Cyclooxygenase-2 Expression during Immortalization and Breast Cancer Progression

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Abstract
Identification of molecular aberrations in premalignant human mammary epithelial cells (hMEC), the precursors for breast cancers, is a central goal in breast cancer biology. Recent studies implicated expression of cyclooxygenase 2 (COX-2) as a marker to identify precursor cells for breast cancer. In this study, we analyzed COX-2 expression in preselection and postselection hMEC cells and observed similar COX-2 levels in both cells. Interestingly, immortalization of postselection cells using various methods leads to a dramatic decrease in COX-2 expression. Similar to immortal cells, the majority of breast cancer cell lines expressed low levels of COX-2 protein. Finally, analyses of COX-2 expression in a series of specimens from reduction mammoplasty, adenosin, ductal carcinoma in situ, and infiltrating ductal carcinoma showed down-regulation of COX-2 expression during tumor progression. Importantly, down-regulation of COX-2 using small interfering RNA in cells showed no effect on cell proliferation, anchorage-independent growth, migration, or invasion. These results show that (a) COX-2 overexpression does not seem to predict a breast cancer precursor cell and does not provide advantage for the cell to be transformed; (b) inhibition of COX-2 does not affect hMEC growth and oncogenic behavior in the conditions analyzed; and (c) COX-2 expression is decreased in breast cancer cell lines and cancer specimens as compared with normal mammary epithelial cells. [Cancer Res 2008;68(2):467–75]

Introduction
Breast cancer is a heterogeneous disease with multiple genetic alterations that influence tumor growth, progression, and metastasis (1). However, breast tumors with favorable clinical and pathobiological features are potentially curable given the availability of several effective treatment modalities and early diagnosis (1). Therefore, defining the biological markers that would provide prognostic assessment and predict treatment response at the time of diagnosis is of critical importance in breast cancer management.

Identification of molecular aberrations in premalignant mammary epithelial cells (hMEC) that are presumed precursors for nearly all breast cancers is a central goal in breast cancer biology. Normal hMECs derived from reduction mammoplasty and cultured in vitro grow for about 15 to 20 population doublings and then senesce (2–6). However, in some cases, an occasional cell population emerges that continue to further grow for 30 to 60 population doublings before senescence (2–6). This process of emergence of cells is also known as self-selection; the cells are termed as preselection cells whereas those that emerge after selection are called postselection cells (2–6). Postselection cells have been reported to lose the expression of p16 INK4a, a cyclin-dependent kinase inhibitor (7). Recent microarray comparison of preselection with postselection hMECs revealed higher levels of cyclooxygenase 2 (COX-2) in postselection cells (8), and COX-2–positive foci have also been observed in healthy mammary tissues (9). Furthermore, postselection cultures showed increased angiogenic and invasion-associated factors (8). Based on these findings, COX-2 was suggested as a marker of precursors and risk of breast cancer (8, 9). The correlation of COX-2 expression and clinical course of breast cancer, however, is quite controversial (9–14). Based on the information of the lack of spontaneous transformation of hMECs in vitro (2–6), which express high COX-2 protein (8), the risks associated with long-term chemoprevention in healthy women, the recent appreciation of cardiovascular risks of COX-2 inhibitor use, and the fact that COX-2 is a stress and inflammation induced protein (15, 16), it is essential to establish whether COX-2 expression in fact contributes to oncogenic conversion of hMECs. Furthermore, the literature presents conflicting reports on the levels of COX-2 expression and breast cancer progression and its use as a marker for disease-free survival or overall survival (9–14).

In this study, we present evidence that COX-2 expression is similar in preselection and postselection hMECs. Importantly, the majority of immortal hMECs and breast cancer cell lines showed a dramatic decrease in COX-2 expression as compared with normal MECs. Importantly, immunohistochemical analyses of breast tissues showed that 100% normal reduction mammoplasty specimens expressed high levels of COX-2 protein, whereas only 60% of adenosin expressed COX-2 protein. More importantly, 49% of ductal carcinoma in situ (DCIS) and 43% infiltrating ductal carcinomas (IDC) showed similar levels of COX-2 as normal mammary epithelium, whereas more than 50% of tumors showed significantly decreased levels of COX-2 protein. Analyses of COX-2 mRNA using quantitative reverse transcription-PCR (RT-PCR) also showed the same pattern. Importantly, knockdown of COX-2 by short hairpin RNA (shRNA) in breast cells did not affect cell proliferation,
anchorage-independent growth, migration, or invasion. These results show that COX-2 expression is not a marker for tumor precursor cells and does not provide advantage for further transformation in hMECs, and its expression is decreased in immortal and tumor cells in vitro and in breast tumor progression in vivo.

Materials and Methods

Cell lines. Normal hMECs (70N, 76N, and 81N), human papillomavirus 16 (HPV16) E6–immortalized hMECs (70NE6, 76NE6, and 81NE6), HPV16 E6–immortalized hMECs (76NE7), HPV16 E6–immortalized hMECs (16A5), milk-derived hMECs (M2E6E7 and M3E6E7), human telomerase catalytic subunit TERT–immortalized hMECs (70NTERT, 76NTERT, and 81NTERT), mutant p53–immortalized hMECs (del239, R175H, and R249S from parental 76N cells), radiation-immortalized hMECs (76R-30), and breast cancer cell lines (21MT, 21MT-1, and 21MT-2) were established in our laboratory as previously described (17–20). All immortalized human hMECs (18A1, 18B5, and MCF-10A) and breast cancer cell lines (MDA-MB-231, MDA-MB-468, MDA-MB-435s, MDA-MB-453, BT474, BT549, ZR75-1, ZR75-30, T47D, SKBR-3, Hs578T, and MCF-7) were obtained from the American Type Culture Collection. Normal and immortalized hMECs were maintained in DFCI-1 medium, as previously described (17). All tumor cell lines were grown in α-MEM or α-MEM supplemented with hydrocortisone and epidermal growth factor (EGF).

Western blot analysis. Sixty micrograms of total protein from cell lysates were run on SDS-PAGE gel (15%, 12%, or 10.5%) and transferred onto polyvinylidene difluoride membrane. Monoclonal antibodies (mAb) against human COX-2 (Cayman Chemical Co.), α-tubulin (Sigma) and p16 (Santa Cruz Biotechnology, Inc.) and rabbit polyclonal antibodies against human HER2 (Santa Cruz Biotechnology), human epidermal growth factor receptor (EGFR; Santa Cruz Biotechnology), and hTERT (Rockland) were used for immunoblotting, followed by horseradish peroxidase (HRP)–conjugated goat anti-mouse antibody (Pierce Biotechnology) or goat anti-rabbit antibody (Bio-Rad Laboratories).

Immunofluorescence. Cells were fixed on coverslips with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked in 5% goat antibody (Bio-Rad Laboratories). Sections were rehydrated and cleared through ethanol and ammonium hydroxide in 1,000 mL of distilled water) for 1 min and rinsing in 7 min, followed by treatment of sections in ammonia water (4 mL 28% hydroxide and 1,000 mL of distilled water) for 1 min and rinsing in distilled water. Sections were rehydrated and cleared through ethanol and xylene and mounted with cover glass using a xylene-based mounting medium. The COX-2 staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong) was evaluated by three independent observers with light microscopy, including two pathologists (Dr. Monica Goswami and Charles Sturgis).

Quantitative PCR to determine relative COX-2 mRNA levels. Specimens were collected from the Brigham and Women’s Hospital without individual identifiers using protocols approved by the Institutional Review Boards of Dana-Farber Cancer Institute. Total mRNAs were extracted from Ber-Ep4 purified normal luminal epithelial cells and macroscopically dissected tumor cells based on adjacent H&E staining slides and cDNAs were synthesized by reverse transcription (21). Quantitative PCR was done on ABI 7500 QPCR machine using ABI SYBR green PCR master mix per manufacturer’s instructions (Applied Biosystems). The cycling conditions were 8 min at 95°C followed by 40 cycles of 15 s at 95°C plus 1 min at 60°C. The relative gene copy numbers toward normal luminal epithelial cells and cancer cells were calculated by the comparative C_{t} method (22) using normalization control ribosomal protein L39 (RPL39). The primer sequences used in this study were COX-2 forward, TGAAGTCCAT- TACGTTTGTCGTG; COX-2 reverse, TCTGTGTCTGAAAGACCTCT; RPL39 forward, CAGCTTCTCCCTCTCCTCT; and RPL39 reverse, CCGAG- GAATCGTATTTAC.

Statvarion and EGFr treatment. Cells were grown in DFCI-1 medium that contains 12.5 ng/mL of EGF (17) and starved in growth factor–deprived DFCI-3 (D3) medium (17) for 72 h and then stimulated with 12.5 ng/mL of EGF; cells were harvested at indicated times and analyzed by Western blotting.

Telomerase and retroviral infections. The COX-2–specific shRNA1 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGATTTGCAACACTTGGTTGACACATAATCGGG-3'; shRNA2 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGCTTACCTCCTCAACACTTGGTTGACACATAATCGGG-3'; shRNA3 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGGTTAAAACCTTACGATGTTCTCTTGAAACATCGTTAAGGTITTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGCTTACCTCCTCAACACTTGGTTGACACATAATCGGG-3'; shRNA2 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGCTTACCTCCTCAACACTTGGTTGACACATAATCGGG-3'; shRNA2 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGCTTACCTCCTCAACACTTGGTTGACACATAATCGGG-3'); shRNA2 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGCTTACCTCCTCAACACTTGGTTGACACATAATCGGG-3'); shRNA2 (oligo sense, 5'-GATCCCCTGATTTGCAAATCCTTTTTGGAAA-3'; oligo antisense, 5'-AGCTTTTCCAAAAAGCTTACCTCCTCAACACTTGGTTGACACATAATCGGG-3') were annealed and cloned into the β-gal and hTERTIII sites of pSUPER retrovector. Retroviruses were produced by transient transfection, as previously described (23). After infection, the cells were selected and maintained in selection medium.

Cell proliferation assay. 18B5 and Hs578T (5 × 10^4) or 21MT (2 × 10^5) cells were plated in T25 flask (in triplicates); 4 days later, cells were counted and plated again in 25-cm^2 flasks at the original density. Cells deprived of growth factors by culturing in D3 were trypsinized and plated in T25 flasks (2 × 10^5 per flask). The cells were grown in D3 plus EGF (12.5 ng/mL) medium.

Transwell migration assay. The migration assay was done using BD BioCoat wells (8 µm pore size; BD Biosciences). Cells expressing COX-2 shRNA and scrambled shRNA were trypsinized and plated in low-serum-containing (1% FCS) α-MEM medium (Hs578T and 21MT-1). Cells [4 × 10^4 (21MT-1) or 2 × 10^4 (Hs578T) per well] were added to the top chambers of the transwell. After 2 h, 10% FCS–containing α-MEM medium was added to the lower chamber and incubated for a further 24 h (21MT-1) or 6 h (Hs578T). The membrane was then stained with Diff-Quik Stain Set kit (Dade Behring, Inc.). Cells that migrated through the membrane were counted using an inverted tissue culture microscope.

Invasion assay. The invasion assay was done using BD Matrigel invasion chamber (8 µm pore size; BD Biosciences). Cells were trypsinized and plated as above. The cells [4 × 10^4 (21MT-1) or 1 × 10^4 (Hs578T) per
well] were added to the top chambers of the transwell as above. Two hours later, 10% FCS–containing αHE medium was added to the lower chamber and cells were incubated for 96 h (21MT-1) or 48 h (Hs578T). Membranes were stained and cells counted as above.

**Anchorage-independent growth assay.** Anchorage-independent assay was done using six-well plates. Each well contained 1 mL of 0.6% agarose in growth medium as the bottom layer and 1 × 10⁶ cells in 2 mL of 0.3% agarose in growth medium as the top layer. The pictures were taken at 21 days after cell seeding.

**Results**

**COX-2 protein is overexpressed in both preselection (p16-positive) and postselection (p16-negative) hMECs.** Based on the recent publication that p16⁻/COX-2⁺ postselection cells represent precursor of breast cancer (8), we assessed the expression of p16 and COX-2 in two different hMEC strains preselection and postselection. Surprisingly, we observed that both p16⁺ (preselection) and p16⁻ (postselection) cells had similar levels of COX-2 expression (Fig. 1A). E7-expressing 70N cells (19) were used as positive control for p16 expression (Fig. 1A).

Considering that preselection and postselection cells can still be a mixture of heterogenous hMECs, we examined expression of COX-2 and p16 by immunofluorescence. As expected, MCF-7 cells used as negative control showed lack of expression of both p16 and COX-2, whereas 70NE7 cells used as positive control showed expression of both p16 and COX-2 proteins (Fig. 1B). Consistent with our Western blotting results (above), preselection hMECs were uniformly positive for both p16 and COX-2 proteins (Fig. 1B). These results show that both p16 and COX-2 proteins were expressed in preselection cells, and therefore postselection cells could arise from COX-2–expressing preselection cells rather than COX-2–negative cells turning into COX-2–positive cells after the selection.

**COX-2 protein is down-regulated in most immortal cell lines.** Next, we examined if indeed COX-2 expression in hMECs can serve as a marker for breast cancer and would thus provide high susceptibility to postselection cells for immortalization and further transformation. Thus, we examined COX-2 expression in immortal cells derived from postselection parental cells using different agents. Surprisingly, nearly all immortal cells had barely detectable

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A similar level of COX-2 protein is expressed in preselection and postselection hMECs. A, preselection and postselection cells from two hMEC strains, 70N and 81N, were analyzed by Western blotting for the levels of COX-2 and p16 protein. 70N.E7 was used as p16-positive control. α-Tubulin immunoblotting was used as a loading control. B, cells [MCF-7, COX-2, and p16 negative, used as negative control; 70NE7, p16-positive cells used as p16-positive control; 70N and 81N (preselection and postselection hMECs)] were immunostained with anti–COX-2 mAb (green) and anti-p16 rabbit polyclonal antibody (red). Cells were observed under a Nikon TE200-U inverted microscope equipped with an ORCA-ER charge-coupled device camera controlled by MetaMorph software. Region Measurements program in MetaMorph software was used for computerized quantification of COX-2 and p16 staining. Right, results shown as average intensity per cell.
levels of COX-2 protein. These included hMECs derived from reduction mammoplasty as well as from milk specimen and immortalized with HPV16 E6, E7, or E6 + E7; mutant p53; TERT; and benzopyrene treatment (Fig. 2; refs. 17–21, 24). Because immortalization by various genes does not involve a common biochemical pathway, immunoblotting with anti-TERT antibody served as a control (Fig. 2A).

Given the fact that hMECs cultures may be heterogeneous, we assessed p16 and COX-2 expression in TERT- or E6-immortalized hMECs by immunofluorescence (Fig. 2B). Correlating with Western blotting results, immunofluorescence analyses showed barely detectable expression of COX-2 and p16 in the cells. These results further show that COX-2 expression in immortal cells is lower than in parental cells.

The majority of breast cancer cell lines have low level of COX-2 protein. Next, we examined a number of breast cancer cell lines including four breast cancer cell lines (21MT-1, ZR75-30, BT474, and SKBR-3) known to express high level of ErbB-2. Similar to immortal cells, the majority (15 of 17) of breast cancer cell lines showed barely detectable levels of COX-2 protein whereas 21MT-1 and Hs578T cells showed relatively high levels of COX-2 expression as compared with postselection normal hMECs (Fig. 2C). Of the four ErbB2-positive breast cancer cell lines, only the 21MT-1 cells showed high COX-2 expression (Fig. 2C). Based on the known status of ErbB2 and EGFR expression in these breast cancer cell lines, immunoblotting with anti-ErbB2 and anti-EGFR antibodies served as controls in these experiments (Fig. 2C). To further substantiate these results, we analyzed COX-2 mRNA levels in selected sets of COX-2–positive and COX-2–negative immortal and cancer cell lines by Northern blot analysis. As expected, COX-2 mRNA levels well correlated with protein expression (Fig. 2D), suggesting that the decrease in COX-2 protein is at the transcriptional level.

COX-2 expression is decreased during tumor progression. Given the fact that we observed decreased levels of COX-2 protein in immortal and tumor breast cell lines, we carried out a stringent analysis of antibody used in our experiments. These included testing several commercially available anti–COX-2 antibodies: rabbit polyclonal (Cayman), rabbit monoclonal (Abcam), goat polyclonal (Santa Cruz Biotechnology), and goat polyclonal (Santa Cruz Biotechnology) in Western blotting and immunofluorescence staining. None of these five antibodies work in Western blotting and also are not specific for cell staining (data not shown). The only antibody that is widely...
used and was used in our experiments above is mouse monoclonal COX-2 antibody from Cayman that showed one specific band (from 16 to 250 kDa) in normal mammary epithelial cells (Supplementary Fig. S1). Next, we overexpressed COX-2 protein in MCF-7 cells and also down-regulated COX-2 protein in Hs578T cells and carried out Western blot analyses. As expected, only one specific band of COX-2 protein was seen in the expected lanes (Supplementary Fig. S1). Not only in Western blotting but also immunofluorescence staining analyses of these cells showed that this antibody recognizes specific COX-2 protein (Supplementary Fig. S2). Furthermore, staining of sections cut from paraffin-embedded MCF-7 cells expressing COX-2 confirmed that this antibody is specific for immunohistochemical staining (Fig. 3A).

Based on this rigorous testing for specificity of the antibody, we assessed the expression levels of COX-2 in a set of breast tissue specimens by immunohistochemistry (Fig. 3A and B). A clear high staining with anti-COX-2 antibody was seen in normal ducts as compared with low COX-2 staining in DCIS and IDC (Fig. 3B), whereas control normal mouse IgG1 showed no staining (Fig. 3A). As summarized in Table 1, reduction mammoplasty specimens showed high expression of COX-2, whereas adenosis, DCIS, and IDC samples showed variable expression with the majority of cases showing low or lack of COX-2 protein. These observations were further strengthened by higher COX-2 expression in normal epithelium adjacent tissues surrounding tumors in three independent specimens. Arrows, the normal adjacent epithelium that illustrates more intense COX-2 staining than the adjacent cancer (arrowheads). Pictures were taken with 20× and 40× objectives. D, total mRNAs were extracted from BerEP4 purified normal epithelial cells and macroscopically dissected tumor cells based on adjacent H&E slides. Quantitative PCR was used to determine the mRNA expression profile of COX-2 in normal breast tissue and tumor specimen. COX-2 mRNA expression of the samples was normalized to RPL39. Normal: N2 and N4; IDC: BWH-T1, BWH-T3, BWH-T8, BOT169, IDC1, MGH-T3, MGH-T4, C2, C3, C6, C7, C10, C22, C26, C27, C35, C36, C39, and C47; invasive lobular cancer (ILC): C13; IDC + ILC: BWH-T7 and BWH-T15; metastasis: C27.

**Figure 3.** COX-2 mRNA and protein expression is decreased in breast tumor specimens in vivo. A, Cayman COX-2 mAb is specific for immunohistochemical staining. MCF-7 cells that stably overexpress COX-2 or vector were fixed with 4% paraformaldehyde overnight. The fixed cells were resuspended in agarose (1% in PBS) at 60°C. The cells in coagulated agarose were embedded in paraffin. Sections cut from paraffin-embedded MCF-7-Vector and MCF-7-COX-2 and sections from normal reduction mammoplasty, DCIS, or IDC tissue blocks were stained with Cayman mAb or normal mouse IgG1 (as negative control). The sections were counterstained in Mayer’s hematoxylin to show nuclear staining (blue color). Pictures were taken with a 40× objective. B, representative examples of immunohistochemical staining of COX-2 in sections from human breast normal reduction mammaryplasty, adenosis, DCIS, or IDC shown in Table 1. Pictures were taken with a 40× objective. C, COX-2 staining intensity was increased in adjacent normal epithelial tissue surrounding tumors in three independent specimens. Arrows, the normal adjacent epithelium that illustrates more intense COX-2 staining than the adjacent cancer (arrowheads). Pictures were taken with 20× and 40× objectives. D, total mRNAs were extracted from BerEP4 purified normal epithelial cells and macroscopically dissected tumor cells based on adjacent H&E slides. Quantitative PCR was used to determine the mRNA expression profile of COX-2 in normal breast tissue and tumor specimen. COX-2 mRNA expression of the samples was normalized to RPL39. Normal: N2 and N4; IDC: BWH-T1, BWH-T3, BWH-T8, BOT169, IDC1, MGH-T3, MGH-T4, C2, C3, C6, C7, C10, C22, C26, C27, C35, C36, C39, and C47; invasive lobular cancer (ILC): C13; IDC + ILC: BWH-T7 and BWH-T15; metastasis: C27.
expression is transcriptionally down-regulated in breast cancers. Taken together, these results clearly show that COX-2 expression does not correlate with breast tumor progression.

**COX-2 expression is regulated by growth condition.** Next, we examined if the expression of COX-2 varies in different culture conditions. For this purpose, we first deprived cells of growth factors by culturing in D3 (17) and then stimulated them with epidermal growth factor. Notably, in each case, when cells were cultured in complete medium or were deprived of growth factors, COX-2 expression was low, whereas addition of EGF dramatically increased the levels of COX-2 protein in a time-dependent manner (Fig. 4A). These results show that COX-2 expression depends on culture conditions and may explain discrepancies in the literature. These results are consistent with recent findings (25).

**COX-2 down-regulation does not influence proliferation or malignant phenotype of cells.** Although there are COX-2 inhibitors available, it is now well recognized that COX-2 inhibitors are not highly specific for COX-2 (26, 27). Thus, to assess if down-regulation of COX-2 expression influences proliferation of cells, we first generated three constructs expressing shRNA against COX-2 and two constructs with scrambled shRNA. As shown in Fig. 4B, cells stably expressing shRNA1 or shRNA2 showed a dramatic reduction in the levels of COX-2 protein, whereas shRNA3 had little effect on COX-2 expression. As controls, both scrambled shRNA-expressing cells showed no change in COX-2 protein expression.

We then assessed the effect of reducing COX-2 expression on cell proliferation. As seen in Fig. 4C, no significant effect on cell proliferation was observed on reduction in COX-2 protein. Next, we examined if decreased levels of COX-2 protein influences growth factor–stimulated proliferation. For this purpose, we first synchronized the immortal 184B5 cells expressing scrambled shRNA or shRNA against COX-2 by growth factor deprivation followed by stimulation with EGF over hours, and then cultured these cells for days and measured proliferation. EGF response was seen by increase in phospho-Akt as expected (Fig. 4D). Consistent with experiments above, no change in proliferation was observed (Fig. 4D). Similar response to colony formation assays was observed (data not shown).

Next, we assessed the effect of COX-2 knockdown on the malignant phenotype of cells. 21MT-1 and Hs578T cells stably expressing COX-2 shRNA or scrambled shRNA were analyzed for their ability to grow in an anchorage-independent manner and their capability to migrate and invade. As shown in Fig. 5, no significant difference was noted in cells expressing COX-2 shRNA in comparison with cells that express scrambled shRNA. Wound healing assay for migration also did not show any difference (data not shown). These results show that down-regulation of COX-2 in breast cancer cells does not influence the malignant phenotypes that we analyzed.

**Discussion**

For more than two decades, normal hMECs established from reduction mammaplasty specimens have provided great models of human epithelial cells to understand the mechanisms of epithelial cell transformation (28, 29). We and others have shown that when hMECs were cultured in serum-free or low-serum conditions, initial outgrowth of cells from organoids is heterogeneous and these cells proliferate for 10 to 15 population doublings followed by senescence (2–6). However, in some cases, a morphologically homogeneous population of cells emerges that then continue to proliferate further for 30 to 50 population doublings before senescence. This process is known as selection or M$_0$ stage and cells before selection are called “early passage or preselection cells” and cells that emerge after selection are called “late passages or postselection cells” (19). Recently, postselection cells are also termed as “variant human mammary epithelial cells” (30). We and others have shown that hMECs do not exhibit spontaneous immortalization (17–20, 29, 31); however, both preselection and postselection cells can be immortalized by a number of viral and chemical agents (19, 32) and activated oncogenes, such as mutant p53 (33) and cellular genes Bmi-1 (34) and ZNF217 (35). It was noticed that in each case, the cells that were immortal had low expression of p16 due to hypermethylation of the promoter of the CDKN2A gene. This led investigators to hypothesize that the cells with p16 hypermethylation may represent precursor cells for breast cancer (7, 30, 36). Further studies showed that cells with hypermethylation of p16 were present in vivo in normal and benign mammary tissues, suggesting that a p16-negative population may be present in preselection cells to begin with. Recently, Crawford et al. (8) carried out microarray analysis of preselection and postselection cells and identified COX-2 gene as one of the genes that were overexpressed in postselection cells. The authors showed that postselection cells have more angiogenic and invasive capability as compared with preselection cells and thus concluded that COX-2 expression may provide susceptibility to transformation.

Considering our long-standing interest in transformation of postselection cells, we examined if indeed COX-2 expression in hMECs can serve as a marker for breast cancer and would thus provide high susceptibility to postselection cells for immortalization and further transformation. Surprisingly, however, we found that cells grown in our culture conditions express similar levels of

### Table 1. COX-2 expression in breast cancer progression

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<td>38 (35.19)</td>
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NOTE: Scoring of staining of various specimens stained with anti–COX-2 antibodies. Specimens were stained as described in Materials and Methods. Staining was independently scored by three investigators, including two pathologists. The samples of various scores are shown in Fig. 3A.
COX-2 in both preselection and postselection stages and this was despite of their being p16 negative or p16 positive. Furthermore, most immortal cells expressed much lower levels of COX-2 protein rather than the expected higher levels if these cells represented precursors of breast cancer. Furthermore, majority of breast cancer cell lines also expressed low levels of COX-2 protein. At present, we cannot explain the discrepancy of our results with that of Crawford et al. (8) except that their culture conditions were different than ours and COX-2 expression is known to change with growth factors, although in our system both preselection and postselection cells are grown under identical conditions.

The role of COX-2 in breast cancer is highly controversial (37, 38). Parrett et al. (39) detected COX-2 mRNA expression in 13 of 13 human breast tumors compared with no detectable expression in normal human breast tissue, and observed a correlation between COX-2 expression and increasing tumor cell mass. On the other hand, Hwang et al. (40) found that only 2 of the 44 tumor samples expressed COX-2. Cejas et al. (41) found that COX-2 mRNA and protein were overexpressed in nontumor ductal epithelium as compared with invasive ductal carcinoma.

Based on such controversial data, we opted to assess the levels of COX-2 protein in a set of specimens. Consistent with our experimental models of normal, immortal, and breast tumor cell lines, we observed that literally 100% of reduction mammoplasty specimens and >60% of adenosin high levels of COX-2, whereas only 50% of DCIS and IDC had similar levels of COX-2 and the other 50% showed barely detectable levels of COX-2 protein. We further confirmed that COX-2 expression was decreased at the level of mRNA by real-time PCR analysis. We thus conclude that COX-2 expression is not higher during breast tumor progression.

Some studies reported COX-2 expression correlates with ErbB2 status. High levels of COX-2 protein were detected in 14 of 15 HER2/neu-overexpressing breast cancers. In contrast, COX-2 was detected only in 4 of 14 HER2/neu-negative breast cancers (42). In DCIS, COX-2 expression was associated with higher cellular proliferation rate, and nuclear grade with estrogen receptor

![Figure 4](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-07-0587)

**Figure 4.** COX-2 expression is growth factor dependent and down-regulation of COX-2 has no effect on cell proliferation. A, immortal breast cell lines (76NE6, 76NTERT, and Mutp53-1, p53del239) were grown in DFCI-1 (D) medium that contains 12.5 ng/mL EGF (17) and starved in D3 medium (17) for 72 h and then stimulated with 12.5 ng/mL EGF, cells were harvested at indicated time points and analyzed for COX-2 and α-tubulin (used as loading control) by Western blotting. B, three shRNAs against COX-2 or two scrambled shRNAs were designed and cloned into pSUPER. retro vector. The immortal hMEC 184B5 and breast cancer cell lines Hs578T and 21MT-1 cells infected with retroviral supernatants. After selection, the cells were analyzed for COX-2 expression by Western blotting (B) or proliferation assays (C and D). For proliferation assay, 5 × 10^4 (184B5 and Hs578T) or 2 × 10^5 (21MT-1) cells expressing scrambled shRNA1 shRNA2, COX-2 shRNA1, COX-2 shRNA2, or COX-2 shRNA3 were plated in 25-cm² flasks (triplicates). 4 d later, cells were counted and plated again in 25-cm² flasks at the original density (such procedure was repeated four times). The number of viable cells was measured by trypan blue exclusion method. Columns, mean of four independent experiments each done in triplicates; bars, SD (C). D, indicated cells were starved in D3 medium for 3 d and then stimulated with 12.5 ng/mL EGF. Cells were then harvested at indicated times and one set of cells was analyzed for COX-2 expression by Western blotting. Phospho-Akt shows starvation and EGF stimulation was as expected. The second set of cells was plated in T25 flasks (2 × 10^4 per flask). The number of viable cells was measured by trypan blue exclusion method at 2, 4, 6, and 8 d. For each time point, cells from six flasks were counted.
negativity and HER2 positivity (13). In contrast, in a study of 57 primary breast carcinomas, 14 DCIS found no significant correlation between COX-2 and HER2 expression. However, overexpression of HER2 in MCF-7 cells was shown to up-regulate COX-2 (43). Davies et al. (12) and Witton et al. (14) did not observe significant correlation between COX-2 and HER2. In our study, 52 of 108 IDC samples were ErbB2 positive. However, 21 of these 52 showed COX-2 expression similar to normal cells, whereas the remaining 31 samples showed decreased levels of COX-2 expression. Mantel-Haenszel $\chi^2$ test (44) showed that there was no correlation between HER2 expression and COX-2 expression. With regard to the overall or disease-free survival of patients, COX-2 overexpression was found in 48.6% of the tumor samples and was predictive for poor disease-free and overall survival in few studies (9–11). In contrast, other studies show no significant correlations between COX-2 expression and breast cancer size and grade (12, 14).

Although studies using COX-2 inhibitors known to be not particularly specific for COX-2 inhibition show reduced cell proliferation and tumorigenic behavior of cells (16, 45), recent knockdown of COX-2 with small interfering RNA (siRNA) showed no effect on cell cycle distribution (46). Another study showed no significant difference in the radiosensitivity of cells in which COX-2 was silenced compared with the cells transfected with negative control siRNAs (47). Similarly, another study using tetracycline-inducible (tet-on) COX-2 antisense showed that COX-2 depletion did not induce cell death (26). We did not observe any effect on cell proliferation or oncogenic behavior after knockdown of COX-2 protein. In mouse model, overexpression of COX-2 can induce mammary gland tumorigenesis (48, 49). However, the mammary tumorigenesis occurred with high frequency in MMTV-COX-2 transgenic mice after multiple rounds of pregnancy, indicating that pregnancy greatly increased the tumorigenesis incidence, which is opposite to those observed in women. We believe that studies that show increased tumorigenicity on overexpression in vivo mouse models may be an indirect effect of COX-2 being involved in inflammatory responses.

We believe that the discrepancies in these studies and our studies could be due to (a) nonspecific reactivity of available anti–COX-2 antibodies for immunohistochemistry of tissue specimens; (b) the sample size in most of these studies is rather small to derive statistical significance; and (c) considering that COX-2 expression is modulated by several factors, such as growth factors, oncogene products, and inflammation, the status of these parameters in a

Figure 5. COX-2 knockdown does not affect migration, invasion, and anchorage-independent growth. A, migration assay using transwell. COX-2 shRNA–expressing cells were trypsinized and suspended in medium and plated on the upper chamber. Cells that migrated through the membrane were counted using an inverted tissue culture microscope. Relative migration was determined by comparing the amount of migration obtained from shRNA-expressing cells to that obtained from scrambled shRNA1-expressing cells. Columns, mean of four independent experiments each done in triplicates; bars, SD. B, invasion assay. The cells were plated as above. The cells that invaded through the membrane were counted using an inverted tissue culture microscope. Relative invasion was determined as above. Columns, mean of four independent experiments each done in triplicates; bars, SD. C, soft agar assay. COX-2 shRNA–expressing Hs578T cells were plated as described in Materials and Methods. The pictures were taken at 21 d after cell seeding. Experiments were repeated at least thrice and a representative experiment is shown.
particular tumor sample could influence the expression of COX-2 protein. The discrepancy in expression of COX-2 in preselection or postselection may vary in different specimens or different cells may grow if established in different culture conditions. This hypothesis is supported by our findings and that of others that expression of COX-2 was changed with culture conditions. Considering that COX-2 expression is influenced by several factors and conditions, the expression data should be considered with caution.

In conclusion, we show that COX-2 overexpression does not predict a breast cancer precursor cell and does not provide advantage for the cell to be transformed, and COX-2 expression is decreased in breast cancer cell lines and cancer specimens compared with normal cells.

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**References**


Correction: COX-2 Expression Decreases in Breast Cancer Progression

In the article on how COX-2 expression decreases in breast cancer progression in the January 15, 2008 issue of Cancer Research (1), there is an error in Fig. 2A. The third lane should be labeled "hTERT" and the fourth lane should be labeled "α-tubulin".

Cyclooxygenase-2 Expression during Immortalization and Breast Cancer Progression

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