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Transgenic Insulin-like Growth Factor-1 stimulates activation of COX-2 signaling in mammary glands

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Abstract

Studies show that elevated IGF-1 levels are associated with an increased risk of breast cancer; however, mechanisms through which IGF-1 promotes mammary tumorigenesis *in vivo* have not been fully elucidated. To assess the possible involvement of COX-2 signaling in the protumorigenic effects of IGF-1 in mammary glands, we used the unique BK5.IGF-1 mouse model in which transgenic (Tg) mice have significantly increased incidence of spontaneous and DMBA-induced mammary cancer compared to wild type (WT) littermates. Studies revealed that COX-2 expression was significantly increased in Tg mammary glands and tumors, compared to age-matched WT. Consistent with this, PGE₂ levels were also increased in Tg mammary glands. Analysis of expression of the EP receptors that mediate the effects of PGE₂ showed that among the four G-protein-coupled receptors, EP3 expression was elevated in Tg glands. Up-regulation of the COX-2/PGE₂/EP3 pathway was accompanied by increased expression of VEGF and a striking enhancement of angiogenesis in IGF-1 Tg mammary glands. Treatment with celecoxib, a selective COX-2 inhibitor, caused a 45% reduction in mammary PGE₂ levels, attenuated the influx of mast cells and reduced vascularization in Tg glands. These findings indicate that the COX-2/PGE₂/EP3 signaling pathway is involved in IGF-1-stimulated mammary tumorigenesis and that COX-2-selective inhibitors may be useful in the prevention or treatment of breast cancer associated with elevated IGF-1 levels in humans.

Keywords

mammary cancer; IGF-1; COX-2; stroma; transgenic mouse

Introduction

Insulin-like growth factor-1 (IGF-1) is a single chain 7 kDa polypeptide that functions as a key regulator of development in mammary glands by stimulating terminal end bud formation and ductal elongation [1]. Numerous studies show that elevated levels of IGF-1 are associated with an increased risk of breast cancer [2,3]. The mechanisms by which IGF-1 contributes to breast carcinogenesis *in vivo* are not yet fully understood, but evidence indicates that they are related to its capacity to induce proliferation and prevent apoptosis [4,5]. Recently a more complex role of IGF-1 in cancer development is beginning to emerge, and reports indicate involvement in modulating other critical events, such as angiogenesis,

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homeostasis of stem/precursor cells, genomic stability and regulation of cellular metabolism [6].

To better understand the role of IGF-1 in mammary tumorigenesis *in vivo*, our laboratory has characterized a novel transgenic (Tg) mouse model in which the overexpression of IGF-1 is under the control of the bovine keratin 5 (BK5) promoter [7]. BK5 directs transgene expression to the myoepithelial cells of the mammary gland, where it acts in a paracrine/juxtacrine manner on the adjacent ductal epithelium [7]. Compared to previous Tg models using whey acidic protein (WAP) or mouse mammary tumor virus (MMTV) promoters [8,9], the BK5.IGF-1 mouse more closely recapitulates the exposure of breast epithelium to stromal IGF-1 observed in women. It thus allows an assessment of the contribution of IGF-1 to breast development and tumorigenesis in a hormonally relevant *in vivo* milieu [7]. Studies with this model demonstrate that there is a significantly higher incidence of mammary cancer in the BK5.IGF-1 Tgs compared to WT mice [7]. When treated with DMBA (20µg/mouse/day), 74% of the Tg mice develop mammary cancer, compared to 29% of the WTs, with a decrease in the overall mean tumor latency from 43.3 wks in WTs to 19.9 wks in Tgs [7].

Interestingly, proliferation of the ductal epithelium, as measured by BrdU incorporation, is significantly increased in mammary glands of prepubertal (4-5 wks) Tg mice compared to WTs, but is not elevated in postpubertal (> 8 wks) Tg glands [7]. Consistent with this finding, cyclin D1 expression is also increased in glands from prepubertal but not in postpubertal Tg mice [10]. Since DMBA treatment begins post-pubertally [7], these unexpected findings indicate that the increased susceptibility to mammary cancer in BK5.IGF-1 mice is not due to post-initiation proliferation [11,12], suggesting that IGF-1 is not acting by a canonic tumor promotion mechanism [13] in this model.

Possible alternative mechanisms of IGF-1-stimulated mammary tumorigenesis can be speculated based on histopathological findings in the mammary glands from Tg BK5.IGF-1 mice, including stromal thickening with moderate desmoplasia, influx of mast cells and increased vascularization [7]. Since inflammation and stromal activation have been shown to play key roles in breast cancer development [14,15], these phenotypic characteristics indicate the possible contribution of inflammatory mediators to IGF-1-stimulated mammary tumorigenesis.

COX-2, a key inflammatory molecule involved in prostaglandin (PG) synthesis [16], has been shown to mediate changes in tissue and tumor stroma [17-19] and to be elevated in a wide variety of cancers, including those of the breast [20,21]. Importantly, recent *in vitro* studies have demonstrated induction of COX-2 by IGF-1 in human pancreatic carcinoma [22], human ovarian cancer [23] and rat gastric mucosal epithelium [24]. These findings, in combination with data showing that the inflammatory COX-2/PG pathway is also associated with increased angiogenesis, evasion from immunologic anti-tumor responses and other pro-carcinogenic processes, lead us to investigate the possible role of the COX-2/PGE2 pathway in mediating mammary tumorigenesis in the novel BK5.IGF-1 model.

Results presented here show that Tg overexpression of IGF-1 induced activation of COX-2/PG signaling in both mammary glands and tumors. In addition, treatment with celecoxib, a selective COX-2 inhibitor, caused a significant reduction in mammary PGE₂ levels and attenuated the pro-tumorigenic, IGF-1-associated mammary phenotype. These findings extend our understanding of the mechanisms by which IGF-1 contributes to mammary tumorigenesis *in vivo* and also reveal the potential for targeting COX-2 for prevention and treatment of IGF-1-stimulated breast cancer.

Materials and Methods

Animals

The generation of the BK5.IGF-1 transgenic mouse model on an ICR background has been described previously [7,11]. All experimental animals were hemizygous for the IGF-1 transgene. Age-matched, WT littermates were used as controls for all experiments. Animals were provided standard lab chow ad libitum and housed in a temperature- and humidity-controlled, AAALAC-certified facility, with a 12 h light/dark cycle. All experiments were conducted in strict accordance with Institutional Animal Care and Use Committee-approved protocols.

For studies of mammary glands, virgin female WT and Tg mice were killed by CO₂ asphyxiation at 4-5 wks of age (prepubertal) or 8 wks of age (postpubertal), and mammary glands were harvested. Carcinogenesis experiments were carried out by administering 20 µg/mouse/day of DMBA by oral gavage starting from 7-9 wks of age as previously described [7]. Tumors were collected at or before reaching 1.5 cm in any dimension.

Celecoxib preparation and treatment

Celecoxib powder (LKT Laboratories, Inc, St. Paul, MN) was dissolved in DMSO, combined with corn oil and vortexed vigorously before each administration. The final emulsion contained 20 mg/ml celecoxib in 1.5% DMSO/corn oil. At weaning (4-5 wks), female BK5.IGF-1 Tg mice were randomly divided into two groups and treated 5 days a week for 4 wks with 100 mg/kg celecoxib or with control vehicle by oral gavage. Mice were sacrificed 6 hours after the last treatment and mammary glands were harvested. Mice were weighed daily during treatment to determine the correct dosage of celecoxib and to monitor for toxicity.

Western blot analysis

Snap-frozen abdominal/inguinal mammary glands or tumors were homogenized with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, and 1 mM NaF) as described previously [7,25]. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (40 µg) were resolved on SDS-PAGE gels and transferred onto PVDF membranes (Pierce, Rockford, IL). After blocking with 5% nonfat milk in PBST, membranes were probed with specific primary antibodies, followed by the appropriate HRP-conjugated secondary antibody, and bands detected using the ECL Plus kit (Amersham, GE Healthcare, Piscataway, NJ). The COX-2 antibody was purchased from Cayman Chemical (#160126, Ann Arbor, MI), and PGE2 receptor 3 (EP3) (sc-16019) and VEGF (sc-152) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH was obtained from Cell Signaling Technology (#2118, Danvers, MA). The bands were scanned with the Typhoon imager (Typhoon 9410, GE Healthcare) and intensity quantified using ImageQuant TL software (Amersham Biosciences, Piscataway, NJ). Target protein bands were normalized to GAPDH bands in the same sample and then normalized to the control group to calculate the fold change.

Double immunofluorescence staining

Mammary tumors were fixed in 10% formalin and embedded in paraffin. To assess expression of COX-2 and K5, slides were deparaffinized, blocked in 10% goat serum for 30 min, and incubated with rabbit anti-COX-2 (1:300, Cayman Chemical) at 4 °C overnight. Sections were then washed and incubated at room temperature (RT) for 40 min with goat anti-rabbit Alexa Fluor® 488 IgG (green immunofluorescence, Invitrogen, Carlsbad, CA). After washing, slides were incubated again with rabbit anti-K5 primary (1:200, #PRB-160P,

Berkeley Antibody Company, Richmond, CA,) at RT for 1 h, followed by incubation with goat anti-rabbit Alexa Fluor® 594 IgG (red immunofluorescence, Invitrogen) for 40 min.

For COX-2 and K5 double staining, slides were blocked in 10% goat serum for 30 min, incubated overnight at 4 °C with a primary antibody solution containing both rabbit anti-COX-2 (1:300) and rabbit anti-K5 (1:200). The following day, sections were incubated with goat anti-rabbit Alexa Fluor® 488 IgG (green) to visualize COX-2 and goat anti-rabbit Alexa Fluor® 594 IgG (red) to visualize K5. Fluorescence-labeled sections were then mounted with VECTASHIELD® mounting medium containing DAPI (Vector Laboratories, Burlingame, CA), coverslipped, and viewed using confocal microscopy at 40x (Olympus Flowview confocal microscope).

ELISA

Abdominal and inguinal glands were homogenized and PGs extracted as described previously [26]. PGE₂ levels were evaluated using an ELISA kit (Cayman Chemicals, cat no. 514010). Data were analyzed using a multiple linear regression program (AssayZap; Biosoft, Cambridge, UK) and levels were expressed as pg/mg protein.

RNA extraction and real-time quantitative PCR (QPCR)

Total RNA was extracted from homogenized abdominal and inguinal glands using the Absolutely RNA® Miniprep kit (Agilent Technologies, Santa Clara, CA), and reverse-transcribed as described previously [7]. Real-time QPCR was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). Primers and probes for analysis of mouse EP1-4 were purchased as a TaqMan® gene expression assay from Applied Biosystems. The TATA box binding protein (TBP) primers and probe used were as follows: forward primer, 5' GGTGGCAGCATGAAGTGACA-3'; reverse primer, 5' -GCACAGAGCAAGCAACTCA-CA-3'; TaqMan probe, 5' -FAM-CCTCTGCACTGAAATCACCTGCAGCA-TAMRA-3'. A relative standard curve was generated for each assay using the whole mammary gland from a young, untreated WT mouse. The C_t (threshold cycle) of each sample was plotted against the log of nanograms of cDNA added to the wells of the standard curve, and the reverse log of that number was normalized to its corresponding TATA box binding protein value. Target gene expression of each sample was normalized to its corresponding TBP gene value. Control tissue (pancreas) from WT and EP3 knockout (KO) mice were kindly provided by Dr. Susan M. Fischer, and were used as positive and negative controls in the EP3 mRNA assay, respectively [27].

Histological analysis and immunohistochemistry

Thoracic mammary glands were fixed in 10% formalin for 24 hours, transferred to 70% ethanol, embedded in paraffin and cut in 5 µm sections. A polyclonal goat anti-mouse CD31 antibody (sc-1506, 1:500, Santa Cruz) was used to analyze microvessels in mammary glands. Sections were incubated with the antibody overnight at 4 °C, followed by HRP-linked anti-goat IgG (Dako, Carpinteria, CA). Color was developed with 3,3'-diaminobenzidine (Dako). To identify mast cells within these tissues, sections were deparaffinized, rehydrated, stained with 0.1% toluidine blue for 10 min, rinsed in water, then dehydrated and mounted. The number of mast cells and CD31-positive vessels were assessed in 8 animals per treatment group. For quantitation, 8 fields per section were counted at a magnification of 40x and the average calculated. The calculated values for 8 individual animals were again averaged to provide the final value for the group.

Statistical analysis

Numerical results reflect mean $\pm$ S.D or SEM, with the n indicated in each figure. Comparisons between groups were determined with two-tailed Student's t test. Statistical analyses were conducted and graphical representations of the data were plotted using GraphPad Prism 5 for Mac (Graphpad Software, Inc, La Jolla, CA), $p < 0.05$ was considered significant.

Results

COX-2 expression and localization in mammary glands and mammary tumors

To investigate whether COX-2 activation is involved in the pro-tumorigenic effects of IGF-1 *in vivo*, we first examined COX-2 expression levels in pre- and postpubertal mammary glands. As shown in Figure 1A & B, Western blot analysis revealed that COX-2 levels were significantly higher in mammary glands from Tg BK5.IGF-1 than in those from WT littermates. This effect was seen in both pre- and postpubertal mammary glands (Figure 1A & B). We also evaluated neoplasms from DMBA-treated Tg and WT mice. Expression of COX-2 was also significantly higher in mammary tumors from BK5.IGF-1 Tgs compared to those from WT mice (Figure 1C).

In order to identify the COX-2 expressing cells, immunofluorescent staining was performed on mammary tumor samples. As shown in Figure 2, COX-2 was expressed in ductal structures in Tg mammary carcinomas. Interestingly, COX-2 expression was found to co-localize with K5 expression, indicating that Tg IGF-1 stimulated COX-2 expression in an autocrine fashion in myoepithelial, but not in ductal epithelial cells, in this model.

PGE₂ levels in mammary glands

COX-2 enzymes are known to exert their effects through generation of eicosanoid products, of which the principal one is PGE₂ [28]. PGE₂ can contribute to tumor development through multiple mechanisms [29] and studies have shown that changes in the abundance of PGE₂ can influence the development of cancer [18,20]. To assess the impact of elevated COX-2 expression in mammary glands of BK5.IGF-1 Tg mice, PGE₂ levels were analyzed by ELISA. As shown in Figure 3, PGE₂ levels were significantly increased in both preand postpubertal Tg mammary glands, compared to those from age-matched WT, indicating activation of the COX-2/PGE₂ pathway by Tg IGF-1.

Expression of PGE₂ receptors in mammary glands

Cellular effects of PGE₂ are mediated through four G-protein-coupled receptors designated EP1-4, which activate specific intracellular signaling pathways, contributing to inflammation, tumorigenesis and metastasis [30,31]. Analysis of receptor expression in mammary glands from WT and Tg mice showed that EP3 mRNA was significantly increased in Tg glands from pre- and postpubertal mice (Figure 4A), although the levels in glands from postpubertal mice were lower. EP2 mRNA expression was significantly elevated in prepubertal Tg glands only (Figure 4D). In contrast, Tg IGF-1 did not affect EP1 or EP4 receptor mRNA levels in glands from either pre- or postpubertal mice (Figure 4C & E).

Western blot analysis of EP1-4 receptors confirmed, for the most part, our QPCR results. EP3 receptor protein expression was significantly elevated in glands from Tg pre- and postpubertal mice (Figure 4B). However, EP2 protein expression was not elevated in prepubertal Tg mammary glands although EP2 mRNA expression was elevated (data not shown). As with the message levels, protein levels of EP1 and EP4 receptors were not increased in Tg glands from either age group (data not shown).

Angiogenesis in mammary glands

PGE₂/EP3 receptor signaling has recently been shown to promote tumorigenesis by stimulating angiogenesis [32-34]. Therefore, we analyzed expression of a proangiogenic growth factor, VEGF and microvessel development in mammary glands from Tg and WT mice to evaluate the effects of EP3 activation. Results from Western blot analyses showed a significant increase in expression of VEGF in prepubertal Tg glands when compared to those from age-matched WT mice (Figure 5A). However, this increase was not observed in postpubertal Tg glands (Figure 5B). Interestingly, CD31 immunostaining was strikingly increased in postpubertal, but not prepubertal, Tg glands, compared to age-matched WT mice (Figure 5C). These observations demonstrate enhanced angiogenesis in glands from Tg mice and also reflect a difference in the timing of VEGF upregulation, which appeared to be an earlier event, and induction development of new vessels, which occurred later in this model.

Effects of celecoxib on COX-2 activity and mammary phenotype in IGF-1 Tg mice

To investigate whether COX-2/PGE₂ contributes to the phenotype seen in susceptible Tg mammary glands, we then treated female Tg mice with celecoxib, a selective COX-2 inhibitor. A daily dose of 100 mg/kg of celecoxib was tolerated well by all mice; there were no remarkable changes in external appearance, behavior and body weights in inhibitor-treated groups (data not shown).

Four weeks of daily oral administration of celecoxib treatment caused a 45% decrease in PGE₂ in Tg mammary glands (Figure 6A). When compared to vehicle-treated Tg glands, glands from celecoxib-treated mice had significant reductions in the number of mast cells and blood vessels (Figure 6B & C). These findings demonstrate that celecoxib effectively reduced PGE₂ levels and that the Tg mammary phenotype was mediated, at least in part, by IGF-1-induced activation of the COX-2/PGE₂/EP3 signaling pathway.

Discussion

It is now generally accepted that the major mechanism involved in IGF-1-mediated carcinogenesis is stimulation of proliferation and inhibition of apoptosis. However, recent studies in both cell cultures and animal models has indicated that the role of IGF-1 in cancer development is more complex and involves multiple pathways that ultimately generate procarcinogenic changes at the cellular, tissue and systemic levels [6]. Consistent with these findings, our results from previous studies in BK5.IGF-1 mice indicate that Tg IGF-1 is not acting as a classical, proliferative, post-carcinogen tumor promotion [7], indicating that other mechanisms are involved in the carcinogenic/co-carcinogenic effects in the mammary gland.

The current study investigated the influences of IGF-1 on COX-2 expression and signaling in Tg mammary tissues. Although COX-2 expression is induced by mitogens, inflammatory cytokines, or growth factors [35,36], the exact role of IGF-1 in regulating COX-2 expression *in vivo* remains incompletely understood. While the induction of COX-2 by IGF-1 has been described in some cell lines [22-24], there are also contradictory studies reporting that IGF-1 treatment fails to increase COX-2 expression [37,38], or even reduces its [39]. These contradictory results indicate that the effects of IGF-1 on COX-2 expression may be cell type or tissue specific.

In the BK5.IGF-1 model, we found that COX-2 expression was significantly and consistently increased in Tg mammary glands and tumors. However, COX-2 mRNA levels in mammary glands from either young or adult mice were not affected by Tg IGF-1 (data not shown), indicating that regulation of COX-2 expression was not at the level of transcription. Other investigators have shown that COX-2 regulation occurs at the level of

translation [40] or protein stability [41-43]. Our results indicate that COX-2 expression in Tg mammary glands was regulated at the level of protein turnover, but additional studies will be needed to fully elucidate this mechanism.

A previous study using the MMTV-COX-2 mouse model demonstrated that COX-2 overexpression alone is sufficient to induce mammary cancer [19]. Interestingly, some of the histopathologic changes found in the COX-2 Tg mice, such as alveolar and intraductal hyperplasia and dysplasia, ductal dilation and pro-tumorigenic stromal alterations [19,44] are similar to the mammary phenotype seen in our BK5.IGF-1 mice [7]. Thus, the present findings, showing a significant increase of COX-2 in mammary glands from Tg mice, indicate that activation of COX-2 signaling contributes to both the IGF-1 phenotype and the associated susceptibility to carcinogenesis.

We also found that COX-2 co-localized with a subpopulation of K5-expressing cells in IGF-1 Tg mammary tumors. Since transgenic IGF-1 is expressed by K5-positive cells and tumors are composed predominantly of K8-positive cells in this model [7], these findings indicate that IGF-1 induces COX-2 expression in an autocrine manner, resulting in increased synthesis of prostaglandins that favor pro-carcinogenic changes in the surrounding stroma.

COX-2 expression in mammary glands from BK5.IGF-1 mice resulted in increased PGE₂ production, which was detected in glands from both pre- and postpubertal animals. PGE₂ is generally considered as the principal downstream prostanoid regulated by COX-2, and many of the pro-tumorigenic effects of COX-2 are attributed to its role in catalyzing PGE₂ synthesis [20]. The upregulation of PGE₂ in the mammary glands from BK5.IGF-1 Tg mice further implicates COX-2/PGE₂ signaling in the effects of IGF-1 *in vivo*.

EP receptor expression studies indicate that among the four G-protein-coupled receptors, EP3 expression alone was elevated in mammary glands from Tg IGF-1 mice. The role of EP3 in tumorigenesis remains incompletely understood, but it has traditionally been linked to growth inhibition and cellular senescence [31,45]. Some studies show that EP3 is downregulated in colon, skin and mammary tumor cells [18,46,47]. In contrast, our analyses showed that EP3 expression was increased in Tg mammary glands at both the protein and message levels, implicating it as a mediator of the cellular effects of PGE₂ in susceptible mammary tissues. Similarly, Amano *et al.* demonstrated that EP3 receptor activation is required for lung tumor-associated angiogenesis and tumor growth [33]. Skin tumor latency is increased in EP3 KO mice and development of squamous cell carcinomas is inhibited in the absence of EP3 expression [48]. These contradictory results suggest that the role of EP3 in tumorigenesis may be variable and depend not only on the tissue and underlying carcinogenic mechanisms, but also on whether studies are conducted *in vitro* or *in vivo*.

EP2 receptor message was upregulated in prepubertal Tg glands without accompanying changes in protein. Inconsistency between QPCR and Western blot analyses of EP receptor expression has been reported previously. In fetal baboon lungs, betamethasone exposure resulted in a twofold decrease in EP2 mRNA that was not accompanied by changes in EP2 receptor protein expression [49]. Similarly, Tg IGF-1 did not increase EP1 and EP4 protein levels in glands from pre- or postpubertal mice, indicating that these receptors were not critical mediators in the pro-tumorigenic effects of IGF-1 in this model. However, it cannot be ruled out that these EP receptors play a role in the progression of IGF-1-associated mammary tumors.

The importance of PGE₂/EP3 signaling is demonstrated by the fact that loss of EP3 is accompanied by a markedly reduced tumor-associated or wound-induced angiogenesis [33,34]. Analyses of the mammary glands from BK5.IGF-1 mice showed that the proangiogenic growth factor, VEGF was also elevated in prepubertal Tg glands.

Interestingly, VEGF expression was upregulated prepubertally, while increased microvessel density was observed postpubertally. This delay may reflect the fact that VEGF-mediated angiogenesis is a multistep process requiring hours, days or weeks, depending on the specific tissue and stimulus[50,51]. The current findings also provide support for the contention that COX-2-stimulated angiogenesis may contribute to tumor susceptibility at a much earlier stage than previously recognized, i.e. before macroscopic tumors are detected [18,52]. Chang, *et al.* showed that the angiogenic capacity of preneoplastic mammary lesions predicts subsequent transformation in both murine and human systems [18]. Therefore, these results indicate that the angiogenic effects of IGF-1 are mediated, at least in part, by COX-2/PGE/EP3/VEGF signaling and identify an alternate and early mechanism by which IGF-1 stimulates mammary tumorigenesis *in vivo*.

COX-2/PGE₂ signaling also modulates inflammatory and immune responses [29,53]. A previous study showed that PGE₂, acting via the EP3 receptor, is a chemotactic factor for mast cells [54]. Recent studies indicate that mast cells contribute to angiogenesis, modulate the immune response and participate in pro-tumorigenic remodeling of the stroma [55]. We previously observed and reported an influx of mast cells in mammary glands of BK5.IGF-1 Tg mice [7]. Results presented here show that celecoxib treatment significantly decreased the number of mast cells in Tg glands, providing another possible mechanism underlying the effects of COX-2 in response to IGF-1 overexpression.

In conclusion, our data show that overexpression of IGF-1 induces COX-2 expression in mammary glands, resulting in increased synthesis of PGE₂ that favors pro-carcinogenic changes in the surrounding stroma. Since IGF-1 is a known breast cancer risk factor and COX-2 inhibitors are gaining acceptance as chemopreventive and therapeutic agents (reviewed in [56,57]), our findings help clarify the relationship between IGF-1 and COX-2 in mediating mammary tumor susceptibility *in vivo*. Additionally, these results may have rapidly translatable applications for breast cancer management or prevention by allowing better stratification of patients and/or identification of women at increased risk.

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Abbreviations

BCL-2	B-cell lymphoma 2
BK5	bovine keratin 5
DMBA	7,12-dimethylbenz[<i>a</i>]anthracene
EP	PGE ₂ receptor
IGF-1	insulin-like growth factor-1
KO	knockout
MMTV	mouse mammary tumor virus
PG	prostaglandin

QPCR	quantitative PCR
RIPA	RadioImmunoPrecipitation Assay
TBP	TATA box binding protein
Tg	transgenic
WAP	whey acidic protein
WT	wild type

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Figure 1.

COX-2 expression in mammary glands and tumors from WT and BK5.IGF-1 Tg mice. Protein expression levels of COX-2 in whole prepubertal or postpubertal (A and B) mammary glands and mammary tumors (C) from WT and BK5.IGF-1 Tg mice were examined by Western analysis. GAPDH was used as a loading control. Numbers of samples analyzed are noted within each bar. Representative blots of 3 different samples per group are shown. Values are expressed as mean $\pm$ S.D. * indicates a p value of < 0.05 as determined by Student's t test.

Figure 2.

Location of COX-2-expressing cells in mammary tumors from BK5.IGF-1 Tg mice. Double-immunofluorescent staining with anti-COX-2 (green) and anti-K5 (red, marker of myoepithelial cells) were performed on mammary tumors from BK5.IGF-1 Tg mice as described in Methods. COX-2 and K5 signals co-localized, as indicated by yellow fluorescence. The far images are of DAPI staining, which detects nuclei. Original magnification, 40x.

Figure 3. PGE₂ expression levels in mammary glands from pre- and postpubertal WT and BK5.IGF-1 Tg mice. PGE₂ levels were quantified using ELISA. Numbers of samples analyzed are noted within each bar. Values are expressed as mean ± S.D. * indicates a *p* value of < 0.05 as determined by Student's *t* test.

Figure 4.

Expression levels of EP receptors in mammary glands from pre- and postpubertal WT and BK5.IGF-1 Tg mice. **A.** QPCR analysis of EP-3 mRNA expression in prepubertal or postpubertal mammary glands from WT and BK5.IGF-1 Tg mice. WT and EP3 KO mouse pancreas were used as positive or negative controls, respectively. Numbers of samples analyzed are noted within each bar. All mRNA levels were normalized to the expression of TBP mRNA of the same sample. The values are expressed as mean $\pm$ SEM. * indicates a p value of < 0.05 as determined by Student's t test. **(B)** Western blot analysis of EP3 protein levels in mammary glands from pre- and postpubertal WT and BK5.IGF-1 Tg mice.

GAPDH was used as a loading control. An extract from mammary tumors of BK5.IGF-1 Tg mice was included as a positive control in right panels. Numbers of samples analyzed are noted within each bar. Representative images of 3 different samples per group are shown. Values are expressed as mean $\pm$ S.D. * indicates a p value of < 0.05 as determined by Student's t test. nd= not detectable. (C) QPCR analysis of EP1 (left panel), EP2 (middle panel) and EP4 (right panel). Number of analyzed samples is indicated within each bar. All mRNA levels were normalized to the expression of TBP mRNA of the same sample. The values are expressed as mean $\pm$ SEM. * indicates a p value of < 0.05 as determined by Student's t test.

Figure 5. Angiogenesis in mammary glands from pre- and postpubertal WT and BK5.IGF-1 Tg mice. Expression levels of VEGF in prepubertal (A) or postpubertal (B) mammary glands from WT and BK5.IGF-1 Tg mice were examined by Western analysis. GAPDH was used as a loading control. Numbers of samples analyzed are noted within each bar. Representative blots of 3 different samples per group are shown. Values are expressed as mean $\pm$ S.D. * indicates a p value of < 0.05 as determined by Student's t test. (C) Immunohistochemical staining with anti-CD31 in paraffin-embedded pre- and postpubertal glands from WT and Tg mice. Original magnification, $\times 20$.

Figure 6.

Effect of celecoxib treatment on PGE₂ levels and mammary phenotype in BK5.IGF-1 Tg mice. Female IGF-1 Tg mice were treated with daily oral gavage of celecoxib at a dose of 100 mg/kg, 5 days per week for 4 wks, starting on the day of weaning. (A) PGE₂ levels, in Tg mammary glands, were determined by ELISA following treatment with celecoxib or vehicle. (B) Toluidine blue staining showing mast cell infiltration in the mammary glands of celecoxib-treated and vehicle-treated Tg mice. Quantitation, as described in the Methods, is shown the right panel. (C) CD31 immunostaining of vehicle- and celecoxib-treated Tg glands. Quantitation, as described in the Methods, is shown the right panel. The number of samples analyzed is noted within each bar. Values are expressed as mean $\pm$ S.D. * indicates a p value of < 0.05 as determined by Student's t test. Original magnification in B and C, 20x.