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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Physiological COX-2 Expression in Breast Epithelium Associates with COX-2 Levels in Ductal Carcinoma *in Situ* and Invasive Breast Cancer in Young Women

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Cyclooxygenase-2 (COX-2) overexpression is implicated in increased risk and poorer outcomes in breast cancer in young women. We investigated COX-2 regulation in normal premenopausal breast tissue and its relationship to malignancy in young women. Quantitative COX-2 immunohistochemistry was performed on adjacent normal and breast cancer tissues from 96 premenopausal women with known clinical reproductive histories, and on rat mammary glands with distinct ovarian hormone exposures. COX-2 expression in the normal breast epithelium varied more than 40-fold between women and was associated with COX-2 expression levels in ductal carcinoma *in situ* and invasive cancer. Normal breast COX-2 expression was independent of known breast cancer prognostic indicators, including tumor stage and clinical subtype, indicating that factors regulating physiological COX-2 expression may be the primary drivers of COX-2 expression in breast cancer. Ovarian hormones, particularly at pregnancy levels, were identified as modulators of COX-2 in normal mammary epithelium. However, serial breast biopsy analysis in nonpregnant premenopausal women suggested relatively stable baseline levels of COX-2 expression, which persisted independent of menstrual cycling. These data provide impetus to investigate how baseline COX-2 expression is regulated in premenopausal breast tissue because COX-2 levels in normal breast epithelium may prove to be an indicator of breast cancer risk in young women, and predict the chemopreventive and therapeutic efficacy of COX-2 inhibitors in this population. *(Am J Pathol 2014, 184: 1219–1229; http://dx.doi.org/10.1016/j.ajpath.2013.12.026)*

In 2010, approximately 13% of all breast cancers in the United States were diagnosed in women age 45 and younger, accounting for nearly 18,600 cases of invasive breast cancer and 6500 cases of ductal carcinoma *in situ* (DCIS).[1] Furthermore, the proportion of advanced breast cancers diagnosed in young American women is increasing at a rate of 2% per year, making young women's breast cancer an emerging concern.[2] Compared with breast cancer in older women, young breast cancer patients have increased recurrence and lower survival rates.[3–8] Although a delayed diagnosis can contribute to poorer survival in some young patients,[6,9] the primary factor driving poor prognosis is tumor biology. Young women's breast cancer has increased hormone-receptor negativity, tumor cell proliferation, and lymphovascular invasion compared with postmenopausal cases.[4,6,10] Moreover, young age at the time of breast cancer diagnosis is an independent poor prognostic factor.[4,6,7,11,12] These data provide compelling arguments to develop novel strategies to reduce breast cancer incidence and poor outcomes in young women.

One potential target for young women's breast cancer is cyclooxygenase-2 (COX-2),[13] a key enzyme in the synthesis of homeostatic and proinflammatory prostanoids.[14] In rodent

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breast cancer models, COX-2 overexpression induces mammary tumorigenesis and is associated with multiple tumor-promotional effects including increased angiogenesis, enhanced tumor cell migration and invasion, and reduced antitumor immunity.[15–20] Conversely, COX-2 inhibition or loss in rodent models reduces mammary tumorigenesis and metastasis.[17,21–23] Clinical data are consistent with similar roles for COX-2 in human breast cancer because COX-2 overexpression in breast cancer is associated with decreased disease-free and overall survival.[24,25] In addition, regular use of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the COX family of enzymes, can reduce overall breast cancer risk.[26,27] To date, the function of COX-2 in young women's breast cancer has not been addressed. In a single study, high COX-2 expression in combination with increased collagen I is reported as a poor prognostic indicator in young-onset breast cancer patients (age, <45 years).[16] One mechanism by which COX-2 may contribute to young-onset breast cancers is through its role in normal breast tissue remodeling. Importantly, windows of active breast tissue remodeling specific to young women, such as those associated with puberty, menstrual cycling, pregnancy, and postpartum breast involution, correlate with an increased risk for incidence and progression of breast cancer.[28,29] Support for this has been shown in rodent models in which postpartum mammary gland involution promotes tissue remodeling, tumor progression, and metastasis, all of which are mitigated by anti–COX-2 treatment.[16,30] Furthermore, COX-2 up-regulation has been observed in rat mammary glands after treatment with the ovarian hormones estrogen and progesterone,[31] which is consistent with a role for COX-2 in physiological breast tissue remodeling associated with pregnancy and the menstrual cycle.

We hypothesized that if COX-2 is involved in breast tissue remodeling, then COX-2 inhibition may represent a particularly efficacious chemoprevention strategy for young women. One important step in addressing this hypothesis is to evaluate COX-2 expression in young women's breast tissue. We used human and rodent mammary tissues to investigate the effect of pregnancy and ovarian hormones on COX-2 expression in normal tissue as well as to explore the link between COX-2 expression in histologically normal adjacent breast tissue, DCIS, and invasive ductal carcinoma (IDC) in young-onset cases. We found that COX-2 expression primarily was epithelial and varied greatly between individual women, with evidence of modulation by ovarian hormones. In addition, analysis of COX-2 expression in paired normal adjacent breast epithelium, DCIS, and IDC within breast tissue from the same woman showed that COX-2 expression in the normal epithelium was associated with COX-2 expression in DCIS and IDC. Altogether, these data suggest that factors regulating COX-2 expression in normal breast epithelium influence COX-2 levels in breast cancer, and indicate that further research is warranted into whether women with high COX-2 expression may benefit preferentially from COX-2 inhibition strategies.

Materials and Methods

Human Tissue Acquisition

Breast specimens from 96 premenopausal women ages 20 to 45 years who underwent a clinically indicated biopsy ($n = 10$) or surgery ($n = 86$) were obtained under University of Colorado Institutional Review Board–approved protocols. Eighty-six specimens were obtained through a Health Insurance Portability and Accountability Act–exempt, consent-exempt retrospective cohort study with Institutional Review Board approval (University of Colorado Institutional Review Board 05-0958), and 10 specimens were obtained through a subsequent prospective full-consent cohort study (University of Colorado Institutional Review Board 09-0583 and 08-0104). Histologically normal tissue, DCIS, and IDC were identified by pathologic review. In 11 cases, COX-2 expression in the normal epithelium adjacent to cancer was compared with expression in a separate quadrant of the breast to determine whether COX-2 expression in the histologically normal epithelium was influenced by location. For reproductive stage and epithelial-stromal analyses, 28 cases with clinical reproductive histories were grouped by reproductive categories of nulliparous ($n = 7$), pregnant ($n = 5$), postpartum involuting (within 2 months of parturition or lactation, $n = 7$), and fully regressed parous (7 to 22 years after parturition, $n = 9$). Thirty-seven cases were used for comparison of paired normal adjacent breast epithelium, DCIS, and IDC within breast tissue from the same woman. For correlations, 46 cases were used for normal and DCIS, and 57 for normal and IDC. To determine COX-2 expression over time, an independent cohort of six premenopausal patients with serial biopsies 2 to 3 weeks apart were analyzed.

Animal Husbandry, Reproductive Staging, and Hormone Stimulation

Animal procedures were approved with ethical consideration by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were housed in static caging with 12-hour light-dark cycles and access to food and water *ad libitum*. To obtain distinct reproductive states, female rats approximately 70 days of age were bred, and mammary tissue was harvested from age-matched virgin, pregnant (days 18 to 20), lactating (days 10 to 11), involuting day 2, 4, 6, 8, and 10 (2 to 10 days after weaning), and fully regressed rats (4 weeks after weaning) as described.[32] For estrous cycle studies, serial vaginal smears were performed and mammary glands were harvested at proestrus, estrus, and diestrus stages 1 and 2 as described.[33] Vaginal smear data were confirmed by cervical histology.[33] For estradiol treatment, virgin female rats approximately 65 days of age were injected subcutaneously with 5 μg 17-β-estradiol (Sigma-Aldrich, St. Louis, MO) in 250 μL sesame oil or sesame oil alone daily for 3 days, and

sacrificed 3 days after treatment. For estradiol plus progesterone treatment, female rats approximately 56 days of age were injected subcutaneously with 5 μg 17-β-estradiol plus 1.5 mg progesterone (Sigma-Aldrich) in 50 μL sesame oil or sesame oil alone daily for 7 days, and sacrificed 24 to 48 hours after treatment.

IHC, Image Acquisition, and Quantification

Four-micrometer–thick, formalin-fixed, paraffin-embedded sections were deparaffinized, rehydrated, and sequentially subjected to Dako TRS Antigen Retrieval Solution (125°C under pressure for 5 minutes; Dako, Carpinteria, CA), Dual Endogenous Enzyme Block (10 minutes; Dako), and Protein Block (10 minutes; Dako). Tissue sections were incubated in primary antibody (1 hour at room temperature; human: Cayman Chemical 160112; rat: Cayman Chemical 160106; Ann Arbor, MI), followed by Envision Plus Rabbit (30 minutes at room temperature; Dako). Immunoreactivity was visualized using 3,3′-diaminobenzidine (Dako). Primary antibody specificity was confirmed in humans by incubating 1:1 with COX-2 blocking peptide before staining (Cayman Chemical) (Supplemental Figure S1A), and in rodents by staining mammary tissue from a COX-2 knockout mouse, kindly provided by Christopher Rivard, University of Colorado AMC (Supplemental Figure S1B).

COX-2–stained slides were acquired using a ScanScope T3 scanner (AperioTechnologies, Leica Biosystems) at 0.46 μ per pixel. Aperio analysis software (Leica Biosystems, Wetzlar, Germany) and a color deconvolution algorithm (thresholds: clear = 240, weak positive = 225, medium positive = 198, strong positive = 150) were used to quantify staining intensity (Supplemental Figure S2). For all COX-2 analyses, the analyst was blinded to study design. For COX-2 quantification of histologically normal adjacent breast tissue, lobules of representative COX-2 staining and size were quantified. Representative lobules were chosen based on pathologic review by a clinical pathologist blinded to the study design. When possible, 10 representative lobules per case were analyzed; however, in cases with limited epithelium as a result of the biopsy method used (ie, surgical versus needle), or high stroma or tumor content, at least five lobules per case were analyzed, for a total of 693 lobules across all cases. To quantify epithelial and stromal COX-2, the stroma within each lobule (intralobular stroma) and the stroma between lobules (interlobular stroma) were analyzed separately. To determine epithelial-only stain, the intralobular stroma signal was subtracted from the whole lobule signal. For alveolar:ductal COX-2 analysis, five to seven ducts and five alveoli per duct were analyzed per case. For quantification of COX-2 expression in rat mammary glands, 10 representative fields per animal were analyzed (n = 4 animals/reproductive stage). To control for changes in adipose tissue, lumen size, and cellular content in the rat mammary gland across the reproductive stages, COX-2 stain was normalized to the total number of nuclei.

Immunoblotting

Pooled rat mammary tissue lysates (n = 6 animals/reproductive group) were prepared as described.[34] Forty micrograms of total protein was separated by SDS-PAGE and immunoblotting was performed using polyclonal rabbit anti–COX-2 (160106; Cayman Chemical) and monoclonal anti-actin (Chemicon, Billerica, MA), followed by anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA, and Santa Cruz Biotechnologies, Santa Cruz, CA, respectively) with detection using ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA). COX-2 antibody specificity was confirmed using mammary tissue from COX-2 knockout mice described earlier (Supplemental Figure S1C). Densitometry was performed using ImageJ software version 1.42q (NIH, Bethesda, MD).

Statistical Analysis

For analysis of COX-2 expression across reproductive stages and after estrogen or estrogen plus progesterone treatment, unpaired one-tailed t-tests were used to test for increased COX-2 expression compared with nulliparous/virgin or vehicle. Two-tailed t-tests were used for comparison of low, medium, and high COX-2 levels in normal adjacent, DCIS, and IDC groups, as well as for analyses of COX-2 expression in the normal epithelium in relation to DCIS grade, tumor grade, tumor stage, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) status. The Welch correction was applied if variance between groups was unequal. For linear regression analyses, Gaussian distributions were assumed and Pearson correlation coefficients were calculated. Statistical analyses were performed using GraphPad Prism software (version 6; San Diego, CA).

Results

Wide Range in COX-2 Expression across Premenopausal Human Breast Tissue

To investigate COX-2 expression in the premenopausal human breast, quantitative COX-2 immunohistochemical analyses were performed on breast tissue obtained from women 20 to 45 years of age. First, the effect of tumor proximity on COX-2 expression in normal adjacent epithelium was assessed because field effects in adjacent normal epithelium are well documented (reviewed by Heaphy et al[35]). In a subset analysis (n = 12), the proximity to tumor did not significantly affect adjacent normal COX-2 expression, indicating that COX-2 expression in normal breast tissue can be assessed in breast cancer cases (Supplemental Figure S3). In all cases containing normal epithelium (n = 66), the normal breast tissue stained positively for COX-2, although the percentage of positivity of the tissue

varied more than 40-fold (1.8% to 71%) (Figure 1A). Furthermore, the mammary epithelium was the dominant source of COX-2, showing a range of low, moderate, and high expression, and accounting for approximately 94% of the total signal (Figure 1, B and C). Based on the wide variation in epithelial COX-2 expression observed, we explored whether the reproductive state contributes to COX-2 expression in normal breast tissue. Cases with clinical reproductive histories (Table 1) were separated into nulliparous, pregnant, postpartum involution, and fully regressed parous groups. A marked approximately sevenfold increase in total lobule COX-2 expression was observed in pregnant cases compared with nulliparous controls (Figure 1, D and E). During pregnancy, COX-2 up-regulation occurred predominantly in the alveoli (Figure 1, F and G). COX-2 expression in the normal breast also increased in

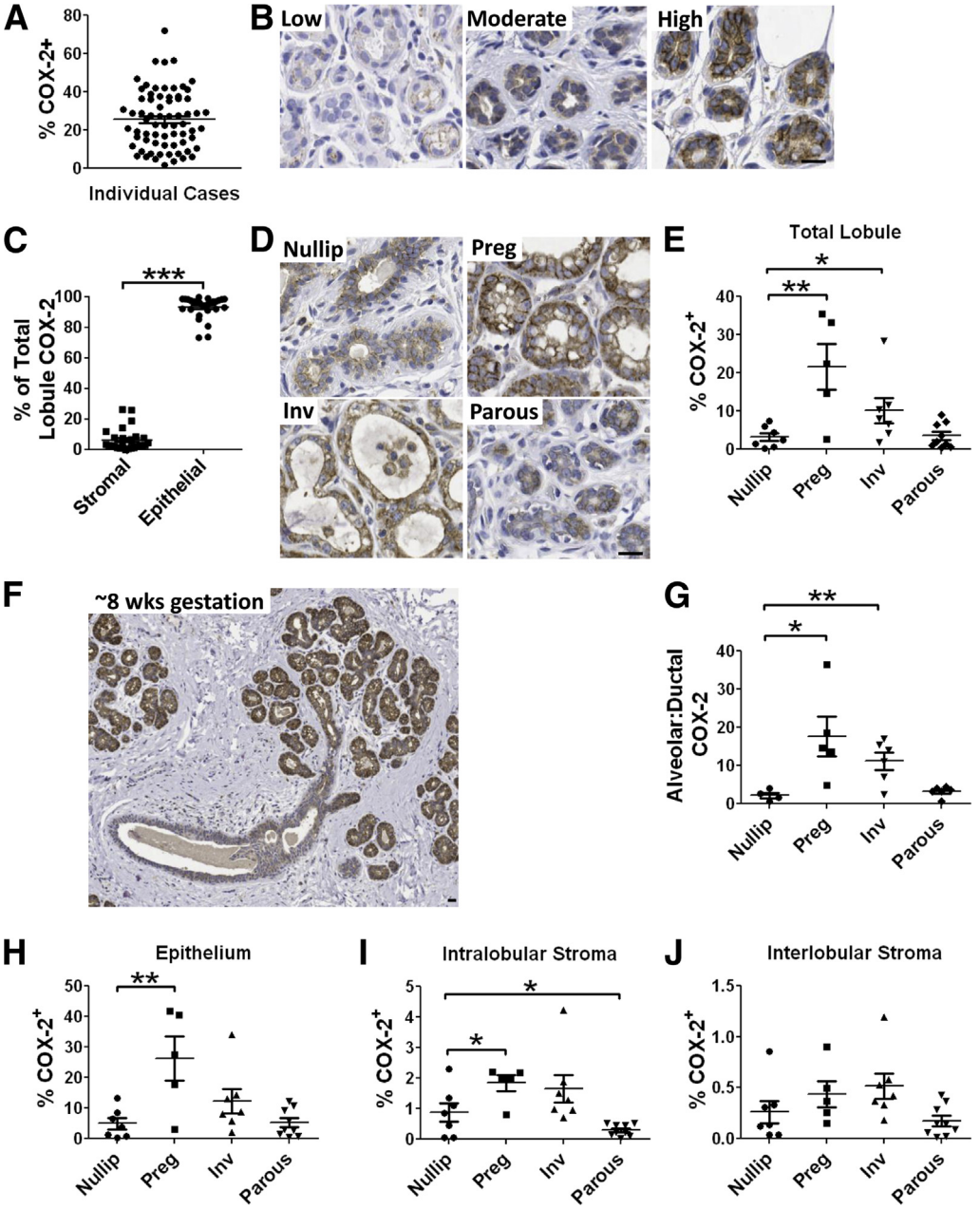

Figure 1 Normal breast tissue COX-2 expression is primarily epithelial and is regulated by pregnancy and postpartum involution. **A:** COX-2 IHC expression varies greatly by case. **B:** COX-2 IHC (brown signal) images are representative of low-, moderate-, and high-expression cases. **C:** Relative stromal and epithelial contribution to the total lobular COX-2 signal. Representative COX-2 IHC (**D**) and percentage of lobular area positive for COX-2 (**E**) in breast tissue from nulliparous (nullip), pregnant (preg), postpartum involution (inv), and parous cases. **F:** COX-2 staining in human breast at approximately 8 weeks gestation shows up-regulation of COX-2 specific to hormone-responsive alveolar epithelium. **G:** Ratio of alveolar to ductal COX-2 stain in the breast across reproductive stages shows preferential up-regulation of COX-2 in alveoli with pregnancy and involution. Percentage of epithelium (**H**), intralobular stroma (**I**), and interlobular stroma (**J**) positive for COX-2. *$P < 0.05$, one-tail unpaired *t*-test; **$P < 0.01$, one-tailed unpaired *t*-test; ***$P < 0.0001$, one-tailed paired *t*-test. Scale bar = 40 μm.

Table 1 Clinical Characteristics of Cases Analyzed by Reproductive Stage

Patient Parameters	Nulliparous	Pregnant	Postpartum involution*	Parous†
Cases (n)	7	5	7	9
Average age (years)	35.6 ± 6.5	34.0 ± 7.7	28.9 ± 7.0	40.9 ± 4.1
Average gravidity	0	1.50 ± 0.7	2.3 ± 0.5	3.3 ± 1.4
Average parity	0	0.50 ± 0.7	1.8 ± 0.5	2.3 ± 1.1
Race				
White	7	1	1	6
Hispanic	0	0	0	2
African American	0	0	0	1
Asian	0	0	1	0
Not reported	0	4	5	0

Data correspond to Figure 1.
*Within 2 months of parturition or lactation.
†More than 7 years after parturition.

postpartum involution cases, but to a lesser extent than during pregnancy (Figure 1, D and E). In fully regressed breast tissue from parous women, expression levels returned to nulliparous baseline levels (Figure 1, D and E). Furthermore, COX-2 expression was independent of patient age or body mass index (Supplemental Figure S4).

Based on the known role for COX-2 stromal cells during wound healing and inflammation (reviewed by Smith et al[36]), we investigated COX-2 expression specifically within the intralobular (within a lobule) and interlobular (between lobules) stromal compartments. These analyses confirmed that COX-2 induction during pregnancy is predominantly epithelial, and also identified a small, but significant, increase in the intralobular stroma (Figure 1, H and I, and Supplemental Figure S5); similar trends were observed during involution (Figure 1, H and I, and Supplemental Figure S5). Quantification of COX-2 in the interlobular stroma showed no significant changes across stages (Figure 1J and Supplemental Figure S5). These data are consistent with coordinated regulation of COX-2 within the epithelium and intralobular stroma during pregnancy, but not within the interlobular stroma.

Evidence for Ovarian Hormone Regulation of COX-2

Having demonstrated that COX-2 is upregulated during pregnancy in women, the Sprague-Dawley rat model was used to investigate mammary tissue COX-2 expression across defined pregnancy, lactation, and postpartum involution time points. COX-2 expression in the mammary

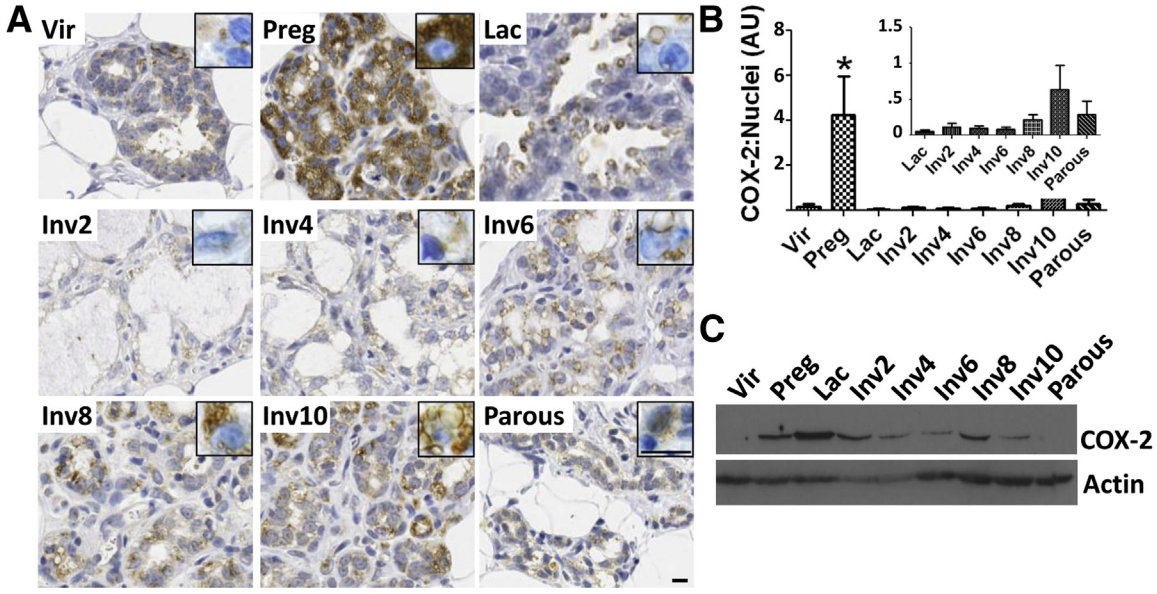

Figure 2 COX-2 is up-regulated in the rat mammary gland during pregnancy and postpartum involution. **A:** Representative COX-2 IHC staining (brown signal) in rat mammary glands from virgin (vir), pregnancy (preg) days 18 to 20, lactation (lac) day 10, involution days 2 to 10 (inv2-10), and parous-4 weeks after weaning. Inset shows epithelial staining at a higher magnification. Scale bar = 10 μm. **B:** Percentage of COX-2$^+$ area normalized to number of nuclei in rat mammary tissue across the reproductive cycle. **C:** Western blot for COX-2 and actin using pooled rat mammary tissue lysates. *$P < 0.05$ versus virgin, one-tailed unpaired *t*-test. AU, arbitrary units.

epithelium was low in virgin (nulliparous) rats, increased significantly during pregnancy, and was lost during lactation (Figure 2, A and B). After lactation, epithelial COX-2 gradually increased across mid- to late-involution (days 6, 8, and 10), and returned to baseline levels in the fully regressed gland (Figure 2, A and B). These data were verified by immunoblot for all stages except lactation (Figure 2C). By immunohistochemistry (IHC), COX-2 stain was low in the lactating gland and was restricted to apical, vesicle-like structures budding off the lactating mammary epithelium (Figure 2A); however, by immunoblot, COX-2 levels were highest during lactation (Figure 2C). Based on these disparate observations, we speculate that COX-2 is localized in the membrane of secreted vesicles during lactation and secreted with milk. Thus, the discrepancy between the IHC and immunoblot data may be explained by the fact that most milk is lost from the gland during IHC preparation, but remains in the tissue lysate used for immunoblot. Of note, a putative role for COX-2 in milk or milk production has not been reported, warranting additional investigation into this observation.

To explore the role of ovarian hormones in COX-2 regulation, COX-2 expression was evaluated by IHC across the rat estrous cycle. COX-2 expression in the mammary epithelium increased modestly during estrus and diestrus stage 1 of the estrous cycle (Figure 3A), consistent with estrogen and progesterone regulation. It is potentially relevant that the range of COX-2 expression levels in the nulliparous rat across estrous stages was similar to that observed in our premenopausal nulliparous and fully regressed parous human cohorts (Figure 1E). In rats, direct evidence for ovarian hormone regulation was shown by COX-2 up-regulation in the mammary epithelium after estradiol and estradiol plus progesterone treatment designed to mimic the biological effects of pregnancy (Figure 3B). The magnitude of COX-2 up-regulation after treatment was comparable with that observed in breast tissue of women during pregnancy (Figure 1, D and E).

Coordinated COX-2 Expression in Normal Epithelium, DCIS, and Breast Cancer

Given the wide range of COX-2 expression in the normal premenopausal human breast (Figure 1, A and B), and the reported poor prognosis associated with COX-2 up-regulation in breast cancer,[24,25] we investigated the relationships between COX-2 expression in normal adjacent breast tissue, DCIS, and IDC. A quantitative assessment of COX-2 expression was performed using matched normal adjacent and DCIS lesions within a single tissue section from the same case ($n = 46$), as well as matched normal adjacent and IDC, also from a single tissue section ($n = 57$), obtained from the cohort described in Table 2. COX-2 expression in the normal adjacent epithelium correlated strongly with DCIS COX-2 expression (Figure 4A), and correlated moderately with COX-2 expression in IDC (Figure 4B). Thirty-seven cases containing normal, DCIS, and IDC on the same tissue section then were stratified into categories of low, moderate, and high normal epithelium COX-2 expression. We found that cases with low adjacent normal COX-2 expression also had low expressing DCIS and IDC (Figure 4C). Similarly, cases with moderate and high adjacent normal COX-2 expression had moderate and high DCIS and IDC expression, respectively (Figure 4C). When comparing normal adjacent, DCIS, and IDC within the same case, COX-2 expression was highest in the adjacent normal tissue, decreased in the matched DCIS, and further decreased in the matched IDC lesion (Figure 4C).

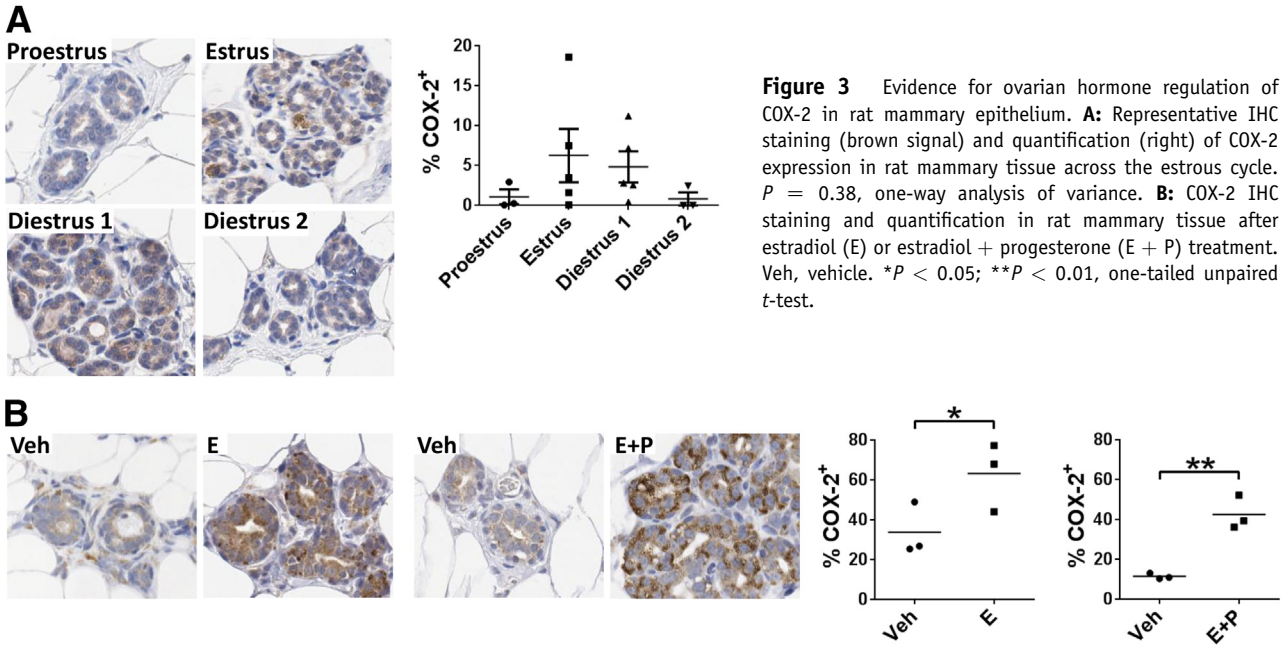

Figure 3 Evidence for ovarian hormone regulation of COX-2 in rat mammary epithelium. **A:** Representative IHC staining (brown signal) and quantification (right) of COX-2 expression in rat mammary tissue across the estrous cycle. $P = 0.38$, one-way analysis of variance. **B:** COX-2 IHC staining and quantification in rat mammary tissue after estradiol (E) or estradiol + progesterone (E + P) treatment. Veh, vehicle. $^{*}P < 0.05$; $^{**}P < 0.01$, one-tailed unpaired *t*-test.

Importantly, COX-2 expression in the normal adjacent epithelium was not associated with DCIS or IDC grade, stage, or estrogen receptor, progesterone receptor, or HER2 status (Figure 4, D–G), indicating that, in our cohort, COX-2 expression in the normal epithelium was not driven by the cancer state. This observation was supported further by evidence that COX-2 expression in the normal epithelium also did not differ significantly with proximity to the tumor (Supplemental Figure S3). These data suggest that women can be segregated into groups of low, moderate, and high COX-2 expression based on the expression levels in their normal breast epithelium, and that factors underlying COX-2 expression in the normal epithelium may influence COX-2 expression in DCIS and IDC.

For COX-2 expression in the normal breast epithelium to be considered a potential risk factor for breast cancer in young women, the distinct baseline levels of low, moderate, and high COX-2 expression in the normal epithelium would need to remain relatively stable over time. However, an important potential caveat raised by our data showing ovarian hormone modulation is whether a single breast biopsy can predict overall COX-2 expression in young premenopausal women. To begin to address this question, COX-2 expression was analyzed in a distinct cohort of premenopausal cases ($n = 6$) who underwent serial breast biopsies 2 to 3 weeks apart, allowing for analysis of COX-2 expression in a single case at two different time points during physiological menstrual cycling. The patient characteristics of this smaller cohort were reflective of the larger cohort used for the normal adjacent, DCIS, and IDC analysis in terms of age, body mass index, parity, histologic and tumor subtype, and stage (Table 2). In five of six cases, the relative COX-2 expression (ie, low, moderate, and high categories) remained within the same category between biopsies, although one case moved from the high to the moderate category (Figure 4H). Although this was a small cohort, these data suggest that COX-2 expression data obtained from a single breast biopsy may prove to be a valid indicator of individual baseline COX-2 expression levels in young women.

Table 2 Clinical Characteristics of Cases Used for Normal Adjacent, DCIS, IDC, and Serial Biopsy Analyses

Patient Parameters	Normal adjacent, DCIS, and IDC analysis	Serial biopsy analysis
Cases (n)	83	6
Average age (years)	37.5 ± 5.6	35.8 ± 10.5
Average body mass index	25.4 ± 6.1	24.8 ± 1.5
Average gravidity	2.2 ± 1.6	1.8 ± 1.3
Average parity	1.7 ± 1.3	1.8 ± 1.3
Race		
White	59	3
Hispanic	13	0
African American	3	0
Not reported/other	8	3
Histologic subtype		
Ductal	74	5
Lobular	5	0
Ductal + lobular	2	1
Other	2	0
Stage		
0	4	1
I	14	1
II	53	2
III	11	2
Unknown	1	0
Tumor subtype		
Luminal A	37	2
Luminal B	17	2
Triple negative	16	0
HER2	7	1
Unknown (including stage 0)	6	1
Tumor grade		
1	4	0
2	31	2
3	39	3
Unknown	5	0
DCIS grade		
1	0	0
2	3	0
3	1	1
Tumor size		
<2 cm	17	3
≥2 cm	51	2
Unknown (including stage 0)	15	1
Lymphovascular invasion		
Present	28	3
Absent	31	1
Unknown (including stage 0)	24	1
Lymph node involvement		
Present	40	4
Absent	37	1
Unknown (including stage 0)	6	1

Discussion

A young women's breast tissue cohort and rat models were used to investigate COX-2 expression in histologically normal mammary epithelium and the relationship between normal adjacent and breast cancer COX-2 expression. This was the first study to address COX-2 expression exclusively in breast tissue of premenopausal women, ages 45 years and younger, and to use computer-based analysis software to quantify COX-2 expression separately in the mammary epithelium and stroma. We found the epithelium to be the dominant source of COX-2 in normal adjacent breast tissue, with an approximately 40-fold range in expression across cases. Cumulatively, our studies implicate differential baseline levels of COX-2 expression between women, which can be influenced by ovarian hormones. Moreover, COX-2 expression in the normal breast epithelium paralleled COX-2 expression in DCIS and IDC. These data are consistent with results obtained from predominantly

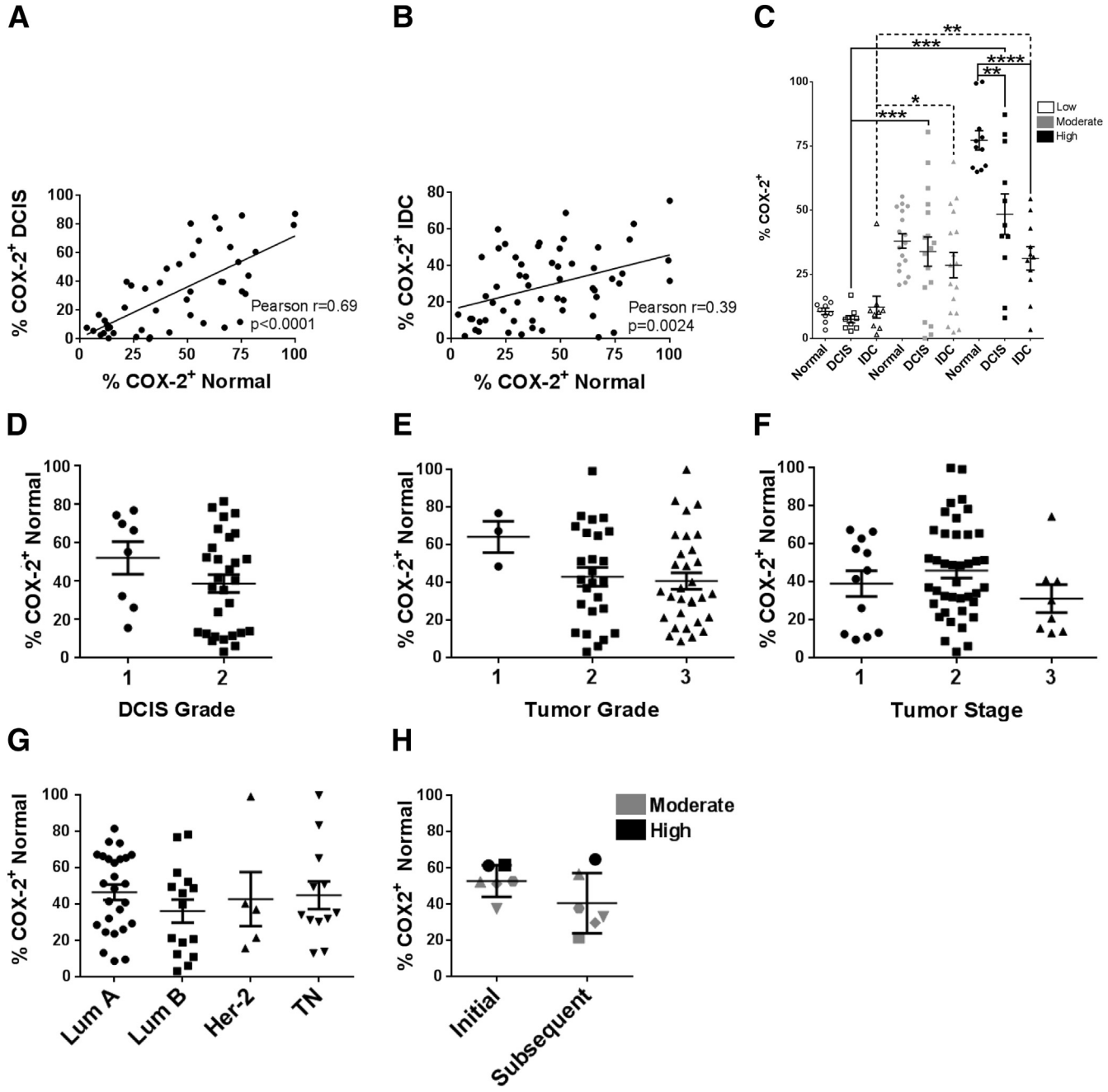

Figure 4 COX-2 expression in normal breast epithelium is associated with COX-2 expression in matched DCIS and IDC. **A:** Correlation between COX-2 expression in matched adjacent normal and DCIS. **B:** Correlation between COX-2 expression in matched adjacent normal and IDC. **C:** Stratification of cases based on low, moderate, and high COX-2 expression in the normal adjacent epithelium. COX-2 expression in normal epithelium is not associated with DCIS grade (**D**), tumor grade (**E**), tumor stage (**F**), or biological subtype (**G**). **H:** COX-2 expression in serial biopsies separated in time by 2 to 3 weeks. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$, two-tailed unpaired *t*-test. Lum A, luminal A; Lum B, luminal B; TN, triple negative.

postmenopausal cohorts in which COX-2 expression also was observed in the breast epithelium and correlated with DCIS and invasive cancer expression.[24,37–39] It previously was proposed that field effects emanating from premalignant tissue or overt cancer are responsible for high COX-2 expression in normal adjacent epithelium[38,40]; however, our data showed that COX-2 expression in the normal epithelium is independent of known clinical prognostic features and support an alternative hypothesis in which physiological regulators of COX-2 expression in the normal breast epithelium influence DCIS and IDC COX-2 expression levels.

The observation that baseline COX-2 levels in the breast vary dramatically between young women raises two

provocative but unanswered questions: whether high baseline COX-2 expression can predict risk of young-onset breast cancer, and whether women with high baseline COX-2 expression would preferentially benefit from COX-2 inhibition strategies (modeled in Figure 5). Consistent with a promotional role for COX-2 in breast cancer risk, increased COX-2 expression in the breast epithelium is associated with premalignancy and induces mammary tumor formation in mice.[20,41] Furthermore, in women, high COX-2 expression in atypia is associated with an increased breast cancer risk.[41] Importantly, a requisite for normal breast COX-2 expression to be predictive for breast cancer risk is that COX-2 expression remains relatively constant across time. Our data showing ovarian hormone modulation of COX-2 in normal breast epithelium would be expected to reduce the predictive value of COX-2 expression in young women. To address this concern, we performed a serial biopsy analysis in a small cohort of individual premenopausal women that showed relative stability in COX-2 expression levels over time. One interpretation of these data is that menstrual cycle–driven fluctuations in COX-2 expression occur within the context of a relatively stable baseline level of COX-2 expression. A larger sample size is necessary to address this key point, as well as to address potential mechanisms determining baseline COX-2 expression levels. Clinical relevance lies in the fact that 70% to 80% of the approximately 1 million clinical breast biopsies per year in the United States alone are given a benign diagnosis.[42,43] For the vast majority of these women, molecular strategies to identify high-risk populations are absent, leaving populations of high-risk women unidentified. Data from our cohort suggest that as many as 30% of young women have high baseline COX-2 expression. The validation of our results showing stable categories of low, medium, and high baseline COX-2 expression would argue for future studies to investigate the relationship between normal epithelial COX-2 expression and breast cancer risk.

The association between COX-2 staining in the normal adjacent epithelium and breast cancer implies that if young women with high baseline COX-2 levels develop breast cancer, their tumors also will express high levels of COX-2 (Figure 5). This is of prognostic significance because COX-2 expression is an independent predictor of decreased disease-free and overall survival.[24,25] Thus, understanding mammary COX-2 regulation and expression in young women may aid in identifying novel treatments for patients whose tumors express increased COX-2. This concept also is supported by other investigators who have proposed that understanding COX-2 expression in the normal breast epithelium is necessary to gain insight into COX-2 expression in cancer.[24,37,38] Our data showing regulation of COX-2 by estrogen and progesterone raise intriguing questions such as whether increased baseline COX-2 expression in young women's breast tissue is associated with early menarche, contraceptive use, or pregnancy history. High COX-2 levels have been implicated in breast cancers diagnosed after pregnancy,[16] providing additional support for understanding how reproductive history influences COX-2 expression in young women's breast cancer. Somewhat surprisingly, a relationship between body mass index and COX-2 expression in the normal adjacent breast epithelium was not observed in our cohort, although additional work is warranted to address this relationship further.

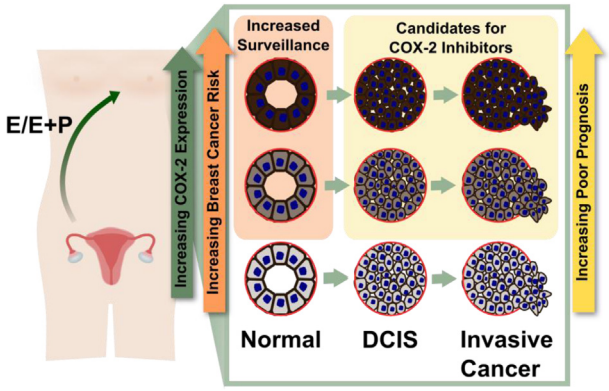

Figure 5 Model of the relationships between COX-2 expression in the normal epithelium, DCIS, and invasive breast cancer in premenopausal women. Baseline COX-2 expression in the normal breast epithelium varies across individual premenopausal women and can be modified by estrogen (E) and progesterone (P) exposure. Young women with increased COX-2 expression in the normal epithelium are predicted to have an increased risk for breast cancers with a poor prognosis, and may be candidates for chemoprevention strategies targeting COX-2.

Data from colorectal cancer indicate that identifying patients whose tumors have high COX-2 expression may be a key step in achieving a survival benefit with NSAID treatment[44]; however, data from the Nurses' Health Study indicate that the survival benefit of NSAID use in breast cancer patients does not depend on tumor COX-2 expression levels.[45] However, it remains unexplored whether young premenopausal breast cancer patients present a unique population in whom baseline COX-2 levels do impact outcomes. Given the high level of physiological tissue remodeling that occurs in the breasts of young women[28] and the links between COX-2, tissue remodeling, and breast cancer, further investigations into the potential benefits of NSAID treatment in young women's breast cancer are warranted.

In summary, we show high variability in COX-2 expression in normal breast epithelium between young women and provide evidence for hormone regulation. Furthermore, we provide evidence that the mediators of physiological COX-2 expression also influence expression in DCIS and breast cancer. These data provide impetus to further determine how COX-2 expression levels are regulated in the normal human breast because baseline COX-2 expression levels may inform breast cancer risk assessment, chemopreventive efficacy of NSAIDs, and utility of COX-2–targeted therapies in young premenopausal women.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.12.026*.

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