Tumorigenesis and Neoplastic Progression

A Reduction of Cyclooxygenase 2 Gene Dosage Counters the Ovarian Morphological Aging and Tumor Phenotype in Wv Mice

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Menopausal ovaries undergo morphological changes, known as ovarian aging, which are implicated in the high incidence of ovarian cancer occurring during the perimenopausal and immediate postmenopausal periods. The germ cell-deficient Wv mice recapitulate these postmenopausal alterations in ovarian morphology and develop tubular adenomas. We demonstrate that a reduction of cyclooxygenase 2 gene dosage rescued the ovarian aging phenotype of the Wv mice, whereas homozygous deletion was accompanied by a compensatory increase in ovarian cyclooxygenase 1 expression and prostaglandin E₂ synthesis. Cyclooxygenase inhibitors also reduced the tumor phenotype in a preliminary study. These findings suggest that increased cyclooxygenase activity contributes to the preneoplastic morphological changes of the ovarian surface epithelium, which can be reversed by a reduction of gene dosage achieved by either genetic or pharmacological approaches. (Am J Pathol 2007, 170:1325–1336; DOI: 10.2353/ajpath.2007.060769)

Menopause is defined as the permanent cessation of menstruation resulting from depletion of germ cells and loss of ovarian follicular activity, and it is accepted to be a by-product of modern health advances and the extension of lifespan that occurred in the last century.¹ By the end of the reproductive age, germ cells and follicles are depleted from the ovaries, and the ovulatory cycle ceases, resulting in menopause. The peri-

menopausal period commences when the first features of menopause begin until at least 1 year after the final menstrual period, generally lasting an average of 5 years. In humans, the transition to menopause is a set of gradual changes, in which ovarian function, reproductive capacity, and hormonal status are altered long before menses stops completely. Menopause generally occurs between 45 to 55 years of age, and the symptoms vary among women.

After menopause, estrogen levels fall, but the gonadotropins including luteinizing hormone and follicle-stimulating hormone (FSH) are elevated and often even higher than before menopause.¹ The incidence of ovarian cancer is highest in the perimenopausal period, which supports the gonadotropin stimulation theory of ovarian cancer etiology. Among the physiological changes associated with menopause, the ovarian tissues undergo morphological transformation, known as ovarian aging, and this is implicated in the high incidence of ovarian cancer that occurs during the perimenopausal and immediate postmenopausal periods.^{1–4} One feature associated with ovarian aging is the accumulation of ovarian morphological changes such as deep invaginations, surface papillomatosis, and inclusion cysts (Supplemental Figure 1, see <http://ajp.amjpathol.org>), which are thought by some to be the histological precursors of ovarian cancer.^{3,4}

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The phenomenon of menopause is not restricted to human females but also occurs in laboratory rats and mice that exhibit postreproductive lifespan preceded by a period of gradual reproductive decline. A naturally occurring mutant, white spotting variant (Wv) mice harbor a point mutation in the kinase domain of the c-kit gene, resulting in developmental defects in germ cells, pigment-forming cells, red blood cells, and mast cells in homozygous mutant mice.^{5–8} The Wv/Wv mice have a similar lifespan as wild type, are sterile, white coated with black eyes, and predisposed to ovarian neoplasms.⁹ The Wv/Wv homozygous mice contain less than 1% of the normal number of oocytes at birth, and the remaining oocytes are depleted by ~8 weeks of age.⁹ Consequently, ovulation ceases, and an increase in pituitary gonadotropins follows because of the lack of feedback inhibition that is normally mediated through progesterone released from the corpora lutea.¹⁰ The females recapitulate and exaggerate the postmenopausal alterations in ovarian morphology and develop ovarian tubular adenomas.⁹ Elevated levels of gonadotropins are believed to be the causative factor of the ovarian neoplasm.^{11,12} The ovarian lesions in the Wv mice are known as complex tubular adenomas.⁹ These ovarian tumors are generally benign and seldom develop malignant features. Nevertheless, the causative factors, the depletion of germ cells, and subsequent increase in gonadotropins, mimic the condition of perimenopausal women, in which gonadotropin levels are elevated and the risk for ovarian cancer is highest.¹³ In addition, the ovarian lesions of the Wv/Wv mice have particular resemblance to morphological changes found in ovaries of women with an increased risk for ovarian cancer. These changes include surface papillomatosis, deep invaginations, and cystadenomas and are considered preneoplastic lesions. Thus, the Wv/Wv mice may constitute a model to investigate how ovulation and gonadotropin stimulation during postmenopause act as etiological factors in ovarian cancer development.

Cyclooxygenase (Cox)-2, encoded by the prostaglandin synthase 2 (*ptgs2*) gene, is a key downstream component of gonadotropin-stimulated signaling in ovulation, and Cox-2 knockout mice are anovulatory.^{14–16} The role of Cox-2 in ovulatory rupture of the ovarian surface is similar to an inflammatory process.¹⁷ Cox-2 is often overexpressed in human cancers,^{18,19} and suppression of Cox-2 by either genetic or pharmacological approaches has been shown to reduce colon tumor development in mouse models^{20–23} and in humans.²⁴ Inhibition of Cox enzymes by nonsteroidal anti-inflammatory drugs also seems to reduce ovarian cancer risk,^{25–27} and a possible mechanism is that inhibition of Cox-2 may reduce the cancer-promoting activity of the inflammation-like ovulatory processes that are stimulated by gonadotropins.²⁸ In this study, we investigated the role of Cox-2 in the development of ovarian tumors in the germ cell-deficient Wv mice, which model postmenopausal ovarian biology.

Materials and Methods

Generation and Genotyping of Wv:Cox-2 Mutant Mice

An inbreeding colony was established by crossing Wv/+ and Cox-2 (+/–) mice in the C57BL/6J background (Jackson Laboratory, Bar Harbor, ME). Littermates with Wv/+;Cox-2 (+/–) genotype were further intercrossed to generate mutant and control mice for analysis. The Wv genotypes of the resulting progeny were identified by the coat color: white, gray with ventral/dorsal spots, or black represents Wv/Wv, Wv/+, or Wv (+/+), respectively. Cox-2 genotypes were verified by polymerase chain reaction (PCR) analysis using the genomic DNA isolated from mouse tails with the following primers²⁹: Cox-2 forward: 5'-GCCCTGAATGAAC-TGCAGGACG-3' and reverse: 5'-ACCTCTGCGATGCTCTTCC-3'; Neo forward: 5'-GCCCTGAATGAACTGC-AGGACG-3' and reverse: 5'-CACGGGTAGCCAACGCTATGTC-3'. PCR reactions were set up in a 25- μ l total volume with a final concentration of 2.5 U of platinum *Taq*DNA polymerase (Invitrogen, Carlsbad, CA), 1 $\times$ PCR buffer (Invitrogen), 1.5 mmol/L MgCl₂, 0.2 μ mol/L primers, 0.2 mmol/L dNTP mixtures (Promega, Madison, WI), and 15 to 20 ng genomic DNA. Cycling conditions were followed as that described by the Jackson Laboratory. In brief, the PCR reactions were performed with 35 cycles consisting of melting at 94°C for 30 seconds, annealing at 66°C for 1 minute, and extension at 72°C for 1 minute. At the end of the PCR reactions, the mixtures were kept for an additional 2 minutes at 72°C and stored at 10°C until analysis. PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide. The presence of wild-type and neo alleles corresponds to 857-bp and 500-bp products, respectively. Approximately 30 to 40% of homozygous Cox-2 mutant mice were lost during the preweaning stage,²⁹ and only limited numbers (28 females were obtained and analyzed so far) of mice with the Wv/Wv:Cox-2 (–/–) genotypes were obtained.

Analysis and Quantitation of Tumor Phenotype

The ovaries of 4- to 5-month-old mice with Wv/Wv, Wv/Wv:Cox-2 (+/–), or Wv/Wv:Cox-2 (–/–) genotypes were harvested for histological analysis of ovarian tumor phenotype. The largest cross-section of an ovary was stained with cytokeratin-8 to identify epithelial components, and a digital image was recorded. The degree of tumor phenotype was defined as the percentage of the ovary penetrated by the cytokeratin-8-positive epithelial tubular structure, which was quantitatively calculated by laying a 20 $\times$ 20 grid over the ovarian image to divide the ovary into 200 to 300 squares. Grids positive and negative for internal epithelial lesions were counted independently by two noninvolved persons (student assistants) and used to calculate the percentage of ovaries infiltrated by the tubular adenomas.

Preparation of Ovarian Lysate and Western Blotting

Mouse ovaries were snap-frozen on surgical removal and maintained at -80°C until the tissues were used for protein analysis. To prepare ovarian lysates, frozen tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mmol/L Tris, pH 7.2, 100 $\mu\text{mol/L}$ sodium orthovanadate, and 50 mmol/L NaF) containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN) using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK) at 5000 rpm, 20-second interval for a total of 4 minutes. The homogenate was centrifuged at $10,000 \times g$ for 10 minutes to remove the particulate material. The protein concentration in the supernatant was measured using the DC protein assay (Bio-Rad, Hercules, CA).

For Western blotting, an aliquot of the total ovarian lysate was separated by electrophoresis on a 4 to 12% gradient gel and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated with antibodies against either Cox-1 or Cox-2 (Cayman Chemicals, Ann Arbor, MI), or β -actin (Sigma, St. Louis, MO) followed by horseradish peroxidase-labeled secondary antibodies (Sigma). The signals were revealed using a chemiluminescence detection system (Pierce, Rockford, IL). Ovarian lysate from Cox-1 (–/–) mice (obtained from Dr. Robert Langenbach, Laboratory of Experimental Carcinogenesis and Mutagenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC) was used to confirm the specificity of Cox-1 antibodies.

Immunohistochemistry

Ovaries were fixed in buffered formalin and embedded in paraffin. The paraffin blocks were cut into 5- μm -thick sections that were placed on positively charged slides. The sections were dewaxed in xylene and hydrated through graded ethanol. Heat-induced antigen retrieval was then performed in 10 mmol/L sodium citrate (pH 6.0) in a microwave initially at high-power setting (no. 10) for 2 minutes followed by low-power setting (no. 2) for 10 minutes. The endogenous peroxidase activity was blocked by immersing the slides in 3% H_2O_2 in methanol for 15 minutes. After 30 minutes of incubation with blocking serum, slides were incubated with rat monoclonal anti-Troma-1/cytokeratin-8 antibodies (Developmental Studies Hybridoma Bank from The University of Iowa, Ames, IA) at 1:600 dilution, Cox-2 rabbit polyclonal antibodies (Cayman Chemical) at 1:600 dilution, Cox-1 rabbit polyclonal antibodies (Cayman Chemical) at 1:500 dilution, or F4/80 rat polyclonal antibodies (Serotec Ltd., Kidlington, Oxford, UK) at 1:200 dilution at 4°C overnight followed by incubating with goat anti-rat horseradish peroxidase-labeled secondary antibodies (BD Pharmingen, Franklin Lakes, NJ) at 1:100 dilution for cytokeratin-8, anti-rabbit labeled polymer horseradish peroxidase (DAKO, Carpinteria, CA) at 1:100 dilution, or biotinylated

anti-rat at 1:200 dilution for F4/80 for 35 minutes at room temperature. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with hematoxylin and mounted in 50:50 xylene/Permount.

Prostaglandin E_2 Assay

The prostaglandin E_2 (PGE_2) level in the ovarian lysates was measured using an enzyme-immunoassay kit (Cayman Chemical). This assay is based on the competition between PGE_2 and a PGE_2 acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibodies. This antibody- PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been attached to the plate. In brief, ovarian lysates together with enzyme-immunoassay buffer, PGE_2 tracer, and antibody were added into the plate. The mixtures were incubated at 4°C overnight. The plates were washed to remove the unbound reagents and developed with the Ellman's reagent (substrate), and the absorption was measured at 412 nm. The amount of PGE_2 in the sample was determined from a standard curve.

FSH Assay

At the time of sacrifice, 100 to 200 μL of sera were collected from each animal and stored at -80°C until testing. The FSH level in the serum was measured by radioimmunoassay through custom service from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA).

Drug Administration

Four-week-old female Wv/Wv mice, weighing ~ 14 g, were randomly allocated into three groups: nontreated control ($n = 5$), indomethacin-treated ($n = 7$), and celecoxib (Celebrex)-treated groups ($n = 8$). Celecoxib was administered through feeding with AIN 76A diet (Dyets Inc., Bethlehem, PA) containing 1500 ppm celecoxib (Fox Chase Cancer Center Pharmacy). Indomethacin was introduced through drinking water (6 mg/ml). During the study, mice were permitted free access to the diet and drinking water. All of the mice were inspected once daily to monitor their general health status. Body weight was measured once per week throughout the experiments. Optimal dosage was estimated to be 100 $\mu\text{g/day}$ for celecoxib and 30 $\mu\text{g/day}$ for indomethacin. Dosages double these amounts resulted in some toxicity such as reduced weight or activity/alertness in the female Wv/Wv mice. Mice were sacrificed at ~ 4 months of age. Ovarian tissues were collected and subjected to histopathological examination.

Statistical Analysis

Basic and standard analytical procedures were applied to examine the statistical significance of the data. Differences in proportions were evaluated by the χ^2 or the

Figure 1. Development of tubular adenomas in Wv/Wv mice. Ovarian morphology was compared between wild-type littermates (**A** and **B**) and Wv/Wv (**C–H**) mice. Cytokeratin-8 (Troma-1) staining was used to highlight epithelial cells. **A:** An ovary from wild-type control at 4 months of age and **B:** at higher magnification. Representative examples of ovaries from Wv/Wv mice at the age of 1 month (**C**), 2 months (**D**), 3 months (**E**), 4 months (**F**), 5 months (**G**), and 9 months (**H**) are shown. At least five mice at each time point were examined. Original magnifications: $\times 40$ (**A**, **C–H**); $\times 100$ (**B**).

Fisher exact test, as appropriate. Student's *t*-test was used to compare the differences in means between two groups. Statistical significance was considered as $P < 0.05$. All *P* values are two-sided.

Results

Ovarian Surface Epithelia Undergo Morphological Transformation and Tumorigenesis in Wv Mice

We have maintained a colony of Wv mice by inbreeding for the last 3 years. Of the more than 200 mature (3 months or older) Wv/Wv females examined, all exhibited ovarian tubular adenomas, whereas none were observed in the wild-type littermates. The progressive changes in ovarian morphology were documented, and representative examples are shown in Figure 1. As a comparison, the wild-type ovary contains developing follicles of all stages and multiple corpora lutea (Figure 1A). The surface epithelia lining the perimeter of the

ovary are the only cells positive for cytokeratin-8, a marker of epithelial cells. Higher magnification reveals the well-organized surface epithelial cells and interstitial stroma cells in the ovarian cortex (Figure 1B). In Wv/Wv mice at 1 month of age, few follicles can be observed in the ovary although the cortex is still enveloped by a smooth layer of cytokeratin-8-positive surface epithelial cells (Figure 1C). We began to observe the presence of tubule-like structures in the ovarian cortex of the Wv/Wv mice by 2 months of age (Figure 1D). By the end of 3 months, the entire ovary was replaced by tumorous lesions. Positive staining for cytokeratin-8 indicates the epithelial origin of the tubular structures (Figure 1E). At 4 and 5 months, the phenotype of tubular adenomas became more complex as shown by the increased penetration and branching of the epithelial tubules (Figure 1, F and G), together with the appearance of scalloping and crowding of epithelial cells into multiple cell layers. We have analyzed the Wv/Wv ovaries for up to 1 year of age when the entire ovaries were completely permeated with the tumor cells. The lesions are somewhat more complex and intense in older mice, but the tumor cells have not further expanded into large masses or acquired malignant features (Figure 1H). Although the ovarian lesions in the Wv mice distribute throughout the ovarian stroma, and are known as stromal tubular adenomas,⁹ the contiguous connection to ovarian surface epithelium is evident. This is especially pronounced in cases of early ovarian lesions in younger (7 to 10 weeks) mice when only a few lesions have developed. Obvious surface origination of the epithelial lesions can be observed (Figure 2A, arrow): the tubular structure is contiguous with the monolayer of the surface epithelium (Figure 2B). We conclude that most if not all of the tubular adenomas in Wv/Wv ovaries are derived from ovarian surface epithelial cells. The majority of the lesions either exhibit inclusion cyst-like structures (Figure 2C) or resemble surface deep invaginations/papillomatosis (Figure 2D). Although dysplastic morphology is evident in some epithelial compartments of the tubular adenomas (Figure 2, E and F), the ovarian epithelial tubular structures in the Wv mice are considered benign tumors and the lesions are confined to ovarian tissues and do not become metastatic. Histopathological evaluation also indicated their benign cytological morphologies without evident mitotic figures. These observations are consistent with the notion that the benign epithelial tubular adenomas are caused by the stimulation of the reproductive hormones gonadotropins rather than oncogenic alterations. Indeed, we confirmed that serum FSH, a key gonadotropin, is greatly increased (~ 10 -fold) in female Wv/Wv mice (Figure 2G). The magnitude of FSH increase is very similar to the elevation found in menopausal women.¹ Thus, it seems that the Wv/Wv mouse model closely mimics the changes in ovarian physiology (follicle depletion), endocrine factor (hormonal increase), and ovarian pathology (morphological changes) in menopausal women. In the investigation of potential ovarian mediators of gonadotropins, we found that ovarian

Figure 2. Ovarian morphological and physiological changes in Wv/Wv mice. Ovarian tissues were harvested and subjected to histological analysis. Cytokeratin-8 staining was used as a marker for epithelial cells. **A:** An example of an ovary from a 7-week-old Wv/Wv mouse is shown for the surface epithelial origin of the tubular adenoma structure. The **arrow** indicates the contiguous links between surface epithelium and the tubular epithelial structure, which are stained for cytokeratin-8, and the area is shown at a higher magnification in **B**. **C** and **D:** Two representative examples of tubular adenomas; and **E** and **F:** two examples of dysplastic epithelial cells from ovarian tubular adenomas of 4-month-old Wv/Wv mice. **G:** Serum was collected from four each of wild-type and Wv/Wv 4-month-old female mice to determine FSH levels in triplicate. Averages of triplicate with SDs are shown. Two-sided Student's *t*-test indicates the difference is statistically significant ($P < 0.001$). **H** and **I:** Ovaries ($n = 10$) from five female mice each, of either Wv/Wv or wild-type littermates at 4 months of age, were collected, dissected to remove the surrounding fat tissues, and pooled. The tissues were homogenized in RIPA buffer, and the lysate was used for measurement of Cox-2 protein by Western blot (**H**) and PGE₂ level by EIA (**I**). Actin level was used as a protein loading control in Western blotting. Averages of triplicate with SDs are shown for PGE₂ levels. Two-sided Student's *t*-test indicates the difference is statistically significant ($P < 0.001$). Examples of ovarian morphology are shown by H&E staining in Supplemental Figure 2 (see <http://ajp.amjpatbol.org>). Original magnifications: $\times 40$ (**A**); $\times 200$ (**B-F**).

Cox-2 protein levels are dramatically increased in Wv/Wv mice compared with those of control littermates (Figure 2H). Accordingly, the ovarian PGE₂ level is threefold to fivefold higher in Wv/Wv mice (Figure 2I).

Wv Mouse Tumor Phenotype Is Suppressed by a Reduction of Cox-2 Gene Dosage

We investigated whether Cox-2 plays a role in the formation of tubular adenomas in Wv mice in which the elevated gonadotropins likely stimulate Cox-2 expression. Cox-2 deficiency was introduced into the Wv mouse colony by crossing Wv/+ with Cox-2 (+/−) mice, and a new inbred colony was established by

crossing Wv/+ : Cox-2 (+/−) siblings. Progenies homozygous for Wv and all genotypes of Cox-2, (+/+), (+/−), or (−/−), were examined for ovarian morphology at ~4 months of age (Figure 3), when the tubular adenoma phenotype seems to be fully presented and fairly uniform in the Wv/Wv mice. The ovarian tubular adenoma phenotype in the 70 Wv/Wv : Cox-2 (+/+) mice produced in this colony was essentially identical to that found in more than 200 female mice of the original colony before introduction of the Cox-2 mutation: all of the ovaries exhibited severe complex tubular adenomas that permeated the entire organ. We observed a significant alleviation of ovarian lesions in the Wv/Wv : Cox-2 (+/−) ovaries analyzed (Figure 3, D–F),

Figure 3. Rescue of Wv/Wv mouse ovarian tubular adenoma phenotype by the Cox-2 knockout. The ovarian morphology from littermates of the Wv and Cox-2 knockout inbred colony was compared. Cytokeratin-8 (Troma-1) staining was used to identify epithelial cells. Representative examples of ovaries from 4.5-month-old mice of Wv/Wv:Cox-2 (-/-) (A-C) and Wv/Wv:Cox-2 (+/-) (D-F) genotypes are shown. The image shown in A is the example of ovary (n = 10) from Wv/Wv:Cox-2 (-/-) mice with significant reduction in ovarian lesions. G: The degree of tumor involvement of each ovary was estimated and the distribution is plotted. n indicates the number of ovaries analyzed. The mean value for ovarian tumor involvement is 0% for wild type, 93% for Wv/Wv, 32% for Wv/Wv:Cox-2 (+/-), and 70% for Wv/Wv:Cox-2 (-/-) genotypes. Student's *t*-tests showed *P* < 0.005 for Wv/Wv versus Wv/Wv:Cox-2 (+/-), and *P* < 0.05 for Wv/Wv versus Wv/Wv:Cox-2 (-/-), indicating a statistically significant difference. The difference between Wv/Wv:Cox-2 (+/-) and Wv/Wv:Cox-2 (-/-) also is statistically significant (*P* < 0.001). Examples of ovarian morphology are shown by H&E staining in Supplemental Figure 3 (see <http://ajp.amjpathol.org>). Original magnifications, $\times 40$.

although the degree to which the tumor phenotype was suppressed varied greatly (Figure 3G). Some ovaries completely lacked epithelial tubular structures inside the cortex (Figure 3, D and F), and some contained only a small number of epithelial tubular structures (Figure 3E). Unlike wild-type ovaries, these Wv/Wv:Cox-2 (+/-) ovaries lacked apparent follicles or cor-

pora lutea. Thus, hemizygous reduction of the Cox-2 gene resulted in a complete (Figure 3, D and F) or partial (Figure 3E) rescue from the epithelial adenoma phenotype. Unexpectedly, of the 10 ovaries from mice of Wv/Wv:Cox-2 (-/-) genotype, only three ovaries exhibited a significant (50% area) reduction in the tubular adenoma phenotype (Figure 3, A and G). All other ovaries showed ovarian morphology indistinguishable from that of Wv/Wv:Cox-2 (+/+) genotype (Figure 3, B and C). The mean value for the area of the ovary covered by lesions is 93% for Wv/Wv, 32% for Wv/Wv:Cox-2 (+/-), and 70% for Wv/Wv:Cox-2 (-/-) genotypes (Figure 3G). Thus, a reduction in Cox-2 gene dosage rescued the ovarian epithelial morphological alteration, but deletion of both copies was less sufficient in reversing the adenoma phenotype in Wv/Wv mice.

Cox-2-Null Deletion Causes a Compensatory Increase in Cox-1 Expression in Ovaries

Measurement of prostaglandin levels in ovaries of these mice showed that Wv/Wv mice exhibited an increase of ovarian PGE₂ (Figure 4A) and Cox-2 (Figure 4B) levels over the wild-type littermates, to 4.2- and 2.1-fold, respectively. The expression of the Cox-1 protein was also elevated over that of wild type (3.5-fold). This increase in the Cox-1 level is unexpected because it is commonly accepted that Cox-1 is a housekeeping gene and generally not regulated.³⁰ However, Cox-1 expression was previously found increased in human ovarian cancer^{31,32} and in ovarian tumors found in a variety of mouse ovarian cancer models.³³

Ablation of one allele of Cox-2 lowered the amount of PGE₂ produced in the ovaries by 45% (compare Wv/Wv:Cox-2 (+/-) to Wv/Wv) (Figure 4A). The prostaglandin level in Wv/Wv:Cox-2 (-/-) mice was higher than that of Wv/Wv:Cox-2 (+/-) and similar to that of Wv/Wv mice (Figure 4A). The ovarian Cox-1 protein amount in Wv/Wv:Cox-2 (-/-) was determined to be 2.8-fold of those in Wv/Wv or Wv/Wv:Cox-2 (+/-) mice (Figure 4C). Presumably, the Cox-2 homozygous deletion causes a compensatory increase in Cox-1 and total prostaglandin level. The Cox-1 compensation was also observed in non-Wv Cox-2-null mice (Figure 4, D and E). In the ovarian extract, the PGE₂ level was increased 25% in Cox-2 homozygous knockout mice, although ovarian PGE₂ was 20% less in Cox-2 hemizygous mice (Figure 4D). Accordingly, ovarian Cox-1 protein was increased 2.1-fold in Cox-2 (-/-) mice but remained unchanged in Cox-2 (+/-) mice (Figure 4E). The expression of Cox-1 and Cox-2 was analyzed by immunostaining in wild-type and Wv/Wv ovaries (Figure 4F). Weak Cox-1 staining distributed evenly throughout the wild-type ovary, and Cox-2 staining was observed in stromal and follicular cells but not in surface epithelial cells. In Wv/Wv ovaries, both epithelial and stromal cells stained strongly for Cox-1 and Cox-2 (Figure 4F).

We studied the epithelial and stromal compartments to determine whether tumor phenotype and Cox-2 expres-

Figure 4. Ovarian PGE₂, Cox-1, and Cox-2 levels in Wv and Cox-2 mutant mice. Ovaries ($n = 4$ to 10) from female mice, of either Cox-2 (-/-), Cox-2 (+/-), Wv/Wv, Wv/Wv:Cox-2 (+/-), or wild-type littermates at 4 months of age were collected and pooled. The tissues were homogenized in RIPA buffer, and the lysate was used for measurement of PGE₂ level by EIA. Relative values compared with wild type (WT) are reported as the percentage of the wild-type control, which is 3.4 pg/ μ g protein. **A:** Three experiments were performed to obtain the representative result, and the averages of triplicate with SDs are shown. Two-sided Student's *t*-test indicates the difference is statistically significant for the comparisons between WT and Wv/Wv ($P < 0.0001$), WT and Wv/Wv:Cox-2 (+/-) ($P < 0.001$), WT and Wv/Wv:Cox-2 (-/-) ($P < 0.0028$), Wv/Wv and Wv/Wv:Cox-2 (+/-) ($P < 0.0001$), and Wv/Wv:Cox-2 (+/-) and Wv/Wv:Cox-2 (-/-) ($P < 0.0002$), but the difference does not reach statistical significance comparing Wv/Wv and Wv/Wv:Cox-2 (-/-) ($P > 0.12$). **B:** Cox-1 and Cox-2 proteins in the ovarian lysate from a pool of 10 ovaries of each genotype were determined by Western blot with actin as loading control. **C:** One of two representative experiments was shown for the determination Cox-1 protein from two ovaries (one mouse) by Western blot. Ovarian lysate from a Cox-1 (-/-) mouse was used for control for the specificity of the anti-Cox-1 antibodies. *Nonspecific protein band that served as a protein loading control. **D and E:** Ovaries ($n = 10$) from five mice each of either Cox-2 (-/-), Cox-2 (+/-), or wild-type (WT) littermates at 4 months of age were collected for PGE₂ assays (**D**) and Western blot analysis for Cox-1 and Cox-2 proteins with actin as a loading control (**E**). Two-sided Student's *t*-test indicates the differences between the PGE₂ levels are statistically significant comparing WT and Cox-2 (+/-) ($P < 0.02$) or WT and Cox-2 (-/-) ($P < 0.04$). **F:** Ovaries from wild-type and Wv/Wv genotypes were stained for Cox-1 or Cox-2. ose, ovarian surface epithelial; s, stroma; f, follicle. Original magnifications, $\times 200$.

sion correlated with inflammation in the ovaries. Infiltration of neutrophils and eosinophilic cells was rarely observed in Wv mouse ovaries and tumors, and no evidence of acute inflammation was found. We also examined macrophage density that associates with chronic inflammation by staining for the F4/80 marker (Figure 5, A and B). F4/80 staining indicated a robust increase in the appearance of macrophages in the tumorous Wv/Wv ovaries over those of the wild-type littermates (Figure 5A). Consistently, the macrophage number was reduced in the recovered ovaries of Wv/Wv:Cox-2 (+/-) genotype

but not of the Wv/Wv:Cox-2 (-/-) genotype (Figure 5, A and C). Moreover, in nontumorous wild-type and Cox-2 (-/-) ovaries, macrophages were distributed in the cortex and stromal compartments but were absent in or near epithelial cells. In the ovarian tumors from Wv/Wv mice, macrophages often infiltrated into the epithelia (Figure 5B, red arrow). The relative macrophage density was quantitated as shown in Figure 5C. The correlation of macrophage density with tumor phenotypes suggests that inflammatory reactions promote the tubular adenoma development in Wv ovaries.

Figure 5. Ovarian macrophage density in Wv and Cox-2 mutant mice. **A:** Macrophages were identified by staining with F4/80. **B:** Examples of a higher magnification of ovaries stained with the F4/80 macrophage marker are shown. The **arrow** indicates macrophages infiltrating epithelia. **C:** Macrophage density was quantitated by counting five fields each of three ovaries from each genotype. Averages of five countings with SDs are shown. Student's *t*-test indicates that macrophage numbers are not statistically significant between WT, Cox-2 (-/-), and Wv/Wv: Cox-2 (+/-) ($P > 0.05$), but when the values of these three genotypes compared with those of Wv/Wv or Wv/Wv: Cox-2 (-/-), the difference is statistically significant ($P < 0.001$). Original magnifications: $\times 40$ (A); $\times 200$ (B).

Pharmacological Inhibitors of Coxs Effectively Prevent Ovarian Epithelial Transformation and Tumorigenesis

We then tested whether the effect of reducing the Cox-2 gene dosage on ovarian tumor phenotype can be achieved by using pharmacological agents. Based on previous studies and our own toxicity study specifically in Wv/Wv female mice, nontoxic dosages of celecoxib, a Cox-2-specific inhibitor, and indomethacin, a nonselec-

tive COX inhibitor, were determined to be 200 and 30 $\mu\text{g/day}$, respectively. When the Wv/Wv female mice received these inhibitors starting at 4 weeks of age, a significant reduction in tumor phenotype was observed at the age of 3 months in all ovaries, and some ovaries were devoid of tubular adenomas (Figure 6). In three of eight Wv/Wv mice, celecoxib feeding prevented the ovarian tumor phenotype; and in the indomethacin-treated group, five of seven mice were rescued. All ovaries of the five control Wv/Wv littermates that were maintained identically

Figure 6. Rescue of Wv/Wv mouse ovarian tubular adenoma phenotype by celecoxib (Celebrex) and indomethacin administration. The representative ovarian morphology from Wv/Wv mice controls (**A**) and those fed with celecoxib (**B**) or indomethacin (**C**) was compared. Cytokeratin-8 (Troma-1) staining was used to identify epithelial cells. The *P* values generated from the χ^2 test (or Fisher's exact test) are 0.2308, 0.0278, and 0.3147, respectively, for the comparisons between celecoxib treatment versus control, indomethacin treatment versus control, and celecoxib versus indomethacin treatment. Thus in this experiment only the difference between indomethacin treatment versus control reaches statistical significance. One example of wild type and two examples of treatment with celecoxib and indomethacin are shown. Original magnifications, $\times 40$.

but did not receive inhibitors showed ovarian tubular adenomas. Only the comparison between controls and indomethacin-treated mice in this experiment reaches statistical significance. However, considering that ovarian tumors were found in all of the more than 300 Wv female mice analyzed previously, the observed reduction in tumor development by both celecoxib and indomethacin is likely significant. Thus in this preliminary study including only a small number of animals, we found that Cox inhibitors are able to prevent ovarian epithelial morphological transformation and tumor phenotypes. Inhibition of both Cox-1 and Cox-2 with indomethacin seems more effective than inhibition of Cox-2 alone with celecoxib, but the difference did not reach statistical significance. The number of animals used in these experiments is relatively small, and additional confirmation is needed. When indomethacin was given for a period of 1 month to Wv/Wv mice at 3 months of age when ovarian tumors were already established, the tumors were not reduced compared with controls (not shown), suggesting inhibition of Cox-2 prevents the development of ovarian tumors but has no suppressive effect on established tumors.

Discussion

In the present study, we demonstrate that a reduction of Cox-2 gene dosage rescued the ovarian aging phenotype of the Wv/Wv mice, whereas homozygous deletion of

Cox-2 gene was less effective in reducing the formation of these epithelial lesions in the ovaries because Cox-2 elimination was accompanied by an increase in ovarian Cox-1 expression and PGE₂ synthesis. The tumor phenotype was also reduced by the use of pharmacological agents to inhibit Cox-1 and/or Cox-2. These findings suggest that increased Cox-1/Cox-2 activity contributes to the preneoplastic morphological changes of the ovarian surface epithelium, and the tumor phenotype can be reversed by a reduction of Cox gene dosage either by genetic or pharmacological approaches. The results suggest the inflammatory environment of the germ cell-depleted ovaries stimulates epithelial morphological changes and tumor development, and this circumstance provides an excellent example of how inflammation promotes cancer. This study provides a model to study the links between reproductive factors, inflammation, and ovarian tumor development.

Inflammatory Environment in Postmenopausal Ovaries

The link between inflammation and cancer is well recognized: chronic inflammation induced by infections or other chemical and pathological factors creates an inflammatory microenvironment in the tissue, which is composed of epithelial cells, stromal cells, leukocytes, and macrophages. These cells are networked by autocrine

and paracrine interactions mediated by proinflammatory cytokines such as tumor necrosis factor- α and interleukin- β .^{34,35} Signal pathways involving nuclear factor- κ B, tumor necrosis factor- α , and Cxcs are known to be involved in both inflammation and cancer. The inflammatory mediators such as proteolytic enzymes, prostaglandins, and diverse reactive oxygen and nitrogen species create a tumor-promoting environment in which transformed (altered by either genetic or epigenetic means) epithelial cells thrive and are selected to expand.^{34,35} Cxcs, Cox-1 and/or Cox-2, play crucial roles in regulation of inflammation and are thought to be targets of the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs for cancer prevention.^{18,19,23}

Gene knockout mice suggest that both Cox-1 and Cox-2 are dispensable for development and have verified the role of Cox-1 and Cox-2 in inflammation that counters host infection by microorganisms.^{29,30} Another established physiological function for Cox activity is in reproduction, especially ovulation,^{14–16} which is considered an inflammatory-like process.¹⁷ Ovarian Cox-2 is induced by the periodical surge of gonadotropins and plays an important and necessary role in the proteolysis and tissue remodeling that precedes the ovulatory rupture of the follicle and ovarian surface to release the ova.³⁶ The elevated gonadotropin level in postmenopause, although it does not cause ovarian surface rupture as in ovulation, still stimulates the ovulation-like inflammatory process and Cox expression.²⁸ The Wv mouse model simulates the postmenopausal ovarian inflammatory conditions as indicated by the increased expression of ovarian Cox-1 and Cox-2 and macrophage infiltration.

We propose that this ovarian inflammatory environment promotes tissue remodeling and perturbation or disruption of epithelial structure, leading to epithelial morphological transformation and the development of the ovarian tubular adenomas in the Wv mice. This mechanism may explain gonadotropin stimulation as an etiological factor for an increased ovarian cancer risk. The ovarian tumor in Wv mouse also represents a unique model in which a physiological inflammatory environment promotes tumor development.

Germ Cell-Deficient Wv Mice as Models for Reproductive Factors in Ovarian Cancer Etiology

Although the ovarian tumors may invade adjacent tissues and fat pads at later stages,⁹ we have not observed any shedding of tumor cells and the formation of ascites in Wv mice. Although the ovarian tubular adenomas are derived from ovarian surface epithelial cells, the histology of the tumors differs from human epithelial ovarian cancer. Thus, the Wv mouse is not a model for human malignant epithelial ovarian cancer.

Nevertheless, the ovarian epithelial morphological transformation in Wv mice resembles ovarian morphological changes associated with reproductive aging and perimenopausal gonadotropin stimulation. Most likely, the Wv mouse model mimics certain biological aspects of

ovarian cancer risk associated with menopause and gonadotropin stimulation. The ovarian tumors in Wv mice are associated with excessive hormonal stimulation but lack genetic mutations that are commonly found in human ovarian cancer, such as Ras and B-Raf mutations in borderline tumors, p53 mutations in malignant ovarian cancer, and pten mutations in the endometrial subtype of ovarian carcinomas.³⁷ In the last few years, a number of technical breakthroughs have led to the establishment of several mouse models for ovarian cancer. First, a genetically defined model of ovarian cancer was established by Orsulic and colleagues,³⁸ in which mouse ovarian surface epithelial cells were transfected with defined genetic changes such as k-Ras, v-Akt, v-myc, and so forth. The cells were then reimplanted into the ovarian bursa and malignant ovarian tumors developed. Using the MIS II R promoter, a mainly ovarian-restricted transcript, Connolly and colleagues³⁹ developed the T-antigen transgenic line that develops malignant bilateral ovarian tumors. Presumably, T-antigen expression results in the inactivation of both p53 and Rb. Indeed, using adenoviral delivery of cre to ovaries of mice with floxed p53 and Rb, Flesken-Nikitin and colleagues⁴⁰ demonstrated the development of malignant ovarian tumors when both p53 and Rb are deleted. Most recently, mice with conditional expression of K-ras and deletion of pten in ovarian surface epithelial cells were found to develop endometriosis and endometrioid carcinomas.⁴¹ Because both mutations are present in endometriosis and endometrioid ovarian cancer in humans, this model seems to recapitulate the genotype and histomorphology associated with the human disease. There are likely additional ovarian cancer animal models that are not mentioned here. These genetic models have provided persuasive evidence for the relevance of these mutations in ovarian carcinogenesis but, nevertheless, have not yet incorporated components related to the etiology of ovarian cancer.

To reconcile the ovarian etiology related to gonadotropin stimulation and postmenopausal biology with genetic mutations in the development of ovarian cancer, it may be proposed that the postmenopausal gonadotropin stimulated epithelial proliferation and morphological transformation may select and promote the expansion of cells with genetic mutations. Mice with a p53 mutation alone do not develop ovarian epithelial tumors, even when ovaries with mutant p53 are transplanted into wild-type hosts to bypass the development of sarcomas and lymphomas in the p53 mutant background.⁴² As a prediction, if additional genetic mutations (such as a p53 mutation) are added to the Wv/Wv females, malignant ovarian carcinomas may develop. Preliminary results of these experiments in our laboratory are very suggestive, but a thorough analysis of these mice with compound genetic mutations will take its course.

Implication of the Cox-2 Gene Dosage Effect

Unexpectedly, a reduction of Cox-2 gene dosage by heterozygous deletion is more effective than homozy-

gous deletion in preventing the ovarian epithelial morphological change and the formation of tubular adenomas in Wv/Wv mice. This seems attributable to a compensatory increase in ovarian Cox-1 when Cox-2 is completely eliminated. Although a compensation between the expression of Cox-1 and Cox-2 was not observed in Cox-2 gene knockout mice initially,^{14,29} it has since been noted,⁴³ and Cox-1 can substitute for Cox-2 in ovulation in certain genetic backgrounds.⁴⁴ Thus, the roles of both Cox-1 and Cox-2 in promoting ovarian tumor development suggest drugs that inhibit both Coxes may be more effective than specific inhibitors of Cox-2 in reducing the development of ovarian tumors.

Moreover, a reduction of the Cox-2 gene copy number is sufficient to reduce the ovarian tumor phenotype in the Wv mice is unique, because reduction of one gene copy number seldom shows a phenotype in gene knockout mice. This dosage-dependent effect of Cox-2 in promoting ovarian tumor development may have crucial implications in strategies using inhibitors as preventive agents for ovarian cancer. It suggests that the use of a low dosage of the drugs to reduce Cox-2 (and Cox-1) activity, rather than a complete suppression of the activity, may be sufficient to reduce tumor incidence.

Interestingly, Cox-1, instead of Cox-2, was found to be overexpressed in human ovarian cancer.^{31,32} The increased Cox-1 expression was observed in several mouse ovarian tumor models,³³ and we also found that Cox-1 is increased in Wv mouse ovarian tumors. Thus, Cox-2 may be important in tumor initiation, and Cox-1 increase may be important for progression and malignancy.

In summary, this study indicates that inhibition of Coxes reduces the morphological perturbation of ovarian surface epithelium induced by increased gonadotropins in Wv mice, which model postmenopausal ovarian biology, and provides further rationale and support for the use of nonsteroidal anti-inflammatory drugs and specific Cox-1 and/or Cox-2 inhibitors for ovarian cancer prevention in peri- and postmenopausal women. In parallel, we found that in human ovaries, peri- and postmenopausal age is the key determinant of ovarian surface epithelial preneoplastic morphological changes in populations with and without BRCA mutations.² Thus, the circumstances in Wv mice are highly pertinent to menopausal women in both biology and pathology. The findings that a reduction instead of complete inhibition of Cox-2 is effective in the suppression of ovarian epithelial lesions and that a compensatory mechanism between the expression of Cox-1 and Cox-2 may offer new strategies for clinical intervention.

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References

1. Gosden RG: Biology of Menopause: The Causes and Consequences of Ovarian Aging. Edited by RG Gosden. New York, Academic Press, 1985
2. Cai KQ, Klein-Szanto A, Karthik D, Edelson M, Daly MB, Ozols RF, Lynch HT, Godwin AK, Xu XX: Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations. *Gynecol Oncol* 2006, 103:719–728
3. Nicosia SV: The aging ovary. *Med Clin North Am* 1987, 71:1–9
4. Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC: Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. *J Natl Cancer Inst* 1996, 88:1810–1820
5. Mintz B: Embryological development of primordial germ-cells in the mouse: influence of a new mutation, Wj. *J Embryol Exp Morphol* 1957, 5:396–406
6. Dubreuil P, Rottapel R, Reith AD, Forrester L, Bernstein A: The mouse W/c-kit locus. A mammalian gene that controls the development of three distinct cell lineages. *Ann NY Acad Sci* 1990, 599:58–65
7. Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P, Besmer P: Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: w37, Wv, W41 and W. *EMBO J* 1990, 9:1805–1813
8. Reith AD, Rottapel R, Giddens E, Brady C, Forrester L, Bernstein A: W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. *Genes Dev* 1990, 4:390–400
9. Murphy ED: Hyperplastic and early neoplastic changes in the ovaries of mice after genic deletion of germ cells. *J Natl Cancer Inst* 1972, 48:1283–1295
10. Eldridge JC, McPherson III JC, Mahesh VB: Maturation of the negative feedback control of gonadotropin secretion in the female rat. *Endocrinology* 1974, 94:1536–1540
11. Murphy ED, Beamer WG: Plasma gonadotropin levels during early stages of ovarian tumorigenesis in mice of the W x/W u genotype. *Cancer Res* 1973, 33:721–723
12. Blaakaer J, Baeksted M, Micic S, Albrechtsen P, Rygaard J, Bock J: Gonadotropin-releasing hormone agonist suppression of ovarian tumorigenesis in mice of the Wx/Wv genotype. *Biol Reprod* 1995, 53:775–779
13. Cramer DW, Welch WR: Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst* 1983, 71:717–721
14. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK: Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* 1997, 91:197–208
15. Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R: Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. *Endocrinology* 1999, 140:2685–2695
16. Reese J, Zhao X, Ma WG, Brown N, Maziasz TJ, Dey SK: Comparative analysis of pharmacologic and/or genetic disruption of cyclooxygenase-1 and cyclooxygenase-2 function in female reproduction in mice. *Endocrinology* 2001, 142:3198–3206
17. Richards JS, Russell DL, Ochsner S, Espey LL: Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol* 2002, 64:69–92
18. Williams CS, Mann M, DuBois RN: The role of cyclooxygenases

- in inflammation, cancer, and development. *Oncogene* 1999, 18:7908–7916
19. Prescott SM, Fitzpatrick FA: Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta* 2000, 1470:M69–M78
20. Oshima M, Dinichuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM: Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996, 87:803–809
21. Lal G, Ash C, Hay K, Redston M, Kwong E, Hancock B, Mak T, Kargman S, Evans JF, Gallinger S: Suppression of intestinal polyps in Msh2-deficient and non-Msh2-deficient multiple intestinal neoplasia mice by a specific cyclooxygenase-2 inhibitor and by a dual cyclooxygenase-1/2 inhibitor. *Cancer Res* 2001, 61:6131–6136
22. Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Smithies O, Langenbach R: Genetic disruption of PtgS-1, as well as PtgS-2, reduces intestinal tumorigenesis in Min mice. *Cancer Res* 2000, 60:4705–4708
23. Subbaramaiah K, Dannenberg A: Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol Sci* 2003, 24:96–102
24. Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B: The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000, 342:1946–1952
25. Cramer D, Harlow B, Titus-Ernstaff L, Bohlke K, Welch WR, Greenberg ER: Over-the-counter analgesics and risk of ovarian cancer. *Lancet* 1998, 351:104–107
26. Sørensen HT, Friis S, Norgaard B, Møller M, Blot WJ, McLaughlin JK, Ekström A, Baron JA: Risk of cancer in a large cohort of nonaspirin NSAID users: a population-based study. *Br J Cancer* 2003, 88:1687–1692
27. Schildkraut JM, Moorman PG, Halabi S, Calingaert B, Marks JR, Berchuck A: Analgesic drug use and risk of ovarian cancer. *Epidemiology* 2006, 17:104–107
28. Smith ER, Daly MB, Xu XX: A mechanism for Cox-2 inhibitor anti-inflammatory activity in chemoprevention of epithelial cancers. *Cancer Epidemiol Biomarkers Prev* 2004, 13:144–145
29. Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O: Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 1995, 83:473–482
30. Smith WL, Langenbach R: Why there are two cyclooxygenase isozymes. *J Clin Invest* 2001, 107:1491–1495
31. Gupta RA, Tejada LV, Tong BJ, Das SK, Morrow JD, Dey SK, DuBois RN: Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. *Cancer Res* 2003, 63:906–911
32. Daikoku T, Wang D, Tranguch S, Morrow JD, Orsulic S, DuBois RN, Dey SK: Cyclooxygenase-1 is a potential target for prevention and treatment of ovarian epithelial cancer. *Cancer Res* 2005, 65:3735–3744
33. Daikoku T, Tranguch S, Trofimova IN, Dinulescu DM, Jacks T, Nikitin AY, Connolly DC, Dey SK: Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. *Cancer Res* 2006, 66:2527–2531
34. Balkwill F, Coussens LM: Cancer: an inflammatory link. *Nature* 2004, 431:405–406
35. Clevers H: At the crossroads of inflammation and cancer. *Cell* 2004, 118:671–674
36. Tsafirri A, Reich R: Molecular aspects of mammalian ovulation. *Exp Clin Endocrinol Diabetes* 1999, 107:1–11
37. Ozols RF, Bookman MA, Connolly DC, Daly MB, Godwin AK, Schilder RJ, Xu XX, Hamilton TC: Focus on epithelial ovarian cancer. *Cancer Cell* 2004, 5:19–24
38. Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE: Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell* 2002, 1:53–62
39. Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC: Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIR promoter develop epithelial ovarian cancer. *Cancer Res* 2003, 63:1389–1397
40. Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY: Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res* 2003, 63:3459–3463
41. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T: Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med* 2005, 11:63–70
42. Chen CM, Chang JL, Behringer RR: Tumor formation in p53 mutant ovaries transplanted into wild-type female hosts. *Oncogene* 2004, 23:7722–7725
43. Kirtikara K, Morham SG, Raghov R, Lauderkind SJ, Kanekura T, Goorha S, Ballou LR: Compensatory prostaglandin E2 biosynthesis in cyclooxygenase 1 or 2 null cells. *J Exp Med* 1998, 187:517–523
44. Wang H, Ma WG, Tejada L, Zhang H, Morrow JD, Das SK, Dey SK: Rescue of female infertility from the loss of cyclooxygenase-2 by compensatory up-regulation of cyclooxygenase-1 is a function of genetic makeup. *J Biol Chem* 2004, 279:10649–10658