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p38 Regulates Cyclooxygenase-2 in Human Mammary Epithelial Cells and Is Activated in Premalignant Tissue

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Abstract

The immediate-early gene, cyclooxygenase-2 (*COX-2*), is induced in a variety of inflammatory and neoplastic processes and is believed to play an important role in tumorigenesis. In this study, we identify an important upstream regulatory pathway of COX-2 expression in variant human mammary epithelial cells (vHMEC), which has been shown to exhibit phenotypes important for malignancy. We find that the stress-activated kinase, p38, is phosphorylated and activated in vHMEC compared with HMEC and is responsible for the expression of COX-2 in vHMEC as cells grow in culture. Furthermore in this capacity, p38 acts to stabilize the COX-2 transcript rather than activate COX-2 transcription. Inhibition of p38 kinase, using a chemical inhibitor, down-regulates COX-2 and decreases cell survival. Examination of archived tissue from women with ductal carcinoma *in situ* reveals epithelial cells that not only overexpress COX-2 but also have an abundance of activated phospho-p38 in the nucleus and cytoplasm, mirroring the expression observed *in vitro*. These epithelial cells are found within premalignant lesions as well as in fields of morphologically normal tissue that surround the lesions. In contrast, low phospho-p38 staining was observed in the majority of normal tissue obtained from reduction mammoplasty. These data help define the regulation of COX-2 expression in early carcinogenesis and provide alternative candidates for targeted prevention of COX-2-induced phenotypes and breast cancer.

Introduction

Two isoforms of prostaglandin endoperoxide H synthase, cyclooxygenase-1 (COX-1) and COX-2, catalyze the conversion of arachidonic acid to prostaglandin endoperoxide H2 in a two-step process carried out by COX and peroxidase activities. COX-1 is constitutively expressed in the majority of tissues and is regula primarily by the availability of arachidonic acid. In contrast, COX-2, although not detected in most norma tissues, is often robustly expressed in neoplastic and inflamed tissues after induction by inflammatory cytokines, mitogenic, or cellular stress signals. Prostaglandins control normal physiologic functions such as the regulation of renal blood flow, mitogenesis, immune function, and ovulation (1). Although prostaglandins have been detected in breast milk, their role in normal breast physiology is not well defined (2, 3).

The regulation of COX-2 protein, in both normal and tumor cells, takes place on numerous levels. The diversity and multiplicity of promoter elements and transcription factors required for COX-2 induction reflect its complex regulation. For example, in C/EBPβ-deficient mice, IFN-inducible expression of COX-2 in fibroblasts was normal, yet completely abrogated in macrophages **(4)**. COX-2 protein levels are not only regulated by *de novo* mRNA transcription, using cis-acting elements such as NFκB, NFIL6, CRE, and Mib-1, but also through mRNA stabilization and protein translation **(5–8)**. As we become more astute in the recognition that COX-2 regulation is cell type and context dependent, it is not surprising that the pathways necessary in one cell type are not always recapitulated in another.

In breast epithelium, COX-2 expression may be an early event in the carcinogenic process. Elevated expression of COX-2 was found in 36% to 56% of invasive tumors **(9–14)** and in an even greater fraction of premalignant lesions such as ductal carcinoma *in situ* (DCIS; refs. **12, 15, 16**). Furthermore, DCIS neighboring invasive breast cancer often stained more intensely for COX-2 than did the malignant lesion itself **(12, 16)**. Of particular interest, COX-2 expression is often elevated in the morphologically normal epithelium adjacent to DCIS, where the levels of COX-2 are equal to or greater than levels in DCIS epithelium **(15)**.

Recently, we reported that COX-2 expression was elevated in a unique subpopulation of variant human mammary epithelial cells (vHMEC) with premalignant properties **(17)**. These cells were identified in explants of reduction mammoplasty tissue obtained from women with no evidence of detectable breast cancer and are not at increased risk of developing breast cancer. The vHMEC population, in addition to accumulating cells that overexpress COX-2 **(17)**, also exhibits gene silencing through hypermethylation of promoter sequences (e.g., $p16^{INK4a}$), loss of specific cell cycle checkpoint controls, and acquisition of chromosomal changes similar to those found in the earliest lesions of human breast cancer **(18)**. We have been able to identify foci of mammary epithelial cells in paraffin-embedded tissue from disease-free women with many of these same characteristics. For example, overexpression of COX-2 in these foci was coincident with the hypermethylation of the $p16^{INK4a}$ promoter sequences **(17)**. The vHMEC provide an ideal model to explore how COX-2 is regulated in mammary tissue undergoing the earliest steps of carcinogenesis. We hypothesize that understanding the pathways by which COX-2 is regulated before overt tumor development may provide insights into tumorigenesis, as well as molecular markers to identify and eliminate potential carcinogenic precursors.

Materials and Methods

Cells and Cell Culture. HMEC and vHMEC were isolated from reduction mammoplasties (RM) of three individuals, RM9, RM15, and RM16 and were propagated in two-dimensional cultures in modified MCBC 170 media (MEGM, BioWhittaker, Walkersville, MD) as previously described **(18, 19)**. All experiments w conducted with exponentially growing HMEC between population doublings 7 to 9, and exponentially growing midpassage vHMEC between population doublings 20 to 34, of which ~15% to 20 % of the population were expressing COX-2. vHMEC, RM9, 15, and 16 ceased to expand in cell number at population doublings 45, 60, and 50, respectively. Three-dimensional cultures were prepared by suspending single cells (5.0×10^4 cells per 100 μL of matrix) in reconstituted basement membrane (rBM; Becton Dickinson, Sunnyvale, CA) in glass capillary tubes. Polymerized rBM was dispensed using a Drummond Digital Microdispenser (Broomall, PA) into media-containing culture plates. After 10 days of growth, three-dimensional cultures were exposed to signaling inhibitors for the times indicated.

Western Blot. Total protein (20-30 μg) lysates from HMEC and vHMEC were electrophoretically separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes according to standard procedures. Antisera against COX-2 (Cayman Chemical, Ann Arbor, MI), total and phosphorylated-p38, total and phosphorylated extracellular signal-regulated kinase1/2 (ERK1/2), total and phospho-AKT, c-jun-NH_2-kinase, phospho-c-jun (Cell Signaling Technologies, Beverly, MA) were used according to manufacturers' protocols.

Prostaglandin E_2 Measurement. Prostaglandin E_2 (PGE_2) was determined using a Prostaglandin E_2-Monoclonal Enzyme Immunoassay kit (Cayman Chemical). Each experiment was carried out in triplicate according to manufacturer's instructions.

Proliferation and Apoptosis Assays. Cells were metabolically labeled with 10 μmol/L bromodeoxyuridine for 4 hours before harvesting. Nuclei were isolated and stained with propidium iodide and FITC-conjugated anti-bromodeoxyuridine antibodies (Becton Dickinson) and analyzed by flow cytometry using a FACS-Sorter (Becton Dickinson) and CellQuest software. Cell death was determined by trypan blue exclusion analysis. Experiments were repeated at least three independent times.

Expression of Cyclooxygenase-2 Construct. COX-2 sense construct was packaged in Phoenix A cells for viral propagation. Viral supernatant was diluted 1:1 with MEGM media and added to vHMEC for 6 hours. The population of vHMEC infected with retrovirus were selected and maintained in 2 μg/mL puromycin.

Immunocytochemistry. Cells cultured on glass coverslips were fixed with ice-cold methanol for 10 minutes and stored in 70% ethanol at 4°C until usage. Cells grown in rBM were mounted in tissue freezing medium (American Mastertech, Lodi, CA), frozen in isopentane cooled in liquid nitrogen, sectioned at 5 μmol/L intervals, and fixed in ice-cold methanol (Fisherbrand, Fisher Scientific, Pittsburgh, PA). All samples were probed with antisera against COX-2 (Cayman Chemical) or phospho-p38 (Cell Signaling Technologies) following manufacturers' instructions. Samples were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR), mounted in Vectashield (Vector Laboratories, Burlingame, CA), and visualized using a LSM450 Zeiss confocal microscope.

Tissue Samples. We analyzed a series of primary human DCIS (n = 30) and normal breast tissue specimens from reduction mammoplasties (n =47) obtained with Institutional Review Board approval fro the surgical pathology laboratory of the University of California, San Francisco and California Pacific Medical Center. Patients were identified through anonymous reference numbers in accordance with federal guidelines.

Tissue Preparation and Immunohistochemistry. Five-micron sections cut from formalin-fixed paraffin-embedded tissue blocks were deparaffinized and rehydrated following standard protocol. Sections were incubated with antisera against phospho-p38 (Cell Signaling Technologies), COX-2 (Cayman Chemical), estrogen receptor (DAKO Co., Carpinteria, CA), and progesterone receptor (Novocastra Lab., Newcastle-upon-Tyne, United Kingdom) following manufacturers' instructions. Antigen-antibody complexes were visualized using the Vectastain Elite avidin-biotin complex kit following standard protocol (Vector Laboratories). Sections were counterstained in hematoxylin dehydrated through graded alcohols, cleared in xylene, and mounted in permount.

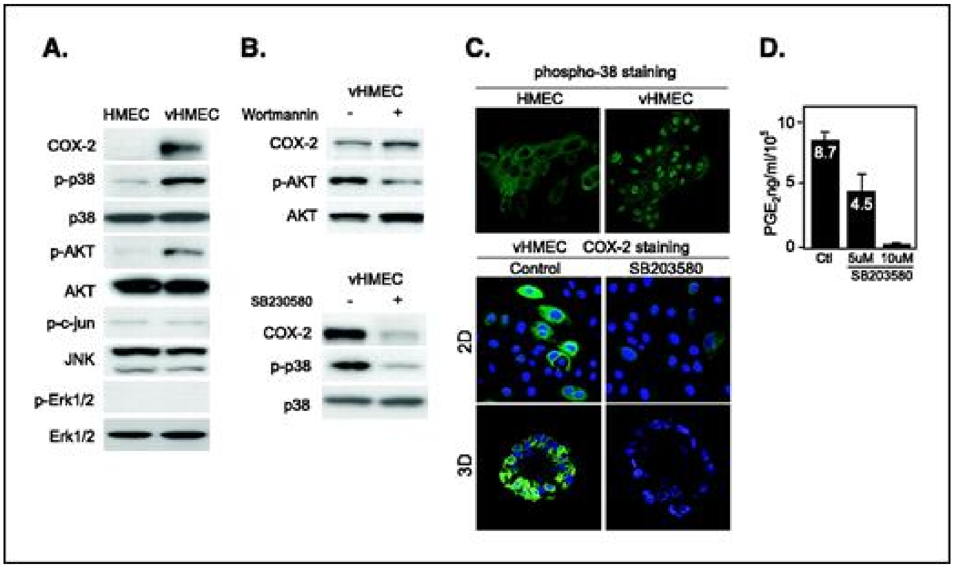
Evaluation of Phospho-p38 Immunostaining. The intensity of phospho-p38 staining was evaluated after examination of the entire slide. Phospho-p38 cytoplasmic staining intensity (1, absent to low; 2, moderate; 3, strong) and phospho-p38 nuclear heterogeneity (1, absent to low; 2, <50% nuclear positivity; 3, >50% nuclear positivity) was evaluated by light microscopy without any knowledge of the patients' clinical data.

Statistical Methods. χ^2 tests were used to test for associations between nuclear or cytoplasmic phospho-p38 levels in DCIS, morphologically normal epithelium adjacent to DCIS, and normal breast epithelium with age, nuclear grade, hormone receptor status, and COX-2 expression. JMP-In statistical package (SAS Institute, Cary, NC) was used for all analyses.

Results

Cyclooxygenase-2 Expression in Variant Human Mammary Epithelial Cells Is Dependent on Phospho-P38 in Two- and Three-dimensional Culture Conditions. We determined the upstream signaling pathways regulating COX-2 in vHMEC by comparing HMEC which have no appreciable expression of COX-2 with midpassage vHMEC which have robust expression of COX-2 in ~20% of the population (17). Isogenic populations of HMEC and vHMEC (RM 16) each express equal levels of ERK1/2, c-jun-NH_2-kinase, AKT and p38 (Fig. 1A), kinases important in the regulation of COX-2 in other systems. Because activation of these kinases is dependent upon their phosphorylated state, we compared their activated phospho-protein kinase levels in HMEC and vHMEC. HMEC do not express COX-2 or any of the four phospho-activated kinases to any appreciable degree (Fig. 1A). Likewise, vHMEC do not express the activated forms of ERK1/2 or c-jun-NH_2-kinase. However, increased COX-2 expression in vHMEC is associated with increased AKT and p38 phosphorylation (Fig. 1A). AKT is not involved in the observed overexpression of COX-2 in vHMEC because treatment with 1 μmol/L wortmannin, an inhibitor of PI3K, down-regulates p-AKT but does not alter COX-2 protein levels (Fig. 1B). In contrast, inhibiting p38 activity (by exposure to SB203580) simultaneously reduces the level of p38 phosphorylation and the level of COX-2 protein (Fig. 1B). Analysis of phospho-p38 in HMEC and vHMEC by immunocytochemistry shows that activated p38 is a uniform characteristic of vHMEC (Fig. 1C). Notably, nuclear-localized activated phospho-p38 is universally seen in all vHMEC at all passages including those that do not express COX-2 (data not shown). Additional immunochemical analysis shows that COX-2-expressing vHMEC lose phospho-p38 staining (data not shown), as well as COX-2 staining, following SB203580 treatment (Fig. 1C). This inhibition of p38 activity also dramatically reduces prostaglandin production (Fig. 1D). Hence, COX-2 protein levels and functional activity are abrogated by phospho-p38 inhibition. These data show that in vHMEC, p38 activation is present before COX-2 expression and is necessary but not sufficient for COX-2 expression.

Figure 1.



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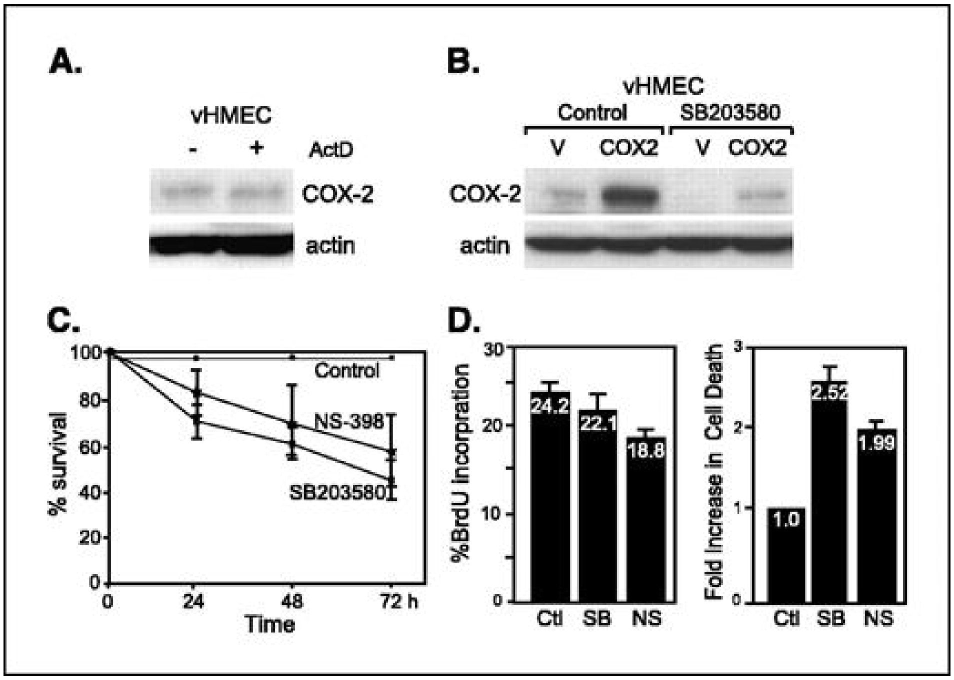
COX-2 expression in variant HMEC is dependent on p38. *A,* we determined the signaling pathways involved in COX-2 expression by probing cell lysates from normal and vHMEC for COX-2 and the phospho-specific and total levels of p38, AKT, c-jun/JNK, or ERK1/2. *B,* cell lysates from vHMEC (RM16) exposed to the PI3-K inhibitor, wortmannin, at 1 μmol/L for 24 hours were probed by Western blot for COX-2, the phosphorylated and total levels of AKT. Similarly, vHMEC exposed to the p38 inhibitor, SB203580, at 10 μmol/L for 24 hours were probed for COX-2, the phosphorylated and total levels of p38 by Western blot. *C,* subcellular distribution of p38 in HMEC and vHMEC was determined by immunostaining for phospho-p38. To determine the individual cellular response to p38 inhibition we stained vHMEC grown on two-dimensional plastic (*2D*) and in three-dimensional (*3D*) culture conditions for COX-2 before and after exposure to SB203580. *D,* levels of PGE_2 production secreted in the surrounding media from vHMEC exposed to 5 and 10 μmol/L SB203580 were measured by ELISA. *Bars,* SD for three experiments. Similar data were obtained from RM9 and RM15.

Because signal transduction pathways are often differentially regulated in two-dimensional versus three-dimensional culture conditions, we evaluated COX-2 regulation in HMEC and vHMEC grown in rBM where they form physiologic three-dimensional structures (mammospheres). The lack of observable COX-2 expression in HMEC grown on two-dimensional plastic is recapitulated in mammospheres (data not shown). Likewise, the observable COX-2 expression in vHMEC grown in two dimensions is also recapitulated in three-dimensional cultures (**Fig. 1C**). Because mammospheres are clonally derived and exhibit characteristics of the founding cell, we observe little heterogeneity of COX-2 staining within a single mammosphere (i.e., negative cells generate negative mammospheres, whereas positive cells generate positive mammospheres. Interestingly, vHMEC mammospheres express COX-2 in a larger fraction of the population than that observed on two-dimensional plastic (64% versus 12%), suggesting either that the basement membrane may contribute to COX-2 expression in vHMEC or perhaps COX-2-expressing cells have a selective advantage when grown in rBM. To determine if p38 inhibition could lower COX-2 levels in three dimensions as was observed in two dimensions, vHMEC were grown in rBM for 10 days and exposed to SB203580 for 24 hours. Inhibition of p38 in three-dimensional culture of midpassage vHMEC results in the down-regulation of COX-2 in all cells of the mammosphere and in all mammospheres (**Fig. 1C**).

We observe that p38 dependence of COX-2 protein expression in vHMEC derived from three individuals (RM9, RM15, and RM16), suggesting that it is a general property of vHMEC. Dose-dependent inhibition of PGE_2 secretion by SB203580 is observed in all samples. Treatment of RM9, RM15, and RM16 with 5 μmol/L SB203580 results in a 48%, 50%, and 40% decrease in PGE_2 production, respectively. PGE_2 levels were further reduced by 89%, 75%, and 77% following treatment with 10 μmol/L SB203580, respectively (data not shown).

Cyclooxygenase-2 Expression in Variant Human Mammary Epithelial Cell Is Regulated at the Post-Transcriptional Level. COX-2 expression can be regulated at both the transcriptional and post-transcriptional levels (i.e., through mRNA stabilization). Exposure of midpassage vHMEC to Actinomycin D inhibits transcription (data not shown) but does not affect the level of COX-2 protein (**Fig. 2A**), suggesting that COX-2 protein level in vHMEC is not regulated at the transcriptional level. To determine if COX-2 expression is regulated post-transcriptionally, we measured COX-2 protein levels in vHMEC engineered to stably express COX-2 under the regulation of an independent, constitutively active promoter. Similar to the parental control, exposure of vector control vHMEC to SB203580 for 24 hours results in the down-regulation of endogenously regulated COX-2 protein (**Fig. 2B**). Surprisingly, we also observed down-regulation of COX-2 protein levels in cells constitutively expressing COX-2 from an exogenous promoter devoid of endogenous regulatory elements (**Fig. 2B**). These data indicate that, independent of transcriptional regulation, activated p38 plays a dominant role in ensuring COX-2 protein expression. Cells expressing activated p38 would be primed for the sustained overexpression of COX-2 after an inducing event. Although we do not yet understand what event is inducing COX-2 in vHMEC, it is clear that activated p38 creates a cellular environment that may stabilize COX-2 mRNA transcripts facilitating COX-2 expression and the ensuing phenotypes **(17)**.

Figure 2.



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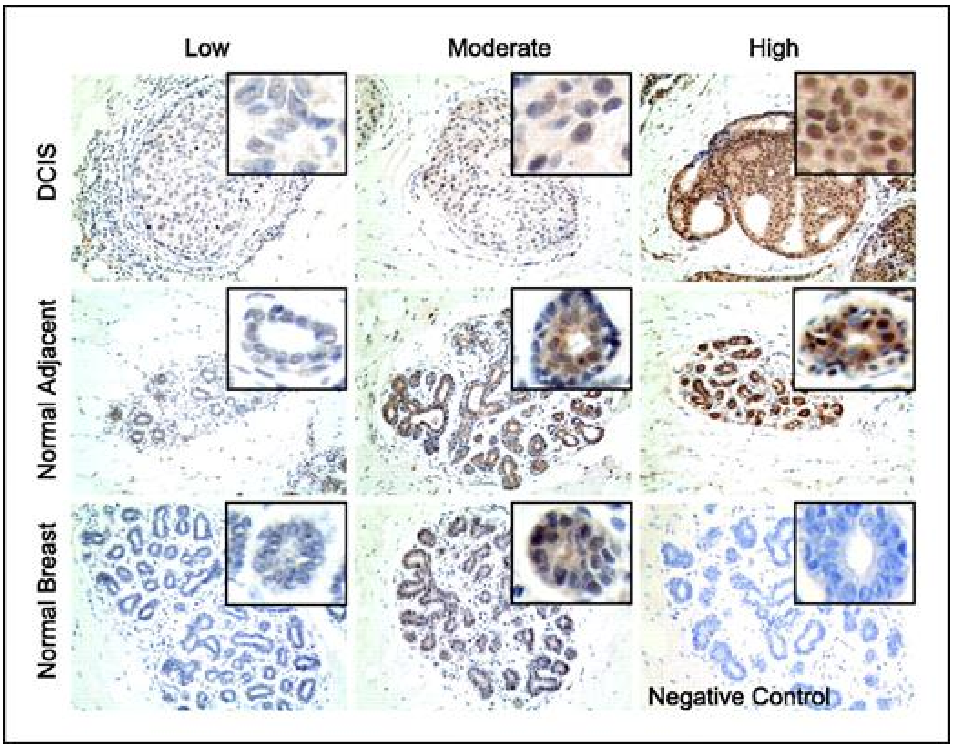
COX-2 expression in vHMEC is regulated at the post-transcriptional level and both p38 and COX-2 protect vHMEC from apoptosis. *A,* to determine if COX-2 expression in vHMEC is dependent on *de novo* transcription, cell lysates from vHMEC (RM16) exposed to 1 μmol/L Actinomycin D were probed for COX-2. *B,* variant HMEC stably transfected with COX-2 or the empty vector with or without 10 μmol/L SB203580 exposure for 24 hours, were probed for COX-2 by Western blot. *C,* variant HMEC (RM16) exposed to 10 μmol/L SB203580 or 25 μmol/L NS-398 were counted at 24, 48, and 72 hours and plotted as percent of plated cells. Cells were pulsed for 4 hours with bromodeoxyuridine (*BrdU*) after 24 hours of exposure to SB203580 (*SB*) or NS-398 (*NS*) and analyzed by flow cytometry following propidium iodide staining (control cells, *Ctl*). *D,* cells collected after 24 hours of exposure to SB203580 or NS-398 were stained with trypan blue and counted to determine the percentage of dead cells. *Bars,* SD of three independent experiments.

p38 Activation and Cyclooxygenase-2 Expression Protect Variant Human Mammary Epithelial Cells from Apoptosis. Because we have previously shown that the COX-2 inhibitor, NS-398, activates apoptosis in vHMEC (**17**), we hypothesized that down-regulation of COX-2 through p38 inhibition might also increase apoptosis. As shown in **Fig. 2C**, both the p38 inhibitor, SB203580, and the COX-2 antagonist, NS-398, decreased cell number by 53% and 41%, respectively. In contrast, neither SB203580 nor NS-398 decreases cell number in HMEC under the same conditions (data not shown). Flow cytometric analysis of bromodeoxyuridine incorporation shows that inhibition of p38 or COX-2 does not appreciably alter the population of cells within the S-phase of the cell cycle (22.1% and 18.8%, respectively) compared with controls (24.2%; **Fig. 2D**). This result suggests that the dramatic decrease in cell number was not due to abrogated proliferation. In contrast, cells treated with either the p38- or COX-2-specific inhibitor exhibited a striking increase in cell death compared with unexposed vHMEC (**Fig. 2D**). Therefore, pharmacologic inhibition of p38 and/or COX-2 decreases cell survival by increasing apoptosis (**Fig. 2**, data not shown and ref. **17**) indicating that activated p38 and subsequent COX-2 expression in vHMEC enhance viability.

Phospho-p38 Is Associated with Premalignant Lesions of the Breast. We evaluated phospho-p38 immunostaining in 30 archival DCIS specimens and 47 reduction mammoplasty specimens from disease-free women. Because phospho-p38 is known to activate downstream targets in both the nucleus and the cytoplasm, special attention was given to subcellular localization of staining. Representative nuclear and cytoplasmic phospho-p38 staining in DCIS and normal breast tissue is shown in **Fig. 3**. High phospho-p38 staining intensity (score of 3), either nuclear or cytoplasmic, is only detected in tissue containing DCIS and its surrounding epithelium (**Table 1**). We found that high nuclear phospho-p38 staining intensity in these tissues was usually accompanied by high cytoplasmic staining, suggesting that the amount of phospho-p38 translocating to the nucleus, as well as the overall amount of activated p38, are both elevated. In contrast to the observations in DCIS-containing tissue, significantly fewer cases of normal tissue from reduction mammoplasty showed nuclear or cytoplasmic phospho-p38 staining (**Table 1**) and when detected did not reach the levels observed in premalignant lesions.

Figure 3.



Phospho-p38 staining is prevalent in DCIS and morphologically normal epithelium adjacent to DCIS. Paraffin-embedded tissue sections of DCIS and normal breast were immunostained with a monoclonal phospho-specific antibody to p38. Because 3,3′-diaminobenzidine was used as a chromogen, tissue sections stained brown indicates positive immunoreactivity. Sections were counterstained with Mayer's hematoxylin to reveal the nuclei. Representative low, moderate and high phospho-p38 staining intensity for DCIS (cases 14232, 1798, and 251, respectively), morphologically normal epithelium adjacent to DCIS (cases 1619, 1468, and 1467, respectively). Normal epithelium from disease-free breast tissue (cases 76 and 14, respectively) did not exhibit high phospho-p38 staining intensity. Normal adjacent epithelium stained with secondary anti-mouse immunoglobulin G only served as a tissue control. We observed that cytoplasmic staining was relatively uniform compared with the heterogeneity of nuclear staining, such that some nuclei are intensely stained whereas others are devoid of staining (*inset*). Representative DCIS lesions that exhibited low, moderate, and high phospho-p38 staining are examples of high-, intermediate-, and low-grade lesions, respectively, illustrating that nuclear staining was more intense in lower-grade nuclei.

Table 1.

Nuclear and cytoplasmic phospho-p38 in DCIS, morphologic normal epithelium adjacent to DCIS, and normal breast

Tissue	No.	Nuclear p-p38									Cy
				1	2	3	No. p38+ (%)	*P*	1	2	3
DCIS	30	5	19	6	25/30 (83%)	Reference	4	12	14	26/30 (87%)	Re
Normal breast around DCIS	26	1	20	5	25/26 (96%)	0.12	3	15	8	23/26 (89%)	0.
Normal breast	47	37	10	0	10/47 (21%)	<0.0001	44	3	0	3/47 (6%)	<(

NOTE: Tissue sections from DCIS, normal breast epithelium adjacent to DCIS, and normal breast tissue were scored (1, absent to low; 2, moderate; 3, maximum intensity) for phospho-p38 staining intensity, with a score >2 considered phospho-p38 positive.

Phospho-p38 and Cyclooxygenase-2 Expression. In a previous study (15), we analyzed 46 DCIS cases and found that 85% of the cases overexpressed COX-2. Because we hypothesize that p38 is necessary to achieve sustained COX-2 expression, we evaluated phospho-p38 staining intensity in these same cases, whenever the DCIS lesion was large enough to provide additional material. Of our original sample group, 30 cases produced slides containing DCIS of which 61% overexpress COX-2. Of these 30 cases, 83% and 87% exhibited nuclear and cytoplasmic phospho-p38 staining, respectively (Table 1). In DCIS, we find that maximum COX-2 expression is always associated with phospho-p38 staining. All cases with intense COX-2 staining (n = 8) exhibit moderate to high nuclear staining of phospho-p38. Conversely, maximum phospho-p38 staining is not always associated with maximum COX-2 staining. Instead, we find that in those cases exhibiting high nuclear phospho-p38 staining, COX-2 staining intensity is equally distributed between absent to low (33%), moderate (33%), and high (33%). Likewise, DCIS cases that are moderate for phospho-p38 staining are also equally distributed among the three levels of COX-2 staining. Thus, high intensity phospho-p38 staining can exist in the absence of COX-2 expression, but high COX-2 expression is not exhibited in the absence of highly active p38.

Phospho-38 in Ductal Carcinoma *In situ* and Other Clinicopathologic Variables. Table 2 illustrates clinical and pathologic variables of the DCIS cases studied. Patient age ranges from 35 to 81 years with mean age of 58. The size of DCIS lesions ranges from 1 to 36 mm, with median size increasing with nuclear grade. Cellular necrosis is present in 48 % of the cases and is more prevalent in higher-grade lesions. The DCIS specimens were predominantly estrogen and progesterone receptor positive. There is no association between phospho-p38 staining (nuclear or cytoplasmic) and patient age (P = 0.31 and 0.59, respectively) or nuclear grade (P = 0.46 and 0.26, respectively; **Table 2**). We find no association between estrogen or progesterone receptor status and nuclear phospho-p38 positivity (P = 0.11 and 0.55, respectively) or cytoplasmic phospho-p38 positivity (P = 0.58 and 0.18, respectively; **Table 2**).

Table 2.

Phospho-p38 and clinicopathologic variables in DCIS

Characteristic	No. patients	% Nuclear phospho-38+	*P*	% Cytoplasmic phospho-p38+	*P*
Age					
<58	13	91	0.31	27	0.59
>58	13	75		67	
ND	4				
Nuclear grade					
Low	8	75	0.46	75	0.26
High	22	86		91	
Estrogen receptor					
Negative	4	50	0.11	25	0.58
Positive	15	87		40	
ND	11				
Progesterone receptor					
Negative	7	71	0.55	29	0.18
Positive	17	82		59	
ND	6				
COX-2					
Negative	11	73	0.29	82	0.64
Positive	17	88		88	
ND	2				

NOTE: Phospho-p38 staining intensity with a score >2 is considered phospho-p38 positive.

Abbreviation: ND; not determined.

Phospho-p38 Is Found in the Field of Morphologically Normal Epithelial Cells Adjacent to Premalignant Lesions. We previously found that morphologically normal epithelium adjacent to a DCIS lesion often had a higher level of COX-2 staining than was seen in the DCIS lesion. Likewise, in this study, for those DCIS lesions with high phospho-p38 staining, 80% have similar or higher phospho-p38 staining intensity in the adjacent normal epithelium (**Fig. 3**; **Table 1**). The remaining 20% have a lower but detectable phospho-p38 staining. Levels of p38 staining, in histologically normal epithelium adjacent to DCIS more closely resembles the DCIS lesion than histologically normal epithelium from disease-free tissue, suggesting that the epithelium adjacent to DCIS and the DCIS lesion share premalignant molecular alterations.

Phospho-p38 Is Found in a Fraction of Normal Disease-Free Breast Tissue. In examining the 47 cases of normal breast tissue obtained from reduction mammoplasty, 21% have phospho-p38 staining in the epithelium. This staining intensity is significantly less than the maximal staining observed in DCIS and its surrounding benign-appearing epithelium (**Fig. 3**). None of the tissues examined, DCIS, normal epithelium adjacent to DCIS nor normal tissue exhibited phospho-p38 staining in fibroblasts of the stromal compartment. Our data shows that whereas activated phospho-p38 is a common molecular characteristic of epithelial cells in a field surrounding and including DCIS (**Table 1**), it is an uncommon characteristic of the bulk of normal mammary tissue.

Discussion

The present study, using cells generated from human breast tissue explants, provides evidence *in vitro* that the differential expression of COX-2 in HMEC versus vHMEC is dependent on activated p38. Whereas considerable evidence has shown the family of mitogen-activated protein kinases, including ERK1/2, p38, and c-jun-NH_2-kinase, or PI3K are involved in COX-2 transcription and/or transcript stabilization in various tumor cell models **(5, 20, 21)**, only p38 regulates COX-2 expression in vHMEC. Unlike many tumor cells, human mammary epithelial cells, either HMEC or vHMEC, do not exhibit endogenous activation of the classic mitogen-activated protein kinases, ERK1/2 kinase nor exhibit c-jun-NH_2-kinase activation through c-jun phosphorylation. The PI3K/AKT pathway also seems not to be responsible for COX-2 induction in vHMEC. Although we observe that the downstream effector of PI3K, AKT, is phosphorylated in vHMEC compared with HMEC, exposure to wortmannin, a known pharmacologic inhibitor of PI3K, slightly elevates COX-2 levels, similar to that described in HT-29 human colon cancer cells and perhaps suggestive of negative regulation **(22)**. We show that the p38 kinase signaling is the predominant pathway for COX-2 regulation in vHMEC as p38 is preferentially phosphorylated in vHMEC and its inhibition leads to down-regulation of COX-2 in cells grown under both two- and three-dimensional culture conditions. All vHMEC have activated phospho-p38 but only a subpopulation expresses COX-2 suggesting that activated p38 is necessary but not sufficient for the increased expression of COX-2 in vHMEC. The mechanisms leading p38 activation and subsequent COX-2 induction remain to be determined.

Our *in vitro* studies establish phospho-p38 as an upstream regulator of COX-2. Our *in vivo* observations indicate that activation of p38 and overexpression of COX-2 characterizes DCIS lesions and adjacent fields of morphologically normal epithelium. Hence, we hypothesize that p38 may be an early molecular event that allows for sustained COX-2 expression in breast tissue, thereby contributing to tumor initiation. We find that phospho-p38 is prevalent in DCIS and in the morphologically normal adjacent epithelium. This is in contrast to the low levels of phospho-p38 observed in normal epithelium from disease-free breast tissue. Notably, all cases of DCIS that exhibit intense COX-2 also exhibit nuclear phospho-p38 staining; however, not all cases with intense nuclear phospho-p38 had coincident COX-2 staining (a finding we also observe in vHMEC grown in culture). Therefore, the activation of p38 may be necessary but not sufficient for the induction of COX-2 in mammary epithelial cells *in vivo* as well as *in vitro*.

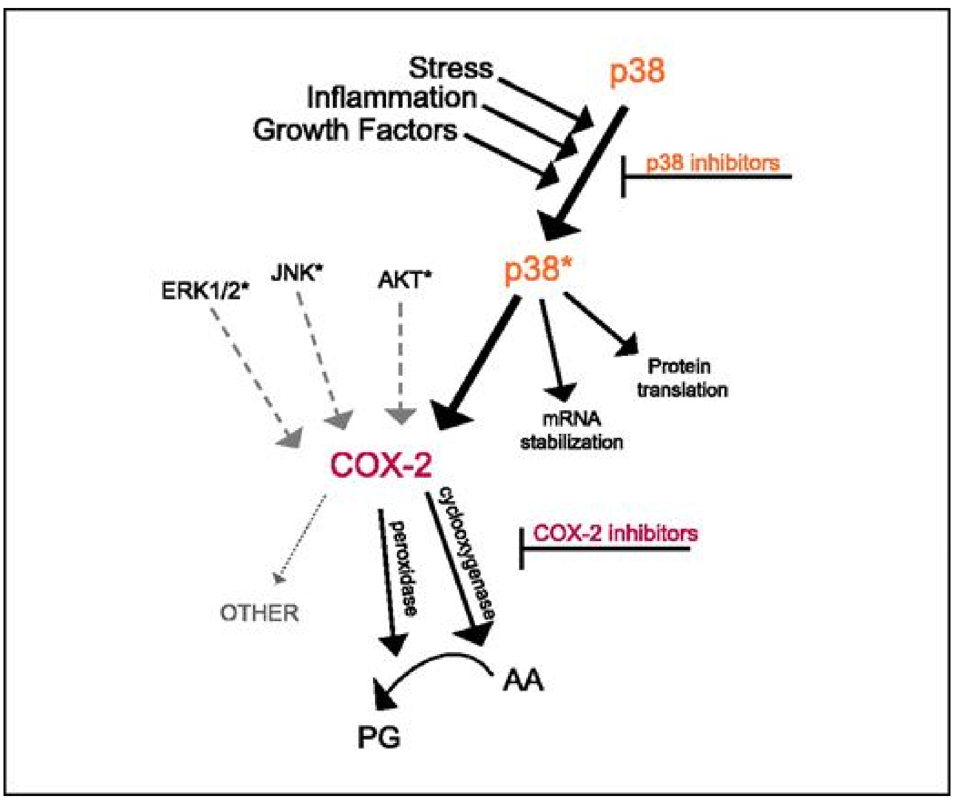
We have previously shown epithelial cells in a subset of normal breast tissue exhibit $p16^{INK4a}$ promoter hypermethylation and COX-2 overexpression **(17, 23)**. We are currently exploring the relationship between phospho-p38 with these molecular characteristics in normal breast tissue as we hypothesize that coincident expression may generate cells susceptible to oncogenic transformation.

Given the potential importance of p38-mediated regulation of COX-2 in early carcinogenesis, we further explored the mechanism of this regulation. In other cell types, p38 has been shown to regulate transcriptional activation of downstream genes as well as transcript stability. In vHMEC, we show that inhibition of transcription with Actinomycin D did not alter the level of COX-2, suggesting that continuous transcription by an autocrine inducer is not responsible for the elevated COX-2 protein levels in vHMEC compared with HMEC. There is growing evidence that post-transcriptional regulation of COX-2 mRNA is important in determining its cellular protein levels **(5, 24, 25)**. We find in vHMEC stably overexpressing exogenous COX-2 that p38 inhibition can dramatically reduce the level of exogenous COX-2 protein, suggesting that p38 may be regulating COX-2 mRNA stability or protein degradation in these cells. A downstream effector of p38 activity, MK-2, mediates COX-2 mRNA stabilization and leads to a decrease in turnover and an increase in protein translation **(5, 24, 26)**. Our findings are consistent with the interpretation that COX-2 expression in vHMEC is not dependent on transcription but instead may require mRNA stabilization through activated p38. The role of p38 in stabilizing labile mRNA may be critical for sustained activity regardless of the source of induction.

Cells with activated p38 and/or COX-2 overexpression may represent an early initiated population with potential to progress to malignancy. Experiments in murine model systems as well as observations in human premalignant breast lesions support the hypothesis that COX-2 overexpression is an early molecular event in breast carcinogenesis **(12, 15, 16, 27)**. COX-2 expression and PGE_2 production have been shown to regulate many of the phenotypes that contribute to tumor initiation and malignant progression such as epithelial cell proliferation, apoptosis, and invasion, endothelial migration and angiogenesis, and host immune evasion **(28–30)**. It is also important to consider that activation of p38 may contribute to carcinogenesis independent of its regulation of COX-2 expression. For example, p38 has been reported to regulate the turnover of several metastatic gene transcripts, such as urokinase-type plasminogen activator receptor and matrix metalloproteinases **(31–33)**. In addition to COX-2, urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor and matrix metalloproteinases have also been shown to associate with premalignant and malignant lesions **(15, 34, 35)**, suggesting p38 may participate in a program that elicits cell survival and stromal remodeling early in carcinogenesis. p38 signaling has also been shown to play a role in murine mammary epithelial-to- mesenchymal transition and human mammary tumor cell migration **(36)**. The role of COX-2 in p38-dependent epithelial-to-mesenchymal transition and tumor epithelial cell migration remains to be investigated. The observed levels of phospho-p38 in DCIS and adjacent fields of morphologically normal epithelium may enable the stabilization of labile gene transcripts induced by relevant oncogenes, such as HER-2/*neu*. In this scenario, the action of p38 would be to stabilize the COX-2 transcript and maintain oncogenic signaling. Additionally, because p38 can stabilize many labile transcripts associated with malignancy, these studies reveal a molecular program activated during early breast carcinogenesis. As shown in this report, this program seems to be activated in a small percentage of normal disease-free breast tissue and may identify cells with oncogenic potential. These cell culture studies show that p38 may be an early event that contributes to the malignant phenotype in epithelial cells in concert with COX-2 and other downstream effectors. Future studies dissecting upstream and downstream pathways of p38 and COX-2 may provide further insights in early events in carcinogenesis and identify novel approaches for chemoprevention.

Studies have shown that the overexpression of COX-2 can elicit phenotypes that are independent of COX activity and prostaglandin synthesis and probably rely on the peroxidase activity exhibited by COX-2 (refs. **37, 38**; see **Fig. 4**). The antineoplastic effects of nonspecific COX-2 inhibitors, such nonsteroidal anti-inflammatory drugs or sulindac sulfone, or selective COX-2 inhibitors, such as celocoxib, inhibit only the COX activity of COX-2 **(39–41)**. Antagonists of p38 may provide an alternative and additional therapeutic target upstream of COX-2, thereby encompassing all COX-2-dependent phenotypes as opposed to only enzymatic inhibition. Therapeutic inhibition of p38, ideally, should selectively eliminate cells possessing activated p38 and its downstream effectors.

Figure 4.



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p38 signaling regulates COX-2 in vHMEC. The signaling mediators, Erk1/2, JNK, AKT, and p38 are involved in COX-2 transcription and/or transcript stabilization in various tumor cell models. However, in vHMEC p38 activation (p38*) by environmental stimuli is the predominant pathway for COX-2 regulation. Antagonists of p38 may provide an alternative and additional therapeutic target upstream of COX-2, thereby encompassing all COX-2-dependent phenotypes as opposed to only enzymatic inhibition.

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