COX-2 mediates tumor-stromal prolactin signaling to initiate tumorigenesis

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Significance

The documented efficacy of COX-2 inhibitors in cancer chemoprevention and in suppression of metastasis is predominantly attributed to inflammatory responses, whereas their effects on tumor-stromal interaction are poorly understood. Through single-cell transcriptome analyses in an immune-compromised mouse xenograft model and in vitro reconstitution experiments, we uncover a tumor-stromal paracrine pathway in which secretion of prolactin by the COX-2 product prostaglandin E2 enhances prolactin synthesis by breast stroma, which activates prolactin receptor expression. Analysis of multiple human cancers confirms differential tumor and stromal cell expression of COX-2, prolactin, and prolactin receptor. Together, these findings may provide novel biomarkers to inform the selective application of COX-2 inhibitors and point to additional targets for suppressing metastasis recurrence.

Tumor-stromal communication within the microenvironment contributes to initiation of metastasis and may present a therapeutic opportunity. Using serial single-cell RNA sequencing in an orthotopic mouse prostate cancer model, we find up-regulation of prolactin receptor as cancer cells that have disseminated to the lungs expand into micrometastases. Secretion of the ligand prolactin by adjacent lung stromal cells is induced by tumor cell production of the COX-2 synthetic product prostaglandin E2 (PGE2). PGE2 treatment of fibroblasts activates the orphan nuclear receptor NR4A (Nur77), with prolactin as a major transcriptional target for the NR4A-retinoid X receptor (RXR) heterodimer. Ectopic expression of prolactin receptor in mouse cancer cells enhances micrometastasis, while treatment with the COX-2 inhibitor celecoxib abrogates prolactin secretion by fibroblasts and reduces tumor initiation. Across multiple human cancers, COX-2, prolactin, and prolactin receptor show consistent differential expression in tumor and stromal compartments. Such paracrine cross-talk may thus contribute to the documented efficacy of COX-2 inhibitors in cancer suppression.

COX-2 | prolactin | NR4A | tumor-stromal communication | metastasis

Invasive localized cancers may shed cells into the bloodstream, circulating tumor cells (CTCs), which become trapped in capillary beds within multiple distant organs, ultimately triggering metastatic disease (1, 2). These disseminated tumor cells may persist as nonproliferative single cells within tissues for prolonged periods of time before a subset initiates proliferation and triggers metastatic recurrence. Early microenvironmental signals that support the initiation of proliferation by disseminated cancer cells are poorly understood. Mouse models of tumor dissemination have pointed to multiple growth suppressive–secreted factors such as TGF-β, BMP, and TSP-1 (3–6), as well as a role for immune surveillance (7, 8), in preventing tumor cell outgrowth. However, early tumor cell growth-enhancing signals are not well defined. Identifying such pathways may support the application of “metastatic chemoprevention,” particularly in cancers that have a long latency before metastatic recurrence.

Microenvironmental signals that modulate the initial proliferation of a single disseminated cancer cell may be shared by single cells that have undergone transforming genetic events within primary tissues (9). Supporting this notion are clinical studies documenting the effectiveness of COX-2 inhibitors in suppressing both cancer initiation and metastatic recurrence. Both randomized controlled clinical trials and large observational epidemiological studies have demonstrated that the COX-2–selective inhibitor celecoxib, as well as nonsteroidal antiinflammatory drugs (NSAIDs) inhibiting both COX-1 and COX-2, reduces the risk of developing multiple cancers (10–13). Initial studies demonstrated a marked reduction in the development of adenomatous precancerous polyps in carriers of familial polyposis receiving COX-2 inhibitors (11). Subsequent trials in individuals at general population risk for developing cancer demonstrated reductions in multiple invasive malignancies, including colorectal and lung cancers, as well as potential reductions in prostate cancer (11, 14–18). In these studies, efficacy in cancer prevention required at least 5 y of daily treatment and cancer risk reduction was maintained for up to 20 y posttreatment (16). In addition to primary chemoprevention, meta-analyses also indicate significant effects of COX-2 inhibitors in reducing
development of metastases following resection of a primary cancer, an effect demonstrated for colorectal and prostate cancers (19). Interestingly, in colorectal cancer, the chemopreventive effects are most striking in reducing tumors that express COX-2 (20) and have mutations in PIK3CA (21), which increase phosphatidylinositol 3-kinase/Akt activity, a known modulator of COX-2-dependent signaling.

The general antiinflammatory effect of NSAIDs and COX-2 inhibitors has led to the assumption that their chemopreventive action may reflect a role for inflammation in enhancing early tumorigenesis. However, a more precise understanding of tumor-stroma–related mechanisms underlying COX-2 cancer chemoprevention is key to try to distinguish potentially beneficial tumor-suppressive pathways from the more global effect of COX-2 inhibitors. Indeed, despite promising epidemiological studies, cancer chemoprevention trials using the COX-2 inhibitor celecoxib were terminated upon the discovery that it also increases the risk for cardiac events, a complication that outweighs its potential benefit in healthy individuals with low cancer risk (22).

The pleiotropic effect of the COX-2 synthetic product prostaglandin E2 (PGE2) on multiple proliferative thrombotic and inflammatory pathways presents a major challenge. This may be addressed, in part, by dissecting the PGE2 pathways that directly modulate tumorigenesis and directing inhibitors to patients at high risk of metastatic relapse, where targeting these pathways may have a more favorable risk/benefit profile.

In pursuing an orthotopic mouse prostate cancer model in which CTCs disseminate to distant organs and persist for weeks as nonproliferative single cells before initiating metastatic proliferation, we identified a pathway involving tumor-stromal interaction linking COX-2 to prolactin signaling. We describe a tumorigenesis-enhancing pathway, whereby cancer cells expressing COX-2 secrete PGE2, which, in turn induces secretion of prolactin by stromal fibroblasts. Up-regulation of prolactin receptor by disseminated cancer cells that are initiating proliferation completes a paracrine loop. The potent inhibition of PGE2 synthesis by celecoxib, independent of its effects on immune responses, abrogates this tumor-stromal cross-talk, and may contribute to the documented cancer-suppressive effects of COX-2 inhibitors.

Results

Single-Cell RNA Sequencing of Individual Cancer Cells and Micrometastases in the Lungs. We generated primary orthotopic tumors by inoculation of GFP-luciferase–tagged mouse prostate cancer cells derived from tissue-specific inactivation of Pten (CE1-4) (23) into the prostate gland (henceforth, prostate) of immunosuppressed NSG mice (Fig. 1 D and SI Appendix, Fig. S1 A). These primary mouse tumor-derived cell lines recapitulate androgenic and epithelial features of primary human prostate cancer, and they display broad metastatic potential following orthotopic injection of 1 million cells into the mouse prostate (23). Proliferation of tumor cells within the prostate is evident by live imaging within 2 wk (SI Appendix, Fig. S1A), and after 6 wk, single tumor cells (STCs) are identified microscopically within multiple tissues, including the lungs (mean = 394 cells per high-power field (hpf)), liver (mean = 54 cells per hpf), bone marrow (mean = 9 cells per hpf), and brain (mean = 1 cell per hpf) (Fig. 1 D and SI Appendix, Fig. S1 B and C). Only 2.2 ± 2.0% (four of 184) of STCs detected at this early time point (6 wk; STC6) are positive for the proliferation marker Ki67, and no micriclellular lesions are identified in the lung, the most accessible organ for detailed analysis (Fig. 1 D and SI Appendix, Fig. S1D). However, in STCs at 9–11 wk (STC9–11) after prostate inoculation, the Ki67-positive proliferative fraction of single cancer cells in the lungs increases to 10.8 ± 3.1% (21 of 195 cells) and rare lesions (<0.1% of all scored events) contain 20 or more cells (Fig. 1 D and SI Appendix, Fig. S1D). Within these early micrometastases, the proliferative Ki67-positive cellular fraction is 38.2 ± 6.4% (124 of 325 cells) (Fig. 1 D and SI Appendix, Fig. S1D). This orthotopic mouse tumor model thus provides a model for time-dependent initiation of metastatic growth.

While single cancer cells isolated from the lungs at the early 6-wk time point are negative for markers of cell proliferation, they are highly proliferative in vitro when collected by trypsin treatment of the whole organ and incubated in standard tissue culture medium. The s.c. inoculation of 106 cells from such in vitro-expanded cultures, derived from cancer cells that were nonproliferative in vivo, readily produces tumors in 16 of 16 NSG mice (SI Appendix, Fig. S1E). Thus, when presented with different environmental signals, apparently quiescent cancer cells that had disseminated to the lungs are competent to proliferate in vitro, and subsequently initiate tumors in vivo.

To trace temporal changes in transcription during early metastatic initiation, we undertook single-cell RNA sequencing (RNA-Seq) of GFP-tagged tumor cells in different tumor compartments: primary cancer cells (n = 29), CTCs isolated by microfluidic capture (24) from blood specimens (n = 12), and individual tumor cells collected at 6 wk (STC9–11; n = 20) and at 9–11 wk (STC9–11; n = 55). We also isolated the multicellular micrometastatic lesions evident at 9–11 wk, subjecting these to cell dissociation and single-cell RNA-Seq (n = 33) (Dataset S1). Transcriptional profiles of these 149 single cells are shown in Fig. 1B. Primary tumor cells are noteworthy for hypoxic and glycolytic signatures (SI Appendix, Fig. S2 A–C), while one early metastatic lesion (Met1) shows up-regulation of epithelial genes and a second (Met2) has high expression of the growth factor binding proteins IGFBP5 and IGFBP7 (SI Appendix, Fig. S2 D–F).

The orthotopic mouse tumor model that we established does not allow for resection of the primary tumor; hence, we could not directly trace the evolution of lung micrometastases from initial STCs. The s.c. inoculation of the primary tumor, which does allow for tumor resection, only produced a very small number of STCs, precluding a detailed transcriptional analysis. Thus, we applied a timeline from orthotopic tumor cell inoculation to dissect pathways potentially involved in the emergence of early proliferation among cancer cells disseminated to the lungs. Compared with the homogeneous pattern observed in the universally nonproliferative cells in the lungs at 6 wk following inoculation, single cancer cells isolated from the lungs at 9–11 wk were heterogeneous, and contained two recurrent pathway clusters (gene set enrichment analysis): a proliferative pathway associated with both G1/S and G2/M cell cycle signatures and a pathway involved in cytokine signaling (Fig. 1 C–E and SI Appendix, Fig. S3 A–C). The emergence of a proliferative signature as STCs transition into micrometastases is consistent with the analysis of Lawson et al. (25) and with the increased Ki67 staining fraction evident in single cancer cells isolated at the 9- to 11-wk time point (Fig. 1 D and SI Appendix, Fig. S1 B–D). The cytokine signaling signature was confirmed through a hypergeometric pathway analysis (13 of 16 BioCarta pathways, with a false discovery rate of <0.2) (SI Appendix, Fig. S3D), and it was selected for further analysis since it represents an unexpected association for early progression of cancer cells disseminated to the lungs.

Induced Expression of Prolactin Receptor Increases Micrometastases in the Lungs. Eighteen cytokine and growth factor receptors passed the statistical threshold for differential expression between single cancer cells isolated at the 6-wk versus 9- to 11-wk time point, with Prolactin receptor (Prlr) being the most abundant (fourfold median induction, grouping 9- to 11-wk single cells and micrometastases compared with 6-wk single STCs and primary tumor cells; P = 1.3e−5, Benjamini–Hochberg ( test) (Fig. 2 A and SI Appendix, Fig. S3E). Primary tumor cells express low levels of Prlr [mean = 224 reads per million (RPM), range: 0–1,796 RPM], as do 6-wk single cancer cells (mean = 325 RPM, range: 0–2,099 RPM). In contrast, 9- to 11-wk single cancer cells express higher levels of Prlr (mean = 679 RPM, range: 0–8,199 RPM), as do micrometastatic cells (mean = 982 RPM, range: 0–5,441 RPM). The fraction of tumor cells expressing >500 RPM of Prlr increases from 17.2% (five of 29)
in the primary tumor and 20.0% (four of 20) in 6-wk single cancer cells to 34.5% (19 of 55) in 9- to 11-wk single cancer cells and 48.5% (16 of 33) in micrometastasis cell populations, a trend evident in all four independent mice analyzed (Fig. 2B). Consistent with RNA-Seq, Prlr protein expression, detected by immunofluorescence (IF), is evident within lung metastases evaluated from multiple mouse models established with three independent Pten-null prostate tumor lines (CE1-4, CE1, and CE2) but not in normal lungs (Ctrl) (Fig. 2C). As expected, RNA-Seq reveals considerable heterogeneity among single cells, consistent with evolution of both the primary tumor and its metastatic derivatives.

The role of prolactin signaling in tumorigenesis is poorly understood, and given the predominant association between early STC proliferation and Prlr expression, we selected this pathway for further analysis. To first test the functional consequences of Prlr expression, we generated doxycycline (Dox)-inducible Prlr expression in mCherry-tagged CE1-4 cells, which demonstrate Prl-induced activation of downstream signaling (Fig. 2D). No such signaling is observed following Dox treatment in the absence of exogenous Prl ligand. Induction of Prlr in the presence of ligand has a modest inhibitory effect on cell proliferation in vitro (Fig. 2E), and CE1-4 cells expressing the receptor generate primary orthotopic tumors of smaller weight, compared with matched noninduced controls.
Prolactin receptor signaling induces metastatic outgrowth. (A) Scatter plot showing the median single-cell RNA-Seq expression for cytokine and growth factor receptors (statistical threshold: \( p < 0.05 \)) versus log-twofold change between STCs collected from the primary tumor and lungs after 6-wk orthotopic inoculation (STCa) versus 9–11-wk orthotopic inoculation (STCa-11) and micrometastases. Prlr is the most abundant differentially expressed receptor. (B) Scatter plot showing single-cell RNA-Seq expression of Prlr in dissociated primary tumor cells, STCs in the lungs after 6 wk (STC n = 20 cells) in Dox-induced (\( \leq 3 \text{ microparticles} \)) versus 8–9-wk orthotopic inoculation (STC n = 20 cells) in uninduced controls; \( * \) statistical significance \( p < 0.05 \), two-tailed Student \( t \) test). (C) Representative IF images showing the expression of Prlr in lung metastases from three prostate cancer orthotopic mouse models expressing Prlr. (D) Western blot analyses showing Dox-inducible expression of Prlr in engineered CE1-4 murine prostate tumor cells and activation of its downstream STAT5 signaling following treatment with 100 ng/mL mouse Prl for 5 or 15 min. (E) In vitro growth curves of CE1-4 cells cultured in the presence or absence of Dox to induce Prlr expression, with or without addition of Prl. A modest antiproliferative effect is evident in cells expressing Prlr and treated with Prl (**\( p < 0.01 \), two-tailed Student \( t \) test). V, vehicle. (F) Schematic representation of Dox-inducible expression of Prlr within prostate tumors derived from orthotopic injection of CE1-4 cells. In the presence of Dox, Prlr is detectable by RNAISH within the primary tumor cells, as well as within small micrometastases in the lungs. (Scale bars, 50 \( \mu \)m.) (G) Bar graph shows reduced size of the primary tumor following the induction of Prlr expression with Dox (+), compared with V-treated controls (–) (\( * p < 0.05 \), two-tailed Student \( t \) test). (H) Bar graph shows quantitation of lung tumor foci across different size categories (one to five cells, six to 20 cells, >20 cells) in Dox-induced (+) versus V-treated (–) mice 6 wk after tumor inoculation (\( n = 3 \) mice per group with at least 10 fields quantified per animal). Post hoc power of 0.16 (**\( p < 0.001 \), ***\( p < 0.01 \); two-tailed Student \( t \) test). n.s., not significant.
Pten-deficient mouse prostate tumor cell lines whose CM either induces [parental CE1-4 and its subclonal lines 3, 4, and 5] or does not induce [subclonal lines 1 and 2] PRL expression by normal human lung fibroblasts (Fig. 3E). Using RNA-Seq, we defined the secretome of these prolactin-inducing versus non-prolactin-inducing cancer cell lines, identifying 40 differentially expressed secretion-related gene products: The top two transcripts highly correlated with PRL induction were COX-2 [prostaglandin-endoperoxide synthase 2 (PTGS2)] and mesothelin (Msln) (Fig. 3F and SI Appendix, Fig. S5D). Treatment of fibroblasts with purified Msln has no effect on PRL expression, whereas the synthetic product of COX-2, PGE2, shows a dramatic dose-dependent (fourfold to eightfold) induction of PRL, recapitulating the effect of CE1-4 tumor cell-derived CM (Fig. 3G). Moreover, the COX-2 inhibitor celecoxib, which blocks synthesis of PGE2 (26), abolishes the ability of CM from CE1-4 cells to induce PRL in human cultured fibroblasts (Fig. 3H). Thus, PGE2 is the main component within prostate cancer cell CM that mediates the induction of PRL by cultured fibroblasts.

**PGE2InducesOrphanNuclearReceptor4A-RetinooidXReceptor-MediatedTranscriptionalActivationofProlactininFibroblasts.** To identify potential mechanisms underlying PGE2-mediated induction of prolactin, we performed both RNA-Seq and genome-wide H3K27-acetylation (H3K27ac) chromatin immunoprecipitation sequencing (ChIP-Seq) using human fibroblasts treated with PGE2 for 6 h. A higher than threefold increase in H3K27ac, a marker associated with increased promotor and enhancer activity (27, 28), is observed at 293 sites across the entire genome, primarily at intragenic sites (45.7% and 51.5% respectively), with a smaller fraction at transcriptional start sites (2.7%) (Fig. 4A and B; n = 2 independent experiments). RNA-Seq of PGE2-treated fibroblasts identifies 162 transcripts up-regulated greater than twofold, compared with untreated controls (Fig. 4C). Remarkably, in this unbiased genome-wide screen for PGE2 targets, the PRL gene is the top hit at the intersection of the RNA-Seq and ChIP-Seq datasets, with 17.19-fold RNA induction after 6 h of PGE2 treatment and dramatic H3K27ac in the gene promoter (Fig. 4D and E). Treatment of CE1-4 cancer cells with PGE2 shows no such induction in the PRL transcript, consistent with an effect specific to stromal fibroblasts (SI Appendix, Fig. S6A).

Motif enrichment analysis across all 293 newly acquired H3K27ac sequences identifies consensus DNA binding sites for the orphan nuclear receptor 4A (NR4A) family (29–31) as by far the most significant (P = 1e-77: Fig. 4F). Remarkably, NR4A binding sites account for 68.6% of all new genome-wide H3K27ac sites acquired following PGE2 treatment (Fig. 4F), suggesting that it may constitute the major transcriptional effect of prostaglandin signaling in fibroblasts. The NR4A gene family is composed of three members with a high degree of...
homology and shared DNA consensus binding sites (29, 30); expression of each family member, NR4A1, NR4A2, and NR4A3, is induced at least twofold upon PGE2 treatment of fibroblasts (SI Appendix, Fig. S6B). These findings are consistent with the modulation of NR4A expression by PGE2 in colorectal cancer cells (32). Analysis of a genome-wide NR4A ChIP-Seq database (33) confirms binding to this specific consensus sequence within the PRL promoter (SI Appendix, Fig. S6C). Of note, the striking PGE2-induced deposition of H3K27ac in the PRL promoter resides within a potential D5R binding site for the NR4A-RXR heterodimer (Fig. 4). This binding motif is confirmed in the NR4A consensus sequence that is separated by five bases from the known NR4A dimerization partner RXR (36) (Fig. 4). Unlike NR4A, RXRa and RXRβ expression is not induced by PGE2 treatment in fibroblasts (RXRβ is not expressed in these cells by RNA-Seq) (SI Appendix, Fig. S6D). Interestingly, NR4A has been linked to expression of prolactin in inflamed joints and in the uterus (37, 38), and our findings now suggest that NR4A-RXR heterodimers constitute key intermediates in the PGE2-mediated induction of prolactin expression in fibroblasts.

To test this model, we first used lentiviral-encoded shRNA constructs to knock down NR4A gene family members and RXRα in human fibroblasts. Knockdown of NR4A1, using either of two shRNA constructs (C8 and C9), suppresses PGE2-mediated PRL induction; individual constructs targeting either NR4A2 (D7 and D9) or NR4A3 (G5 and G7) abrogate both baseline and PGE2-induced expression of PRL (Fig. 4H). While the seed sequences targeting each NR4A transcript are unique (SI Appendix, Fig. S6E), coregulation of gene family members appears evident following knockdown of individual genes (SI Appendix, Fig. S6F), suggesting that all gene family members contribute to PRL regulation. Knockdown of RXRα (F4 and F5) also dramatically suppresses baseline and PGE2-induced PRL expression, consistent with a functional role for the NR4A-RXR heterodimer (Fig. 4I and SI Appendix, Fig. S6F). We also tested the effect of well-characterized small-molecule inhibitors of NR4A1 [Dim-c-pPhCo2Me (39)] and RXRα [HX531 (40)]. Treatment of fibroblasts with either drug suppresses PGE2-mediated induction of PRL (Fig. 4I). Thus, an unbiased genome-wide screen for all transcriptional and promoter/enhancer (H3K27ac) changes induced by treatment of human fibroblasts with PGE2 identifies PRL as a lead transcriptional target, providing independent and orthogonal evidence in support of the PGE2-prolactin pathway uncovered through single-cell analyses in our mouse prostate model (Figs. 1–3). Furthermore, activation of NR4A binding sites is the predominant genome-wide transcriptional consequence of PGE2 treatment in human fibroblasts, and suppression of the NR4A-RXR heterodimer, through either knockdown or small-molecule inhibition, abrogates prolactin induction by PGE2 in these cells.

**Tumor-Stromal Expression Patterns of COX-2, Prolactin, and Prolactin Receptor in Primary Human Cancers.** The tumor-stromal paracrine signaling involving PGE2, prolactin, and prolactin receptor is evident in multiple independently isolated mouse primary prostate-tumor-derived cell lines generated following in vivo Pten deletion, and it is also recapitulated in the induction of prolactin by PGE2 in cultured human fibroblasts. To test whether compartmentalized prolactin–prolactin receptor signaling is shared across other tumor types, we used arrayed primary tissue sections

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**Fig. 4.** PGE2-mediated activation of prolactin in fibroblasts is mediated by NR4A-RXR heterodimers. (A) Composite plot showing genome-wide H3K27ac ChIP-Seq signals in control (black) and PGE2-treated dermal fibroblasts (DFs; orange). Data represent two biological replicates. The x axis represents a 10-kb window centered on increased H3K27ac sites in PGE2-treated cells (10 ng/mL, 6 h; n = 293 sites). (B) Distribution of increased H3K27ac sites in PGE2-treated DFs among transcriptional start sites (TSS) and intragenic and intergenic genomic loci. Promoters are annotated using the RefSeq promoter database. (C) Venn diagram comparing genes with greater than threefold increased H3K27ac activation peaks (ChIP-Seq) and those with greater than twofold up-regulated expression (RNA-Seq) in DFs after 6 h of PGE2 treatment. The 293 H3K27ac sites enriched after PGE2 treatment correspond to 265 unique genes. (D) Six genes at the intersection of the ChIP-Seq and RNA-Seq datasets are listed, with prolactin (PRL) as up-regulated in the RNA-Seq dataset. (E) ChIP-Seq data showing increased H3K27ac modification in the promoter region of the PRL gene, 6 h after PGE2 treatment of DFs. (F) Motif analysis of sites with increased H3K27ac following PGE2 treatment. The motif recognized by NR4A family members (Gene Expression Omnibus accession no. GSM77637) is the most highly enriched and accounts for the majority of sites marked by H3K27ac. (G) Nucleotide sequences between the two H3K27ac peaks in the promoter of PRL contain a potential DR5 binding site for the NR4A-RXR heterodimer. (H) Suppression of PRL mRNA induction in DFs by PGE2, following infection with shRNA constructs targeting NR4A1 (C8 and C9), NR4A2 (D7 and D9), NR4A3 (G5 and G7), RXRα (F4 and F5), or scrambled control (shSCR). Baseline and PGE2-induced mRNA levels are shown relative to the full induction with shSCR (qPCR). Knockdown efficiency of the knocked-down constructs is shown by ChIP-Seq signals in control (black) and PGE2-treated dermal fibroblasts (DFs; orange). Data represent two biological replicates. The two H3K27ac peaks in the promoter of PRL are marked in orange. (I) Small-molecule inhibitors suppress PGE2-mediated induction of PRL mRNA. Dim-c-pPhCo2Me (10 μM) abrogates NR4A1 (39), while HX531 (1 μM) suppresses RXRα (40). Cells were treated for 18 h before PGE2 exposure. Bars represent the average of at least two biological replicates, normalized to the control-PGE2-treated expression for comparison among experiments. Error bars are 1 SD (**P < 0.01, two-tailed Student t test; individual comparison with shSCR + PGE2).
(prostate, colorectal, and lung cancers) to quantify expression of COX-2 (PTGS2), prolactin, and prolactin receptor in human clinical specimens. We used either immunohistochemistry (IHC; Protein Atlas database (41)) staining or quantitative RNA-in situ hybridization (ISH) (branched chain technology RNA-ISH; Advanced Cell Diagnostics, Inc.). High-intensity COX-2 expression is detectable by IHC in the tumor compartment of 12 of 12 prostate cancers (100%; one specimen with low signal (10–30% positive cells) and 11 specimens with high signal (>60% positive cells)), 11 of 11 colorectal cancers (100%; all with high signal), and eight of 12 lung cancers (66.7%; two with low signal, one with intermediate signal (31–59% positive cells), and five with high signal). In all cases, expression of COX-2 is exclusively localized to tumor cells (Fig. 5 and SI Appendix, Fig. S7 A and B).

PRL mRNA expression has been reported in unfractionated bulk human prostate tumors (42), but using microscopic imaging, we find expression to be largely restricted to the stromal cell compartment; PRL expression is detectable by IHC in 77 of 100 primary and metastatic prostate cancers (77%; 13 with low signal, 15 with intermediate signal, and 49 with high signal), in 24 of 58 colorectal adenomas and cancers (41.4%; 12 with low signal, six with intermediate signal, and six with high signal), and in eight of 11 non-small cell lung cancers (72.7%; three with low signal, two with intermediate signal, and three with high signal) (Fig. 5 and SI Appendix, Fig. S7 A and B). Since expression of the prolactin receptor in human cancers is not well established, we used quantitative RNA-ISH, demonstrating expression of the PRLR transcript within tumor cells, but not neighboring stromal cells, in tissue arrays of human primary and metastatic prostate cancers [24 of 89 cases (27%): 11 with low signal, four with

![Fig. 5.](https://www.proteinatlas.org) Localization of COX-2–prolactin signaling components in multiple human cancers. (A) Representative images and quantitation of COX-2 (PTGS2) expression in 20 prostate and 24 colorectal human cancers using IHC (proteinatlas.org), with high-magnification images demonstrating expression in the tumor cell compartment. Representative images and quantitation of prolactin (PRL) expression in 12 prostate and 12 colorectal human cancers using IHC (https://www.proteinatlas.org), with high-magnification images showing expression in the stromal compartment. Representative images and quantitation of prolactin receptor (PRLR) are shown in 66 prostate and 32 human colorectal cancers, detected using RNA-ISH using the ACD probe against human PRLR (542558). S, stromal compartment; T, tumor. (Insets) High-magnification images demonstrate transcripts limited to the tumor cell compartment. (Magnification: 3×.) Scale bars, 20 μm. Additional tumor types are shown in SI Appendix, Fig. S7 A. (B) Bar graphs showing the percentage of human tissue samples positive for COX-2 (PTGS2) and PRL by IHC or for PRLR by RNA-ISH, divided into categories of low, medium, and high signal. Samples were considered positive if at least 10% of cells in the appropriate compartment (tumor or stromal) of the section stained positive (10–30% of cells stained was considered low signal, 30–60% of cells stained was considered medium signal, and >60% of cells stained was considered high signal). Tissue samples examined are primary tumors. For PRL staining, data from this study (n = 67) and The Human Protein Atlas (n = 12) were combined (41).
Functional consequences of Cox-2 inhibition in a prostate mouse model. (A) Representative fluorescent whole-mount images of mouse tibia and IHC staining of mCherry-labeled tumor cells from vehicle (V)- or celecoxib (CEL)-treated mice (24-h pretreatment followed by daily treatment with CEL (25 mg/kg, oral) for 8 wk [n = 6 mice (12 tibias) per treatment]). (Scale bars, 200 μm.) (B) Bar graph showing the occurrence of histologically confirmed tumors in V- versus CEL-treated mice (***P < 0.001, Fisher’s exact test; odds ratio = 0.045). (C) Schematic model illustrating the paracrine signaling network involving PGE2, NR4A-RXR, prolactin, and prolactin receptor in the initiation of tumor growth. PGE2 secretion by cancer cells, through activation of NR4A-RXR binding to PRL gene regulatory sequences, induces the production and secretion of prolactin by neighboring stromal fibroblasts. Prolactin, in turn, activates signaling through the prolactin receptor whose expression is up-regulated in tumor cells, thereby promoting their proliferation. This pathway is interrupted by Cox-2 inhibitors (e.g., CEL), which abrogate the initial production of PGE2 in tumor cells.


discussion

Our data suggest a model whereby secretion of PGE2 by tumor cells induces expression of NR4A family members within neighboring fibroblasts. NR4A-RXR heterodimers bind to the PRL promoter, inducing its expression and secretion by fibroblasts. Prolactin then activates signaling in single cancer cells that appear to recapitulate PGE2-dependent signaling and in the setting of very early tumor initiation from a small cancer cell inoculum, Cox-2 inhibition efficiently suppresses the initiation of tumorigenesis in vivo. We next tested the consequences of disrupting this paracrine pathway in our model of prostate cancer metastasis. Starting 1 wk following orthotopic injection of 1 million CE1-4 cells into the prostate, weekly administration of docetaxel (10 mg/kg administered intravenously weekly for 5 wk) reduced the size of primary tumors (from 0.8 to 0.6 g; P < 0.001) and the lung metastatic burden for all categories of lesion sizes (SI Appendix, Fig. S8). Adding celecoxib (20 mg/kg daily for 6 wk) to docetaxel significantly abrogated the number of metastatic foci in the lungs (P < 0.001), while having no effect on the primary tumor (SI Appendix, Fig. S8). Importantly, use of this immune-compromised NSG tumor model uncovers a tumor-suppressive effect of Cox-2 inhibitors that appears to be mediated through tumor-stromal interactions, independent of the inflammatory effects prominently elicited by PGE2 (48).

early tumorigenesis, and identify biomarkers and potential therapeutic targets that ultimately may enable more specific targeting of PGE2-mediated enhancement of tumorigenesis.

The orthotopic mouse prostate cancer model that we studied allows for early and large-scale dissemination of STCs to multiple organs, along with a defined period during which these cells remain viable but nonproliferative, before initiating micrometastases. While dormant bone metastases are typical in human prostate cancer, such disseminated cancer cells are evident in multiple organs in various cancer types, and the number of disseminated cancer cells and the ease of isolation from the lungs led us to focus on these cells. Similarly, the frequent expression of COX-2 and prolactin receptor in different human cancer types and the common expression of prolactin itself in tumor-associated stromal cells suggest that this PGE2-driven paracrine pathway may contribute to diverse tumors. Our use of an immunosuppressed mouse model allowed us to identify a tumor-stromal signaling pathway that is distinct from the classical antiinflammatory effects mediated by COX-2 inhibitors. However, we cannot exclude additional contributions to tumor initiation from immune cells as a consequence of PGE2 exposure. In immune-competent models with already established primary tumors, PGE2 has been reported to increase some endogenous antitumor immune responses (48, 49), while reducing therapeutically induced inflammatory responses (50). The relative contributions of immune cells versus stromal fibroblasts to the PGE2 effect in enhancing
the earliest steps in tumor cell proliferation remain to be determined.

In this context, the unbiased genome-wide transcriptional annotation of nontransformed human fibroblasts exposed to PGE2 is remarkable in identifying the PRL gene as the major target for such a pleiotropic signaling pathway. NR4A has been identified as a downstream effector of PGE2 in LS-174T colorectal cancer cells (52) and our data extend these findings by identifying NR4A binding sites as the most common chromosomal sites with H3K27ac changes following PGE2 treatment of untransformed fibroblasts (68% of all up-regulated H3K27ac sites). The NR4A pathway itself has been implicated as an intermediate in immune signaling, but its role in normal fibroblasts has not been defined, and its heterodimerization with RXR provides for a degree of complexity and specificity in target gene activation (51). While the PGE2-induced, NR4A-RXR-dependent induction of PRL is the most striking transcriptional target of PGE2 identified here, the relative expression patterns of NR4A and RXR family members may lead to additional PGE2 targets in diverse cell types within the cancer microenvironment.

Physiological studies of prolactin have focused on its role as a peptide hormone normally secreted by the pituitary gland and critical to pregnancy-associated lactation (52). However, extrapituitary prolactin, expressed from a distinct promoter, has been reported in bulk unfractionated tumor specimens, including breast and prostate cancers (53–55). The restricted localization of extrapituitary prolactin to the tumor-associated stroma, as opposed to tumor cells, has not been previously appreciated. In addition, prolactin is detectable in blood specimens from patients of both genders with multiple different types of cancers (56), and, recently, it scored as one of the serum proteins most highly correlated with different tumor types in a blood-based multicancer screening platform (57). It is noteworthy, however, that stress and situational anxiety are known to result in marked fluctuations in serum prolactin levels (58), a caveat that will need to be considered in blood-based analyses. At the cellular level, the consequences of prolactin signaling on tumorigenesis are not well established, and diverse effects have been reported following treatment of different cancer cell lines with ectopic prolactin (59–61). In the model that we tested here, prolactin signaling had an inhibitory effect on proliferation of cancer cells, both in vitro and in the primary tumor, but it mediated a striking increase in the ability of single cancer cells that had disseminated to the lungs to produce micrometastases. Understanding the specific prolactin-mediated pathways that underlie this phenomenon may provide additional insight into the early signals that regulate tumor cell dormancy within their microenvironment and the initiation of cancer cell proliferation.

Despite considerable promise in cancer chemoprevention (10–13, 16, 18, 19), COX-2 inhibitors were withdrawn after the discovery that they increase the risk of cardiac events (62), a complication that nullified their potential benefit in healthy individuals at low risk of developing cancer. The application of COX-2 inhibitors to prevent late recurrences of metastatic cancer, following surgical resection of a primary localized but invasive cancer, remains an area of investigation (19). In this context, the risk/benefit profile of COX-2 inhibitors is likely to vary significantly according to both clinical stage and the molecular composition of individual tumors.

Our observations may have clinical implications both in terms of diagnostic biomarkers and potential therapeutic targets in suppressing metastatic recurrence. Future clinical studies will be required to test whether markers of active COX-2–prolactin signaling may identify patients with high-risk primary tumors in whom COX-2 inhibitors could be most effective in preventing metastatic recurrence. In addition, directly targeting key components of the NR4A-RXR signaling pathway might conceivably provide more specificity than COX2 inhibitors, whose suppression of PGE2 synthesis leads to effects on both tumor and cardiovascular systems. Taken together, the dissection of the PGE2–prolactin paracrine signaling axis between the tumor and stroma provides mechanistic insight into pathways that are targeted by COX-2 inhibition, the most compelling and epidemiologically validated chemoprevention strategy in human cancer.

Materials and Methods

Materials, including cell lines and mouse models used and generated by this work, are available in SI Appendix. Animal experiments were performed in accordance with institutional guidelines at the Massachusetts General Hospital and approved by the animal protocol (Institutional Animal Care and Use Committee protocol 2010N000006). Human normal and tumor tissue microarrays were generated from discarded excess tissue obtained from the Massachusetts General Hospital (MGH) and after being deidentified per MGH Institutional Review Board-approved protocols (prostate protocol 2000P002109 and colon protocol 2016P002541). Methods used for single-cell collection and in vitro reconstitution experiments are described in SI Appendix. Methods for single-cell RNA-Seq, bulk RNA-Seq, ChIP-Seq, IF, IHC, RNA-ISH, qPCR, ELISA, and all associated data analyses are standard and are described in SI Appendix. RNA-Seq and ChIP-Seq data that support the findings of the study have been deposited in GEO with the GSE96676 accession code (63).

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