc^{Min/+}$ $Cox2^{\Delta M}$ mice will be referred to as $Apc^{Min/+}$ mice. *Cox2* gene deletion in myeloid cells and in intestinal epithelial cells, as a result of cell-type

specific Cre recombinase expression, was examined as previously described (Ishikawa et al., 2011).

2.2. Genotyping of mice

DNA was isolated from tail snips. Cre and *Cox2* allele genotypes were determined as previously described (Ishikawa et al., 2011). A PCR-based protocol from the Jackson Laboratory was adapted to genotype the *Apc* locus. PCR reactions for *Apc* wild type or *Min* alleles were carried out separately with appropriate positive, negative and no template controls. All PCR reactions were carried out using an MJ Research thermal cycler.

2.3. Tumor number and size measurements

$Apc^{Min/+}$ $Cox2^{\Delta M}$ mice and their littermate $Apc^{Min/+}$ controls were sacrificed at 150 days of age; $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice and their littermate $Apc^{Min/+}$ controls were sacrificed at 180 days of age. The entire intestinal tract was removed, flushed with PBS several times, and the small intestine was cut into 4–6 pieces. Each section was opened longitudinally and kept flat between bibulous papers. The tissues were fixed in 10% formalin overnight, transferred to 70% alcohol and stored at 4 °C until use. Intestine sections were stained with 1% methylene blue, and tumors were counted using a dissecting microscope at 10× magnification. Maximum tumor diameter was measured with a calibrated eye piece reticle. Tumors were classified into the following size groups: <1 mm, 1–2 mm, 2–3 mm, 3–4 mm, 4–5 mm, and >5 mm (Oshima et al., 1996).

2.4. Immunohistochemistry

COX2 immunohistochemical detection on formalin-fixed, paraffin-embedded tissues was performed as described previously (Ishikawa et al., 2011). Briefly, 4 μm thickness sections were used for immunostaining, and COX2 protein was detected with a polyclonal anti-COX2 antibody (Thermo Scientific).

2.5. Statistics

Variation in tumor counts between mice was investigated utilizing two-way ANOVA models. These models contained the main effects of gender (m/f), Cre (+/−), and the interaction effect of gender and Cre. This model was run separately for both cell types ($LysM^{Cre}$ and $VillinCre^{Tg}$). Average tumor counts are reported as mean ± 1 standard error. Residual analysis yielded no evidence that the normal model was an inadequate fit. The significance threshold was set at 0.05. Statistical analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Myeloid cell-specific Cox2 gene deletion in $Apc^{Min/+}$ mice does not affect polyp formation

Macrophages are among the several cell types in which COX2 is reported to be expressed in $Apc^{Min/+}$ intestinal tumors (Hull et al., 1999). To investigate whether COX2 expression in myeloid cells, including macrophages, contributes to tumor burden in $Apc^{Min/+}$ mice, we created $Apc^{Min/+}$ mice with a targeted deletion in the myeloid cell *Cox2* gene. $Cox2^{flox/flox}$ $Apc^{Min/+}$ mice were crossed with $Cox2^{flox/flox}$ $LysM^{Cre/+}$ mice to create both $Apc^{Min/+}$ $Cox2^{\Delta M}$ mice with a myeloid-specific *Cox2* gene deletion and their littermate $Apc^{Min/+}$ controls. $Apc^{Min/+}$ $Cox2^{\Delta M}$ mice ($n = 8$) developed 60 ± 7.9 polyps at 150 days of age (Figure 1A). Littermate control $Apc^{Min/+}$ mice ($n = 9$) developed 54 ± 4.6 polyps. There was no significant difference ($p = 0.35$, group effect from two-way ANOVA model) in intestinal polyp frequency in $Apc^{Min/+}$ $Cox2^{\Delta M}$ mice and littermate control $Apc^{Min/+}$ mice. In addition, there was no difference in size distribution in the polyps found in $Apc^{Min/+}$ mice and $Apc^{Min/+}$ $Cox2^{\Delta M}$ mice (data not shown). Thus we did not detect any effect on intestinal tumorigenesis in $Apc^{Min/+}$ mice unable to express COX-2 in myeloid cells.

3.2. Intestinal epithelial cell-specific Cox2 gene deletion in $Apc^{Min/+}$ mice reduces polyp formation

COX2 expression is observed in dysplastic epithelial cells of the intestinal tumors of $Apc^{Min/+}$ mice (Williams et al., 1996), and in epithelial cells of polyps from FAP patients (Brosens et al., 2008; Jungck et al., 2004). To determine whether intrinsic intestinal epithelial cell COX2 expression modulates polyp formation in $Apc^{Min/+}$ mice, we created $Apc^{Min/+}$ mice with a targeted *Cox2* deletion in their intestinal epithelial cells. $Cox2^{flox/flox}$ $Apc^{Min/+}$ mice were crossed with $Cox2^{flox/flox}$ $Villin\text{-}Cre^{Tg}$ mice to generate both $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice with targeted intestinal epithelial cell Cox2 deletions and their littermate control $Apc^{Min/+}$ mice. The control $Apc^{Min/+}$ mice ($n = 15$) developed 40 ± 6.3 tumors at 180 days of age (Figure 1B). In contrast, $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice ($n = 17$), in which the *Cox2* gene is deleted in intestinal epithelial cells, developed 27 ± 3.0 polyps. The 31% reduction in polyp formation as a consequence of deleting the intestinal epithelial *Cox2* gene is – in contrast to the targeted deletion of the *Cox2* gene in myeloid cells – highly significant ($p = 0.004$, group effect from two-way ANOVA).

In addition to reducing the number of polyps that develop in $Apc^{Min/+}$ mice, targeted *Cox2* gene deletion in intestinal epithelial cells also altered the polyp size distribution (Figure 2). "Small" polyps, i.e., polyps less than 2 mm in diameter (Oshima et al., 1996), were similar in number in $Apc^{Min/+}$ mice and $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice. However, there was a substantial reduction in "large" polyps; e.g., polyps 2 mm or greater in diameter (Oshima et al., 1996). These data on polyp frequency and size distribution suggest that intrinsic intestinal epithelial cell COX2 expression is a strong driving force in polyp promotion in $Apc^{Min/+}$ mice.

Immunostaining in the intestinal tumors of control $Apc^{Min/+}$ mice showed COX2 expression in many, but not all, intestinal epithelial cells and in cells of the lamina propria (Figure 3A and B). In the normal intestinal mucosa adjacent to the tumors, COX2 expression was present in lamina propria cells, but not in the epithelium (Figure 3C). In contrast to the results for control littermate $Apc^{Min/+}$ mice, few if any dysplastic epithelial cells in $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice intestinal tumors stain positive for COX2 (Figure 3D and E). However, COX2 expression was detected in the cells of the tumor lamina propria.

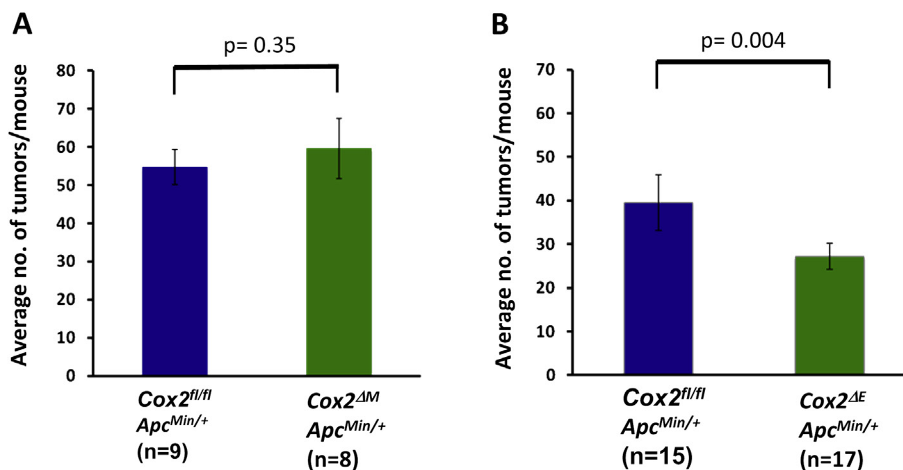

Figure 1 – Intestinal tumor formation in *Apc*$^{Min/+}$ mice with cell-type specific *Cox2* gene deletions. (Panel A) The *Cox2* gene was deleted in myeloid cells of *Apc*$^{Min/+}$ *Cox2*$^{\Delta M}$ mice. Intestinal tumors in *Apc*$^{Min/+}$ *Cox2*$^{\Delta M}$ mice and littermate control *Apc*$^{Min/+}$ mice were counted at 150 days of age. (Panel B) The *Cox2* gene was deleted in intestinal epithelial cells of *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice. Intestinal tumors in *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice and control littermate *Apc*$^{Min/+}$ mice were counted at 180 days of age. *p*-values for the Cre effect were computed from the two-way ANOVA models. Means ± S.E. are shown.

As in the normal intestinal mucosa of Apc$^{Min/+}$ tumors (Figure 3C), cells of the lamina propria, but not epithelial cells, were positive for COX2 expression in the normal mucosa adjacent to tumors of Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice (Figure 3F).

3.3. *Gender-specific differences in polyp formation in control* Apc$^{Min/+}$ *mice*

We observed an unexpected (to us) gender difference in polyp formation in the control Apc$^{Min/+}$ mice. For both control Apc$^{Min/+}$ mice in the myeloid Cox2 gene deletion study (Figure 4A) and the control Apc$^{Min/+}$ mice in the intestinal epithelial cell Cox2 gene deletion study (Figure 4B), we found significantly more polyps in the female Apc$^{Min/+}$ mice than in the control male Apc$^{Min/+}$ mice. Although there are hundreds of publications studying characteristics of Apc$^{+/-}$ mice, we found only two other reports of gender-based differences in polyp frequency (McAlpine et al., 2006; Yoo et al., 2008).

3.4. *Intestinal epithelial cell-specific* Cox2 *gene deletion reduces polyp formation in female* Apc$^{Min/+}$ *mice, but not in male* Apc$^{Min/+}$ *mice*

Once we observed the gender-based differences in polyp formation in control Apc$^{Min/+}$ mice (Figure 4), we revisited the effect of myeloid cell- and intestinal epithelial cell-targeted Cox2 gene deletion, to determine whether there might be gender-based differences in the roles of myeloid or intestinal epithelial cell COX2-driven polyp formation in Apc$^{Min/+}$ mice. Deletion of myeloid cell Cox2 in Apc$^{Min/+}$ Cox2$^{\Delta M}$ mice had no effect on intestinal tumorigenesis, in either male or female mice (data not shown).

Deletion of the Cox2 gene in intestinal epithelial cells of male Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice had no effect on the frequency of polyps, when compared to their control Apc$^{Min/+}$ littermates (Figure 5). In contrast, Cox2 gene deletion in intestinal epithelial cells of female Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice caused a greater than two fold, highly significant ($p < 0.001$, pairwise difference in two-way ANOVA model) decrease in polyp formation when compared to their female Apc$^{Min/+}$ littermates (Figure 5). Perhaps coincidentally, the observed polyp frequency in female Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice was reduced to the same frequency observed in male Apc$^{Min/+}$ and Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice.

We analyzed polyp size distribution in male and female Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice and their littermate male and female Apc$^{Min/+}$ control mice in two ways. Deletion of the Cox2 gene in intestinal epithelial cells of female Apc$^{Min/+}$ mice affects the size of polyps (Figure 6A), as well as the total number of

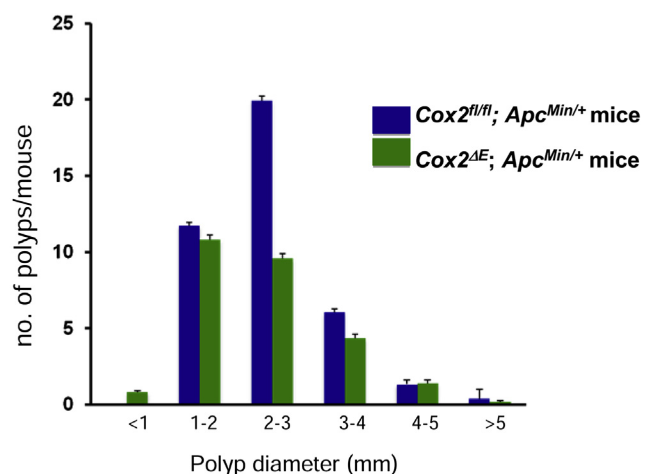

Figure 2 – Sizes of intestinal tumors in *Apc*$^{Min/+}$*Cox2*$^{\Delta E}$ mice (n = 17), in which the *Cox2* gene is specifically deleted in intestinal epithelial cells, and of littermate *Apc*$^{Min/+}$ mice (n = 15). Data are presented as average number of tumors per mouse in each group. Means ± S.D. are shown.

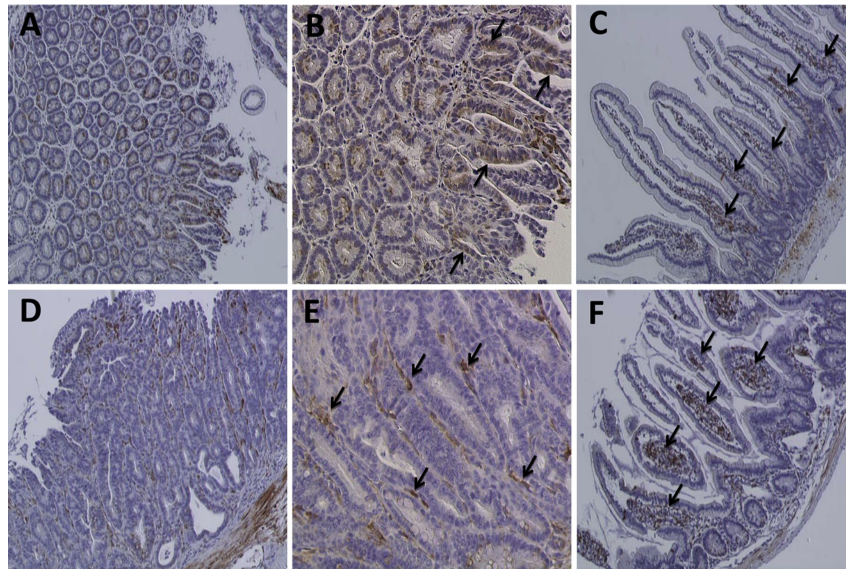

Figure 3 – COX2 protein expression in intestinal tumors of *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice. (A) Control littermate *Apc*$^{Min/+}$ mouse intestinal tumor, with extensive COX2 expression in tumor epithelial cells (100×). (B) Higher magnification of control littermate *Apc*$^{Min/+}$ mouse intestinal tumor (200×). Arrows indicate positive COX2 protein staining in epithelial cells. (C) Tumor-adjacent normal mucosa of control littermate *Apc*$^{Min/+}$ mouse (100×). Arrows indicate positive COX2 staining in lamina propria cells. (D) *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mouse intestinal tumor, with COX2 expression present predominantly in non-epithelial cells (100×). (E) Higher magnification of *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mouse intestinal tumor (200×). Arrows indicate positive COX2 protein staining in stromal, non-epithelial cells. (F) Tumor-adjacent normal mucosa of *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mouse (100×). Arrows indicate positive COX2 staining in lamina propria cells.

polyps (Figure 5). Comparing the numbers of polyps of different sizes (Figure 6A) demonstrates that there is a striking (greater than 75%) reduction in the numbers of polyps of diameter 2 mm or greater in female Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice when compared to littermate female Apc$^{Min/+}$ mice. Figure 6B shows the percentage (rather than the numbers, as in Figure 6A) of polyps of differing diameters in female Apc$^{Min/+}$ and Apc$^{Min/+}$ Cox2$^{\Delta E}$ mice. Many authors classify polyps of Apc$^{Min/+}$ mice as either “small” (less than 2 mm in diameter), or “large” (2 mm or greater in diameter). By these criteria, not

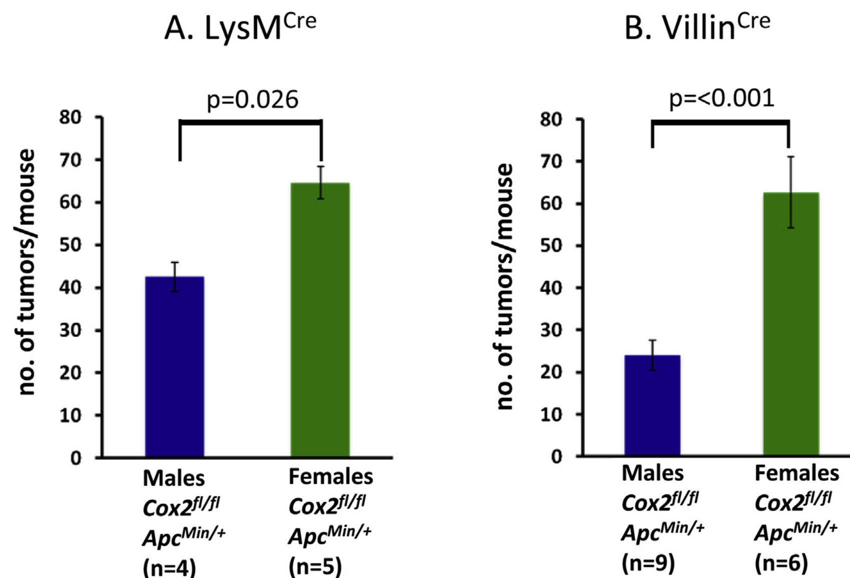

Figure 4 – Gender effects on the frequency of intestinal tumors in control *Apc*$^{Min/+}$ mice for targeted deletion studies with both *Apc*$^{Min/+}$ *Cox2*$^{\Delta M}$ mice and *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice. (Panel A) intestinal tumors in male versus female control littermate *Apc*$^{Min/+}$ mice for the experiment in which the *Cox2* gene was deleted in myeloid cells. (Panel B) intestinal tumors in male versus female control littermate *Apc*$^{Min/+}$ mice for the experiment in which the *Cox2* gene was deleted in intestinal epithelial cells. *p*-values were computed from the pairwise differences estimated by the two-way ANOVA models. Means ± S.E. are shown.

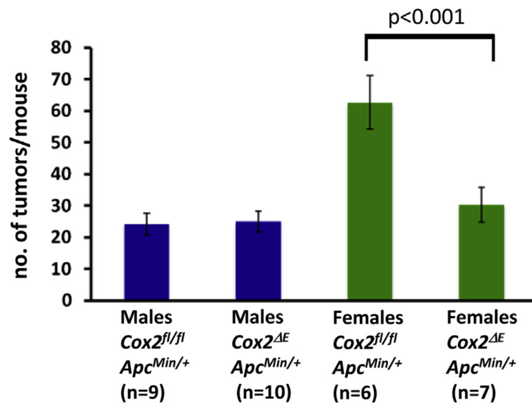

Figure 5 – Intestinal epithelial *Cox2* deletion as a result of targeted Cre recombinase expression has no effect on male $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice, but significantly decreased tumor formation in female $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice. The *p*-value was computed from the pairwise difference estimated by the two-way ANOVA model. Means ± S.E. are shown.

only is the number of polyps reduced in female $Apc^{Min/+}$ mice by targeted deletion of the Cox2 gene in intestinal epithelial cells (Figure 5); in addition, the polyps that do form in female $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice are skewed heavily toward "small" polyps, in contrast to the predominantly "large" polyps found in control littermate female $Apc^{Min/+}$ mice.

In contrast, to the results for female $Apc^{min/+}$ mice, neither the number (Figure 5) nor the size distribution (Figure 6C and D) of polyps in male $Apc^{Min/+}$ mice was affected by targeted deletion of the Cox2 gene in intestinal epithelial cells. In summary, our results demonstrate that intestinal epithelial COX2 expression plays a strong modulatory role in tumor development in female $Apc^{Min/+}$ mice, but not in male $Apc^{Min/+}$ mice.

4. Discussion

Increased COX2 expression has been reported in human intestinal tumors of multiple origins, and in intestinal tumors that occur in a variety of animal models. A causal role for COX2 expression in the progression of intestinal cancer has been most clearly defined in hereditary familial adenomatous polyposis (FAP) patients, in whom the APC gene is mutated, and in $Apc^{+/-}$ mice. Treatment of FAP patients with sulindac (Giardiello et al., 1993), celecoxib (Steinbach et al., 2000) or rofecoxib (Higuchi et al., 2003), all selective COX2 inhibitors, reduces polyp frequency and size, strongly suggesting a causal role for COX2 expression as a driver for FAP. Similarly, both celecoxib (Jacoby et al., 2000) and rofecoxib (Oshima et al., 2001) inhibition of $Apc^{Min/+}$ mouse intestinal polyp induction reinforce the suggestion for a COX2 causal role in $Apc^{+/-}$ intestinal cancer. The reduction in intestinal tumor number and size in $Apc^{\Delta 716/+}$ (Oshima et al., 1996) and $Apc^{Min/+}$ (Chulada et al., 2000) mice with global Cox2 gene deletions provided conclusive proof that COX2 expression, in some cell type(s), plays a major role in $Apc^{+/-}$ tumor progression.

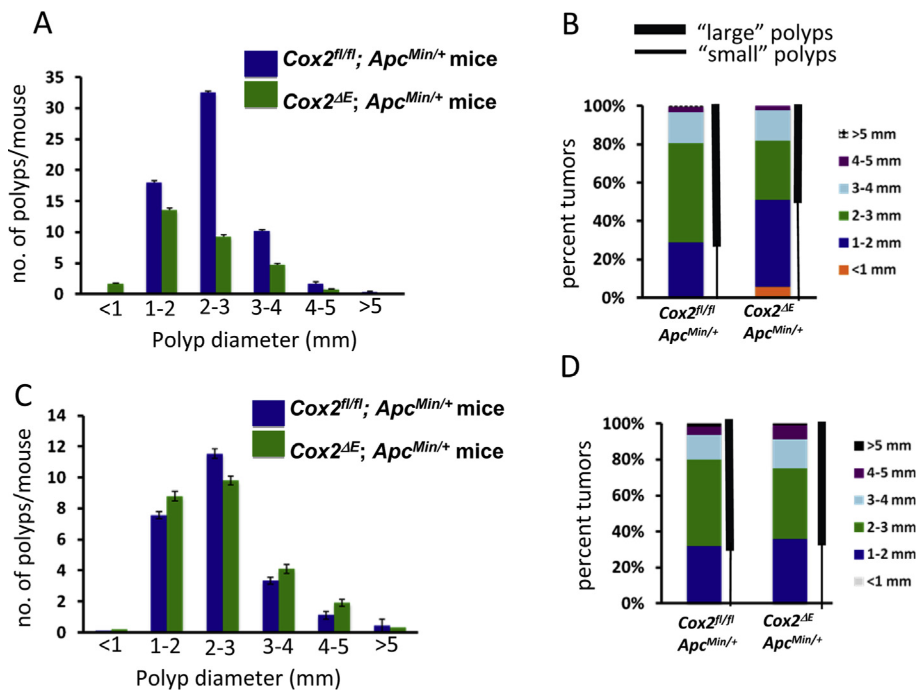

Figure 6 – Sizes of intestinal tumors in male and female $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice, in which the *Cox2* gene is specifically deleted in intestinal epithelial cells, and in littermate $Apc^{Min/+}$ mice. (Panel A) Numbers of intestinal tumors per mouse in female $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice ($n = 7$) and their female littermate $Apc^{Min/+}$ mice ($n = 6$). Data are shown as number of tumors per mouse in each group. (Panel B) Percent of tumors in each size class for female $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice and their female littermate $Apc^{Min/+}$ mice. Small polyps are defined as less than 2 mm in size; large polyps are 2 mm or greater in size. (Panel C) Numbers of intestinal tumors per mouse in male $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice ($n = 10$) and their male littermate $Apc^{Min/+}$ mice ($n = 9$). (Panel D) Percent of tumors in each size class for male $Apc^{Min/+}$ $Cox2^{\Delta E}$ mice and their male littermate $Apc^{Min/+}$ mice. Means ± S.D. are shown.

Dysplastic intestinal epithelial cells, stromal fibroblasts, endothelial cells and macrophages are all reported to express COX2 (Oshima et al., 1996; Williams et al., 1996; Sonoshita et al., 2002; Hull et al., 1999) in intestinal tumors of *Apc*$^{+/-}$ mice. Each cell type has been suggested as a (and/or the) cell type in which COX2 must be expressed to drive tumor progression. However, neither pharmacologic studies with COX2 inhibitors nor studies of mice with global *Cox2* gene deletions can conclusively identify the cell type(s) in which COX2 expression is required for *Apc*$^{+/-}$ tumorigenicity, since both interventions eliminate COX2 function in all cells.

Hull et al. (1999) demonstrated COX2 expression in macrophage of the lamina propria of *Apc*$^{Min/+}$ mice, and suggested a paracrine effect of macrophage COX2 function on epithelial cells in *Apc*$^{Min/+}$ mouse adenomas. However, myeloid cell-specific *Cox2* gene deletion has no effect on the number or size of *Apc*$^{Min/+}$ intestinal tumors (Figure 1A); myeloid/macrophage derived, COX-2 dependent prostanoids do not modulate tumor formation in *Apc*$^{Min/+}$ mice. In contrast, *Cox2* intestinal epithelial cell-specific deletion significantly reduced the frequency of *Apc*$^{Min/+}$ tumors in our cohort of *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice (Figure 1B), initially suggesting a required role in these cells for COX2 function in *Apc*$^{+/-}$ tumor progression.

When we considered gender as a variable in *Apc*$^{Min/+}$ intestinal tumor induction we found that control *Apc*$^{Min/+}$ female mice, both for the myeloid-specific *Cox2* knockout (*Apc*$^{Min/+}$ *Cox2*$^{\Delta M}$) mice and for the intestinal epithelial cell-specific *Cox2* knockout (*Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$) mice, developed more tumors than did their male *Apc*$^{Min/+}$ counterparts (Figure 4). Although there are hundreds of papers reporting studies with *Apc*$^{+/-}$ mice, we found only two other studies that report on the role of gender in tumor formation. McAlpine et al. (2006) and Yoo et al. (2008) also observed greater intestinal tumor frequency in female *Apc*$^{+/-}$ mice than in male *Apc*$^{+/-}$ mice. However, it is clear that this gender distinction is not widely appreciated.

When we analyzed gender-specific effects in our *Cox2* targeted deletion studies, we found that intrinsic COX-2 expression in intestinal epithelial cells of male *Apc*$^{Min/+}$ mice plays no role, in either tumor frequency or tumor size, in intestinal polyp development; these characteristics were the same in male *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice and their littermate *Apc*$^{Min/+}$ control mice. In contrast, both polyp frequency and size were reduced in female *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice when compared to their littermate control *Apc*$^{Min/+}$ mice. Despite the reduction in polyps in the female *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice, there remained a cohort of intestinal polyps that formed in these animals; i.e., in female mice unable to make COX2-dependent prostanoids in their intestinal epithelial cells.

In studies with *Apc*$^{\Delta 716/+}$ *Cox2*$^{-/-}$ mice and *Apc*$^{Min/+}$ *Cox2*$^{-/-}$ that had homozygous global *Cox2* gene deletions, intestinal polyp formation was reduced by 84–86% (Oshima et al., 1996; Chulada et al., 2000). Although gender differences were not considered in these studies, the data suggest that COX2-derived prostanoid product(s) play a role in the development of a great majority of the polyps that occur in *Apc*$^{+/-}$ mice. The most likely interpretation of the frequency differences in tumor formation in *Apc*$^{Min/+}$ mice in which there is a global *Cox2* knockout (*Apc*$^{Min/+}$; *Cox2*$^{-/-}$ mice) and tumor formation in *Apc*$^{Min/+}$ mice in which there is a targeted epithelial cell-specific *Cox2* knockout (*Apc*$^{Min/+}$; *Cox2*$^{\Delta E}$ mice) is that COX2 expression in cells of the tumor microenvironment, and thus a paracrine effect(s) of COX2-derived prostaglandins from these cells, is also required for tumor development in *Apc*$^{+/-}$ mice. A less likely possibility is that a subset of intestinal tumors that develop in these animals does not require COX2 function in any cell type. In light of our results, unequivocal clarification of these alternative possibilities would require re-examination of the occurrence of tumors in *Apc*$^{+/-}$; *Cox2*$^{-/-}$ mice. If intestinal tumors do, in fact, occur in *Apc*$^{+/-}$; *Cox2*$^{-/-}$ mice, the result would suggest a COX2-independent pathway to (at least some) intestinal tumors in *Apc*$^{+/-}$ mice. If tumor frequency is totally (or even largely) eliminated in *Apc*$^{+/-}$ *Cox2*$^{-/-}$ mice, identification of a cell type(s) in which COX2 expression is causally needed in a paracrine fashion for intestinal tumorigenesis would require analysis of *Apc*$^{+/-}$ mice with targeted *Cox2* gene deletions in alternative candidate cell types (e.g., fibroblasts, myofibroblasts, and endothelial cells).

In contrast to the results for male *Apc*$^{Min/+}$ mice, in which tumor formation appears to be independent of COX-2 expression in intestinal epithelial cells (Figure 5), tumor formation is reduced by nearly two-thirds in female *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice, in which the *Cox2* gene is specifically deleted in intestinal epithelial cells (Figure 5). Tumor frequency in female *Apc*$^{Min/+}$ *Cox2*$^{\Delta E}$ mice is similar to tumor frequency in male *Apc*$^{Min/+}$ mice; intestinal epithelial cell COX2 expression is largely, if not completely, responsible for the greater intestinal tumor frequency in female *Apc*$^{Min/+}$ mice.

The frequency (Figure 6A) and percentage (Figure 6B) of "small" tumors, less than 2 mm in diameter, do not differ in female *Apc*$^{Min/+}$ mice and *Apc*$^{Min/+}$; *Cox2*$^{\Delta E}$ mice. However, "large" tumors are less frequent in female mice with an intestinal epithelial cell-targeted *Cox2* gene deletion (*Apc*$^{Min/+}$; *Cox2*$^{\Delta E}$ mice) than in their female *Apc*$^{Min/+}$ littermates (Figure 6A); the large tumors are a greater percentage of the intestinal epithelial tumors that form in female *Apc*$^{Min/+}$; *Cox2*$^{\Delta E}$ mice (Figure 6B). In contrast, the distribution of tumor sizes in male *Apc*$^{Min/+}$; *Cox2*$^{\Delta E}$ mice and their littermate *Apc*$^{Min/+}$ control mice are similar (Figure 6C and D). Oshima et al. (1996) report that COX2 expression could not be detected in "small" tumors of *Apc*$^{\Delta 716/+}$ mice, but was easily detectable in larger tumors. Collectively, these data support the suggestions (i) that tumor frequency and size are driven, to a much greater extent in female *Apc*$^{+/-}$ mice than in male *Apc*$^{+/-}$ mice, by an intrinsic intestinal epithelial cell COX2 product and (ii) that tumor formation in *Apc*$^{+/-}$ mice of both genders is also dependent on the paracrine action of a COX2 product of a cell type in the microenvironment. Seno et al. (2002) suggest that stromal cell COX2 expression results in PGE_2-driven angiogenesis and consequent tumor promotion in *Apc*$^{\Delta 716/+}$ mice.

In addition to genetic studies of the *Apc*$^{+/-}$ mouse model of hereditary human APC colon cancer, the role of COX-2 has been examined genetically in other mouse colon cancer models. Ulcerative colitis in patients is a frequent precursor to colon cancer. Colitis-dependent colon cancer has been modeled by exposing mice to a single injection of the carcinogen azoxymethane (AOM), followed by colon tumor promotion with dextran sodium sulfate (DSS) oral administration.

Colon tumors induced in mice by AOM/DSS administration express increased COX-2 levels relative to surrounding colon tissue (Ishikawa and Herschman, 2010). However, despite the presence of elevated COX-2 protein in the AOM/DSS-induced tumors, and in contrast to results with $Apc^{+/-}$ mice, global *Cox2* gene deletion had no effect on AOM/DSS colon cancer induction; COX2-derived prostanoids are not required for tumor induction in this mouse model of colitis-associated colon cancer (Ishikawa and Herschman, 2010). Clearly, one cannot generalize from one type of colon cancer to another with respect to roles for COX2 modulation of colon tumor progression.

We have not found a previous clinical study in patients or preclinical study in animal models in which gender-specific differences in COX2 expression have been correlated with intestinal cancer detection, prognosis, survival or therapeutic outcome, nor have we found another report in any animal model in which differential COX2 expression mediates gender differences in tumor development. However, there are a few clear examples of sexual dimorphism in cyclooxygenase-modulated biological processes. Estrogen-driven COX2 expression in female mice leads to prostacyclin-dependent atheroprotection in female mice (Egan et al., 2004). In chronic Freud's adjuvant-induced arthritis and inflammatory pain, reduced edema and joint destruction were observed in female $Cox2^{-/-}$ mice relative to appropriate controls, but not in males (Chillingworth et al., 2006). Naor et al. (2009) report that indomethacin was more effective in blocking LPS-promoted lung colonization in male rats than in female rats. Elucidation of gender-mediated, COX2-dependent tumor number and progression in $Apc^{+/-}$ mice will require both endocrine and genetic studies.

Conflict of interest statement

None declared.

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