RESEARCH PAPER

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COX-2 dependent regulation of mechanotransduction in human breast cancer cells

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Abbreviations: COX-2, cyclooxygenase-2; CSK, cytoskeleton; ECM, extracellular matrix; FACS, fluorescence-activated cell sorting; FTTM, Fourier transform traction microscopy; MTC, magnetic twisting cytometry; PGE2, prostaglandin E2; PLA2, phospholipase A2; RGD, arginine-glycine-aspartic acid

The ability of living cells to exert physical forces upon their surrounding is a necessary prerequisite for diverse biological processes, such as local cellular migrations in wound healing to metastatic-invasion of cancer. How forces are coopted in metastasis has remained unclear, however, because the mechanical interplay between cancer cells and the various stromal components has not been experimentally accessible. Current dogma implicates inflammation in these mechanical processes. Using Fourier transform traction microscopy, we measured the force-generating capacity of human breast cancer cells occupying a spectrum of invasiveness as well as basal and inducible COX-2 expression (MCF-7<SUM-149<MDA-MB-231). Compared with non-invasive MCF-7 and moderately-invasive SUM-149, poorly-differentiated MDA-MB-231 cells showed increased cellular dispersion on collagen matrix that was accompanied by emergent distribution of contractile stresses at the interface between the adherent cell and its substrate, defined herein as the traction field. In metastatic MDA-MB-231 cells, the local tractions were precisely tuned to the surrounding matrix rigidity in a physiologic range with the concomitant expression of mechanosensitive integrin β_1. These discrete responses at the single-cell resolution were correlated with PGE_2 secretion and were ablated by shRNA-mediated knockdown of COX-2. Both COX-2-silenced and COX-2-expressing cells expressed EP2 and EP4 receptors, but not EP1 and EP3. Exogenous addition of PGE_2 increased cell tractions and stiffened the underlying cytoskeletal network. To our knowledge this is the first report linking the expression of COX-2 with mechanotransduction of human breast cancer cells, and the regulation of COX-2-PGE_2-EP signaling with physical properties of the tumor microenvironment. Drug treatments aimed at reducing this mechanical interplay may have therapeutic potential in the treatment of breast cancer.

Introduction

Breast cancer is the most common malignancy diagnosed in women[1] and is the second leading cause of cancer-related death among women in the United States.[2] It is estimated that up to 10% of breast cancer in Western countries is due to genetic predisposition with many extrinsic factors contributing to the disease progression, presumably via genetic and/or epigenetic changes.[3] The prognosis of metastatic breast cancer remains dismal, however, and current surgical and medical treatments are entirely ineffective in eradicating metastatic spread of primary tumors–the major cause of morbidity and mortality in cancer patients. There is an unmet need for understanding the mechanisms governing metastasis and developing new effective treatments for breast cancer.

A classical view of tumor metastasis begins with the acquisition of traits that allow malignant cells to escape from the primary tumor, to invade the local parenchyma constituting the extracellular matrix (ECM), and to enter the circulation.[4,5] Metastasis then progresses with the transportation of cancer cells via blood circulation to distant target organs, whereupon individual cancer cells adhere, spread and migrate through the ECM in the surrounding tissue forming secondary tumors.[4,5] In this metastatic-invasion framework, the ability of an individual cancer

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cell to invade its local microenvironment, to evade shear stresses imposed by circulation, and to migrate through the surrounding ECM all comprise mechanical properties.[6] Current dogma implicates inflammation within the tumor microenvironment in these mechanical processes.[7,8] However, the mechanical interplay between cancer cells and their physical tissue microenvironment is poorly understood. Further, it remains unclear how an individual cancer cell senses, integrates and initiates its network of mechanical forces during cellular metastasis.

Cyclooxygenase-2 (COX-2), the inducible isoform of prostaglandin (PG) H_2 synthase, is a key mediator of inflammation and converts PLA_2-mobilized arachidonic acid into biologically active lipids.[9] Expression of COX-2 is elevated in various human malignancies,[10-13] and COX-2 catalyzed PGE_2 has been implicated in angiogenesis[14] and metastasis of various cancers, including that of the breast.[15,16] We have previously shown that highly metastatic MDA-MB-231 human breast cancer cells stably expressing a COX-2-specific short hairpin RNA interference molecule exhibit marked reduction in mRNA expression of the classical invasion-related matrix metalloproteinases.[17] Corroborating these changes, cells lacking COX-2 showed decreases in their ability to degrade and infiltrate reconstituted ECM *in vitro*.[17,18] Further, the loss of COX-2 significantly delayed tumor onset as well as inhibited extrapulmonary metastasis *in vivo*.[18] To what extent this loss-of-function of metastatic-invasion in human breast cancer cells is attributable to the regulation of cellular mechanics remains unexplored, however. Here we report that COX-2 expression and activity are precisely tuned to the local rigidity of the collagen matrix and, in the absence of inflammatory milieu, regulate cell tractions in a feed-forward mechanism involving the COX-2-PGE_2-EP signaling axis. To our knowledge this is the first report linking the expression of COX-2 with mechanotransduction of human breast cancer cells.

Results

Force-generating capacity of individual human breast cancer cells with different COX-2 expression and invasiveness. We first interrogated the force-generating capacity of human breast cancer cell lines occupying a series of invasiveness, including the classical luminal-like, non-invasive MCF-7 and basal-like, moderately invasive SUM-149 and highly invasive MDA-MB-231. We focused on these cell lines because they exhibit low-to-high expression of basal and inducible COX-2 (MCF-7<SUM-149<MDA-MB-231).[18,19] This was undertaken using Fourier transform traction microscopy that provides for measuring individual cell mechanical properties in a precise manner, in cells growing on matrices with varying degrees of stiffness across physiological extremes.[20-22] For this study, we used collagen-coated, elastic gel substrate (~8 kPa) that recapitulates the elastic modulus of human breast tissues ranging from fibroadenoma and low-grade invasive ductal carcinoma (IDC).[23]

On this moderately stiff matrix *in vitro*, MCF-7 and SUM-149 cells showed a more rounded morphology in comparison to MDA-MB-231 cells: MDA-MB-231 cells were elongated and polarized, exhibiting a characteristic mesenchymal phenotype. As shown in **Figure 1**, highly invasive MDA-MB-231 cells were bigger in size and exerted upon their surrounding greater tractions than moderately invasive SUM-149 and non-invasive MCF-7 cells. Strikingly, concordant with a ranked-order based on metastatic-invasion potential,[19,24] the net contractile moment, which is a scalar measure of the cell's contractile strength,[25] was ~2.1-fold and ~2.9-fold higher in MDA-MB-231 cells than the respective SUM-149 ($P = 0.0069$, Wilcoxon Scores) and MCF-7 cells ($P = 0.0004$, Wilcoxon Scores) (**Fig. 1C**). Although SUM-149 cells trended toward increases in projected cell area and net contractile moment than MCF-7 cells, there were no significant differences between the 2 cell lines in terms of cell size (**Fig. 1B**; $P = 0.0702$, Wilcoxon Scores) and contractile strength (**Fig. 1C**; $P = 0.2458$, Wilcoxon Scores). These results suggest a close correlation between COX-2 expression, force generation, and the metastatic abilities of human breast cancer cells.

Role for COX-2 in cell tractions? COX-2 and its secondary lipid byproducts are critical determinants of breast cancer invasion and metastasis.[15,16,26] We have previously shown that poorly differentiated MDA-MB-231 cells stably expressing COX-2 shRNA showed marked attenuation in their abilities to secrete secondary lipid mediator products of the COX-2 reaction (i.e. PGE_2) in response to the proinflammatory cytokine interleukin (IL)-1β. [17] We performed extensive quality control analyses of individual clones expressing a short hairpin RNA complementary to COX-2 mRNA (data not shown) and selected clone 2 which could not be induced by IL-1β to secrete PGE_2 for detailed studies.[18] Cell ensembles of clone 2 were relatively more packed with their neighboring cells (**Supplementary Fig. 1**) and showed restricted cellular motions than that of parental MDA-MB-231 counterpart (**Supplementary Video 1**). As denoted by colors in **Supplementary Figure 1**, cells lacking COX-2 exhibited ~37% slower average speed (0.80 μm/min *vs.* 1.27 μm/min). Furthermore, as probed by *spontaneous* motions of microbeads functionalized to the cytoskeleton (CSK) through cell surface integrin receptors,[27,28] COX-2-silenced cells exhibited in turn marked decreases in the rate of cytoskeletal remodeling than COX-2-expressing MDA-MB-231 cells (**Supplementary Fig. 2**). Corroborating these changes in the mechanical properties, COX-2-silenced cells expressed appreciable decreases in the levels of transcripts involved in the cytoskeletal regulation pathways, including ras homolog gene family U and J (RhoU and RhoJ), Rho GTPase activating protein 24 (RhoGAP), and CDC42 effector protein 5 (CDC42EP5) (**Supplementary Fig. 2**). When we measured tractions within the cell monolayer using Monolayer Traction Microscopy,[29] root-mean-square (RMS) traction in cell ensemble of clone 2 trended toward decreases compared to that of parental MDA-MB-231 counterpart; however, the decrease was not statistically significant (data not shown). Herein, we performed the complementary experiments at the level of individually dispersed cells using Fourier transform traction microscopy.

At the single-cell level, MDA-MB-231 clone 2 stably expressing COX-2 shRNA exhibited marked reduction in the dispersion of cell size on collagen matrix than the COX-2-expressing parental counterpart (**Fig. 2**). Compared with parental MDA-MB-231 cells, COX-2-silenced cells showed ~35% reduction in projected cell

area (1364.47 ± 78.51 μm^2 COX-2-silenced *vs.* 2903.37 ± 225.50 μm^2 COX-2-expressing, Mean ± SE) while showing ~60% reduction in net contractile moment (1.20 ± 0.19 pNm COX-2-silenced *vs.* 3.58 ± 0.13 pNm COX-2-expressing, Geometric Mean ± SE). These striking differences in cell size and contractile strength were persistent and long-lived across physiologic range of matrix rigidity–i.e., mimicking the stiffness of human breast tissues of normal fat to intermediate-grade IDC (**Fig. 3A, B**).[23] Interestingly, with increasing matrix rigidity, MDA-MB-231 cells showed progressive increases in cell spreading (**Fig. 3A**) and net contractile moments (**Fig. 3B**). In addition, MDA-MB-231 cells showed increased expression of mechanosensitive integrin β1, but not β3, with increasing matrix rigidity (**Fig. 3C, D**; **Supplementary Fig. 3**). These discrete cellular responses to matrix rigidity were absent in COX-2-silenced MDA-MB-231 cells. These results, taken together, support the conclusion that expression and/or activity of COX-2 are critical for the mechanotransduction of human breast cancer cells and suggest that physical changes in the tumor microenvironment may affect metastatic-invasion of human breast cancer *via* mechanisms involving COX-2 and its lipid byproducts.

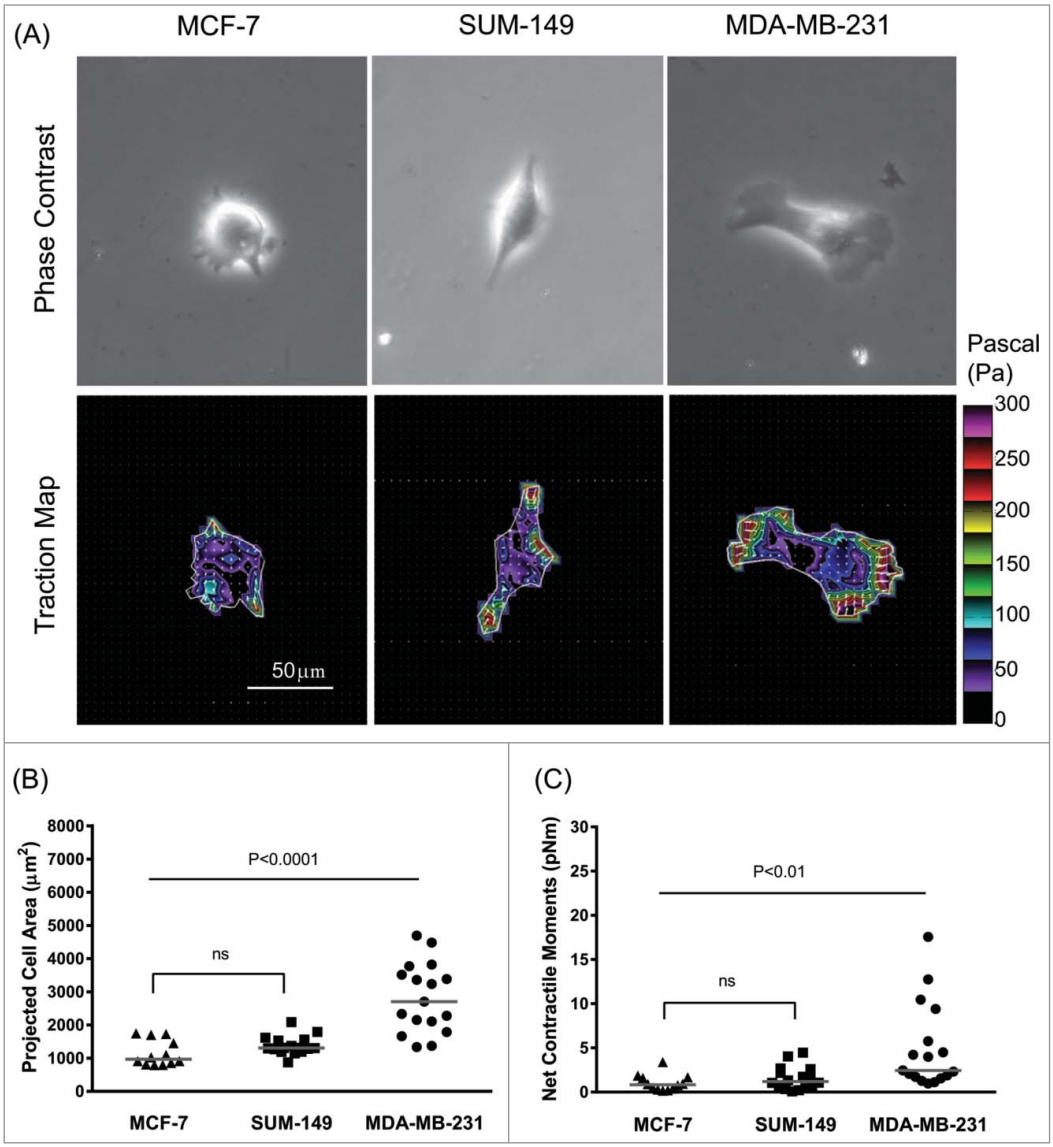

Figure 1. Association between invasiveness and COX-2 status on the generation of traction force in human breast cancer cells. (**A**) Representative phase contrast and traction map images of MCF-7, SUM-149 and MDA-MB-231 cells. White lines show the cell boundary, colors show the magnitude of the tractions in Pa (see color scale), and arrows show the direction and relative magnitude of the tractions. Scale bar, 50 μm. The projected cell area (**B**) and computed net contractile moments (**C**) of individual breast cancer cells (MCF-7, SUM-149, and MDA-MB-231). The line bars represent the median for each group (n = 12 -17 cells for each group).

Propagation of cellular tractions is mediated by a feed-forward mechanism involving COX-2-PGE_2 axis. The induction of COX-2 expression stimulates the biosynthesis and secretion of PGE_2.[9] In COX-2-expressing, parental MDA-MB-231 cells, we found progressive and appreciable increases in the levels of PGE_2 production with increasing matrix rigidity (**Fig. 4A**). These cellular responses were entirely ablated in MDA-MB-231 clone 2 cells stably expressing COX-2 shRNA. PGE_2 acts through G protein-coupled receptors (EP1–4) and initiates a complex downstream signaling that regulates, in turn, cell proliferation, migration and invasion.[16,30] Both parental and COX-2-silenced MDA-MB-231 cells showed transcript levels of EP2 and EP4, but not EP1 and EP3 (**Supplementary Fig. 4**). Whereas exogenous addition of PGE_2 did not influence the dispersion of human breast cancer cells on collagen matrix (data not shown), it appreciably increased their ability to exert tractions (**Fig. 4B**). In order to further validate this unique mechanical responsiveness to PGE_2, we applied *forced* motions of ferrimagnetic microbeads functionalized to the CSK using Magnetic Twisting Cytometry (**Fig. 4C** and **Supplementary Fig. 5**).[31-33] As expected, PGE_2 caused

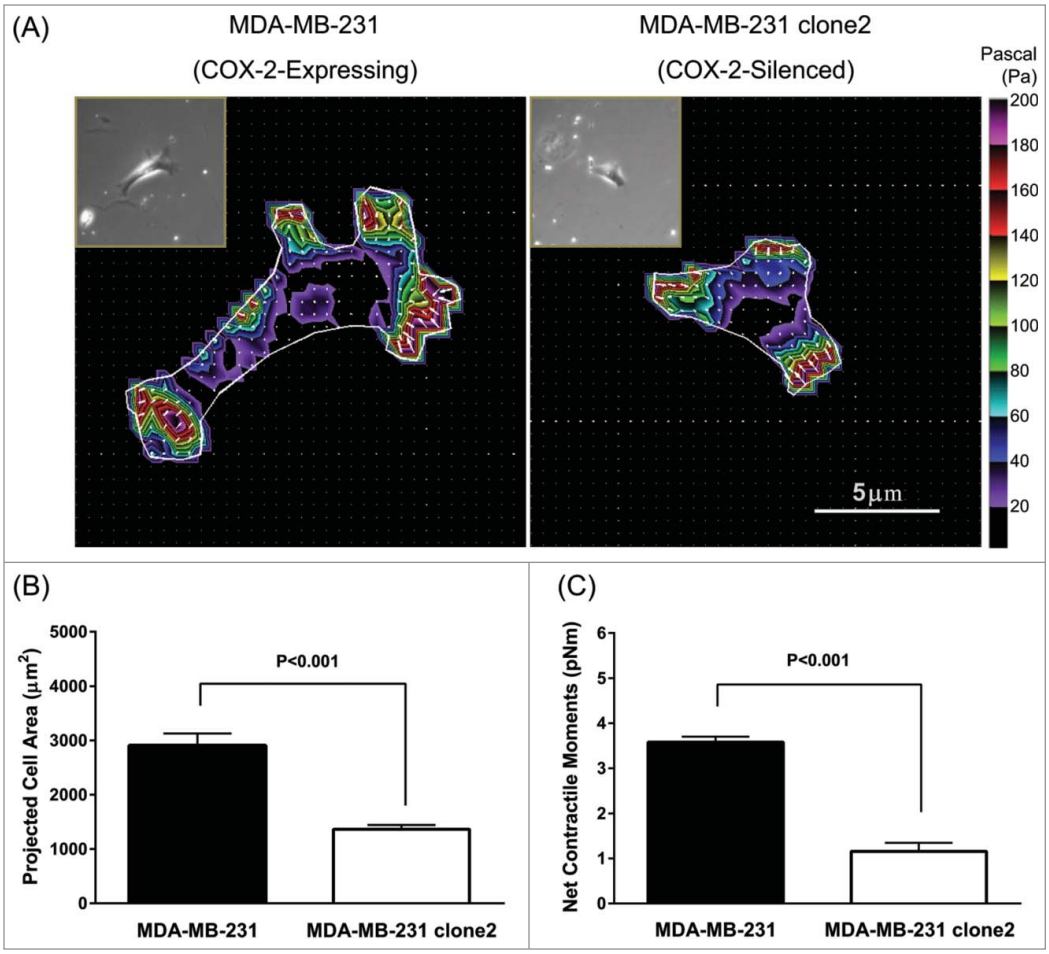

Figure 2. Effect of COX-2-silencing on the generation of traction force in invasive MDA-MB-231 cells. (**A**) Representative traction maps of MDA-MB-231 (COX-2-expressing *vs.* COX-2-silenced) cells. The projected cell area (**B**) and computed net contractile moments (**C**) of COX-2-expressing and COX-2-silenced cells (n = 31 cells for each group). Herein, projected cell area is presented as Mean ± SE, and net contractile moment is presented as Geometric Mean ± SE.

time- and dose-dependent decreases in the stiffness of isolated human airway smooth muscle cells (**Supplementary Fig. 5**)[34]. In contrast, exogenous addition of PGE_2, both acutely and chronically, stiffened the underlying cytoskeletal network of metastatic human breast cancer cells, including moderately-invasive SUM-149 cells (**Fig. 4C** and **Supplementary Fig. 5**). PGE_2 had no effect on the stiffness of non-metastatic MCF-7 cells, however (**Fig. 4C**). Taken together, these findings show that the physical nature of the tumor microenvironment, independent of immune inflammatory responses, may also impact COX-2 expression and/or activity in human breast cancer cells and that propagation of physical forces and cell metastasis may be mediated by COX-2-driven PGE_2 in a feed forward manner.

Discussion

Mechanotransduction is the most pervasive element of the metastatic cascade but is incompletely understood, especially within the context of the wound-like inflammatory environments that exist within tumors. Most solid tumors, including breast cancers, exhibit inflammatory properties characterized by increased levels of prostaglandins and other proinflammatory cytokines that are secreted by tumor cells, stromal cells, and specialized immune cells.[35] Eicosanoids formed by the action of COX-2 on arachidonic acid have been shown to impact cell motility, invasion, vascular characteristics and metastatic dissemination.[36-38] A major product of the COX-2-catalyzed reaction is prostaglandin E_2 (PGE_2), an inflammatory mediator that participates in various biological processes,[39] and plays multiple roles in cancer aggressiveness.[40,41] Here for the first time we have identified a role for COX-2 expression, signaling and function in the induction of physical forces in human breast cancer. We also identified, for the first time, the effect of matrix rigidity on the propagation of these forces, demonstrating the role of the physical microenvironment on the inflammatory response of cancer cells.

We observed a robust association between invasiveness and force-generating capacity of human breast cancer cells. The most invasive and metastatic MDA-MB-231 cells displayed the highest net contractile moment–a scalar measure of a cell's contractile strength (**Fig. 1**). The association between invasiveness and contractile strength is consistent with 2 recent studies that reported increased tractions in more invasive breast cancer cells.[42,43] Increased local cell tractions–the force exerted upon its surrounding area–may provide a biophysical marker of the cell's ability to adhere, spread and migrate through the ECM, as well as invade and metastasize through the endothelial cell barrier during intravasation and extravasation. The three cell lines used in this study also expressed low-to-high basal and inducible COX-2 (MCF-7<SUM-149<MDA-MB-231). Because of the different lineages of these cells we could identify an association between COX-2 and cell tractions but not a direct role of COX-2 in driving these parameters. Further validation was provided by the marked

reduction in cell spreading and tractions observed in MDA-MB-231 clone 2 cells stably expressing COX-2 shRNA than COX-2 expressing parental counterpart cells (**Fig. 2**). MDA-MB-231 clones stably expressing COX-2 shRNA are poorly invasive and lack the ability to metastasize.[17,18]

In order to show the loss-of-function, here we applied shRNA-mediated knockdown of COX-2 in the highly invasive and metastatic MDA-MB-231 cell line. This approach was chosen, rather than using pharmacological inhibitions, however, because COX-2 inhibitors are known to have several COX-2-independent functions and, like most drugs, have off target effects. For example, many of the celecoxib-associated effects observed *in vitro* and *in vivo* are not related to COX-2 inhibition, but to COX-2-independent actions of celecoxib.[44] MDA-MB-231 cells treated with celecoxib also exhibited a trend toward decreases in net contractile moment but, unlike the dramatic effect of COX-2-silencing on cell spreading, had no effect on the extent of cell spreading on the elastic substrate (**Supplementary Fig. 6**). These findings, taken together, suggest that COX-2-mediated induction of effector protein expression might be required to fully realize the propagation of mechanotransduction in human breast cancer cells. Toward this end, increased COX-2 can mediate the upregulation of several oncogenic pathways including proteolytic enzymes.[18] Interestingly, in pancreatic cancer, COX-2 has been recently identified as a novel target of the transcription regulator Yes-associated protein (YAP).[45] There is also increasing evidence to suggest that YAP is a mechanosensor in various cell types, suggesting that COX-2 can be induced mechanically.

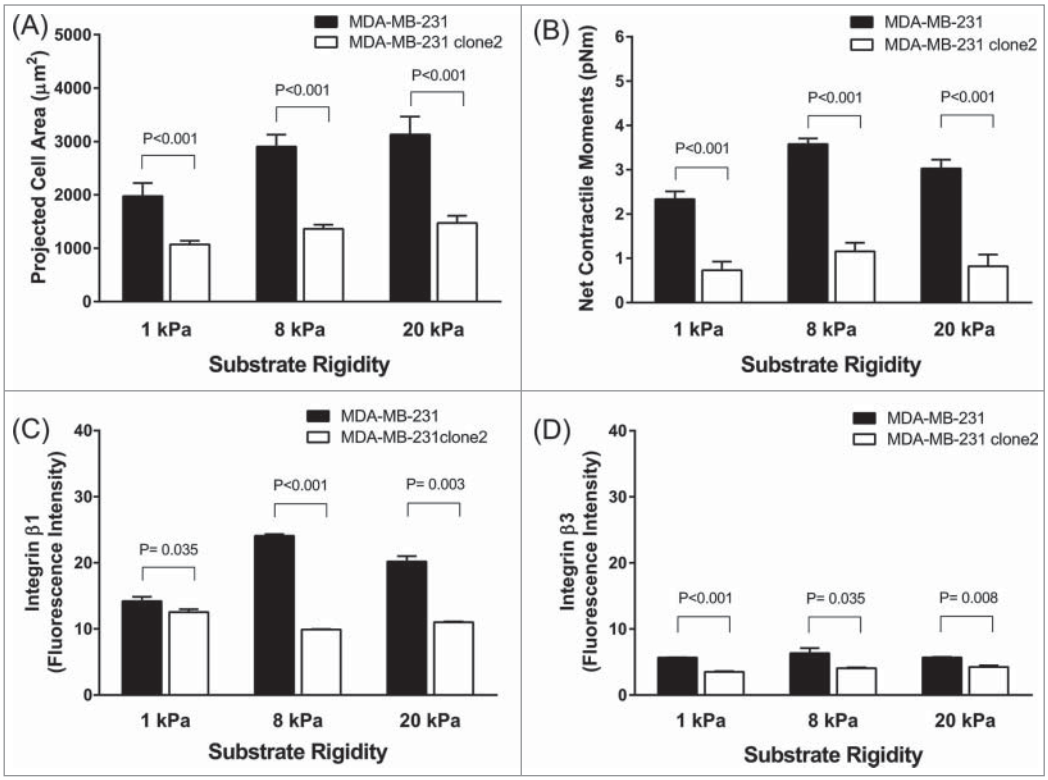

Figure 3. Effects of COX-2-silencing on the mechanobiology of invasive MDA-MB-231 cells. The projected cell area (**A**) and computed net contractile moments (**B**) of MDA-MB-231 cells (COX-2-expressing *vs.* COX-2-silenced) measured on elastic substrate with varying stiffness (1 kPa, n = 32 -37; 8 kPa, n = 31 ; 20 kPa, n = 18 -25 for each group). Expression levels of integrin β1 (**C**) and β3 (**D**) in MDA-MB-231 cells (COX-2-expressing *vs.* COX-2-silenced). Data are presented as Mean ± SE (n = 3 experiments).

Cancerous tissues typically exhibit increased ECM rigidity compared to normal tissues. Breast and other solid tumors are denser than surrounding stroma because of the increased deposition and differential remodeling of collagen fibers that surround the growing tumor.[46] A high density of collagen 1 fibers in the ECM has been identified as a predictor of increased metastasis.[47,48] High collagen density has also been observed to increase tractions by breast cancer cells.[43] Increased metastasis associated with high collagen density may be mediated in part by this increased tractions. Previous studies have identified the effects of ECM stiffness on migration and proliferation of glioma cells, and the effect of matrix stiffness and confinement on cell migration speed of glioma cells.[49-50] Here, with increasing matrix rigidity, MDA-MB-231 cells showed progressive increases of cell spreading that was accompanied by increased expression of mechanosensitive integrin β1 (**Fig. 3**). These mechanical responses to increasing matrix rigidity were absent in COX-2 silenced MDA-MB-231 cells. Our data are consistent with earlier observations that cancer cells on substrates of increasing stiffness exhibit increased tractions,[43] and demonstrate for the first time that expression and/or activity of COX-2 are critical in this mechanotransduction of human breast cancer cells. Further support for the role of COX-2 is evident from the increase of PGE_2 secretion with increasing substrate rigidity in MDA-MB-231 cells, but not in COX-2 silenced cells (**Fig. 4**). Exogenous addition of PGE_2 to these COX-2 silenced cells resulted in a partial recovery of tractions whereas further increased responses are noted in their parental counterpart, MDA-MB-231 cells (**Fig. 4B**). The increase of PGE_2 secretion with increasing substrate rigidity suggests mechanical induction of COX-2 in these cells.

Using MTC, we further validated the effects of PGE_2 across breast cancer cell lines: non-invasive and non-metastatic MCF-7, moderately invasive and metastatic SUM-149 cells, and most

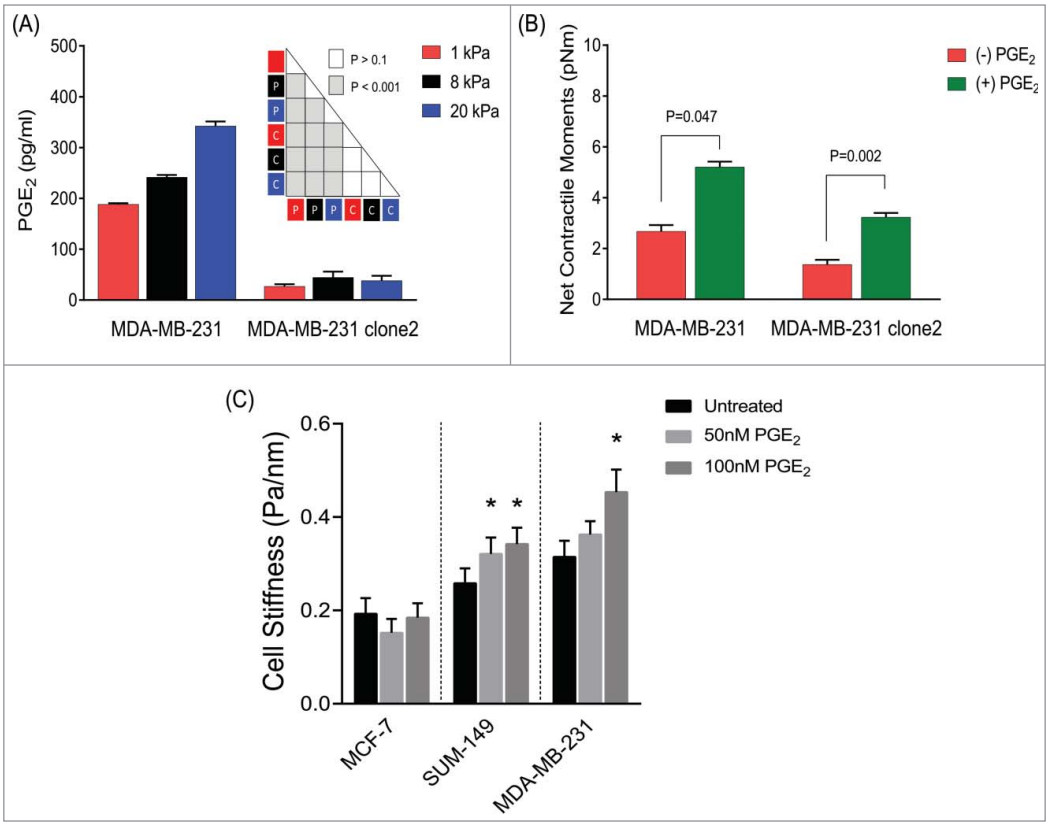

Figure 4. A feed-forward mechanism involving COX-2-PGE_2-EP signaling on the mechanotransduction of human breast cancer cells. (**A**) Effects of matrix rigidity on COX-2 activity. MDA-MB-231 (COX-2-expressing and COX-2-silenced) cells were cultured on elastic substrates with varying stiffness, and the secreted levels of PGE_2 in the culture media were measured after 24 h. Data are presented as Mean $\pm$ SE (n = 3 experiments); ANOVA test (P = parental ; C = clone2). (**B**) COX-2-expressing and COX-2-silenced cells were seeded onto an elastic substrate with Young's modulus of 1 kPa, and treated for 24 h with or without increasing concentrations of PGE_2. On the one hand, cell spreading area increased progressively with increasing concentrations of PGE_2 (50 nM < 100 nM < 500 nM < 1 μM). On the other hand, while cell traction force increased with PGE_2 at 50 nM and 100 nM, PGE_2 concentrations above 100 nM led to decreases in cell traction force (data not shown). Here we report the effects of 50nM PGE_2 on cell traction force. Data are presented as Geometric Mean $\pm$ SE (COX-2-expressing cells, n = 19–21; COX-2-silenced cells, n = 14–22). (**C**) Effects of PGE_2 on cytoskeletal stiffness of human breast cancer cells. For this MTC study, cells were plated at 30,000 cells/cm^2 on plastic wells previously coated with collagen type I for 24 h in media alone or media supplemented with 50–100nM PGE_2. Data are presented as Geometric Mean $\pm$ SE (MCF-7, n = 183 -200 cells; SUM-149, n = 296 -310 cells; MDA-MB-231, n = 171 -397 cells).

invasive and metastatic MDA-MB-231 cells (**Fig. 4C** and Supplementary **Fig. 5**). Consistent with the ranked order differences in the net contractile moments measured by Fourier transform traction microscopy (**Fig. 1**), we found appreciable ranked order differences in the basal cytoskeletal stiffness across breast cancer cells (MCF-7<SUM-149<MDA-MB-231). Compared with MCF-7 cells, both SUM-149 and MDA-MB-231 cells exhibited significantly higher cytoskeletal stiffness (**Fig. 4C**). There were no statistical differences between SUM-149 and MDA-MB-231 cell stiffness, however. More strikingly, PGE_2 treatment increased the stiffness of moderately invasive SUM-149 and highly invasive MDA-MB-231 cells whereas it had no effect on non-invasive MCF-7 cells. Taken together, our results using two independent single-cell analyses confirmed the unique physical properties of metastatic human breast cancer cells, and further validate the role for COX-2-generated PGE_2 on the mechanotransduction and progression of cell metastasis. In conclusion, our findings provide a new mechanistic framework in which changes in the physical properties of the tumor microenvironment may affect metastatic-invasion of human breast cancer *via* mechanisms involving COX-2 and its lipid byproducts. As such, drug treatments aimed at reducing this mechanical interplay may have therapeutic potential in the treatment of breast cancer.

Materials and Methods

Cell lines and culture conditions. All human breast cancer cell lines, with the exception of SUM-149, were cultured with DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY). SUM-149 cell line was maintained in RPMI 1640 media (Sigma-Aldrich) supplemented with 8.25% fetal bovine serum, 0.1mM HEPES (Sigma-Aldrich) and 10 mg/ml pancreatic insulin (Sigma-Aldrich). Cells were maintained at 37°C in humidified air containing 5% CO_2.

Preparation of elastic matrix. As previously described,[50] a mixture of acrylamide (5–10%) and bis-acrylamide (0.03–0.3%) was used to vary the rigidity of elastic gel blocks in the physiological range.[23] For elastic gels used in the detection of COX-2 activity and mechanosensitive integrins, gel blocks were fashion onto individual 35 mm glass-bottom wells with 20 mm inner glass diameter (P35G-0–20-C; MatTek Inc., Ashland, MA). For gels used for gauging cell traction forces, fluorescent microbeads (0.2 μm in diameter, Molecular Probes, Eugene, OR) were added to the mixture of acrylamide and bis-acrylamide and were fashioned onto individual 35 mm glass-bottom wells with 14 mm inner glass diameter (P35G-0–14-C). All gel blocks were coated with collagen type I (0.2 mg/ml) using a photo-activating cross-linker sulfo-SANPAH (Pierce, Rockford, IL).

Fourier transform traction microscopy (FTTM). The contractile stress arising at the interface between each adherent cell and its substrate was measured with traction microscopy.[20-22,25] Cells were plated sparsely on elastic gel blocks, and allowed to adhere and stabilize for 24 h. For each adherent cell, images of fluorescent microbeads embedded near the gel apical surface were taken at different times; the fluorescent image of the same region of the gel after cell detachment with trypsin was used as the reference (traction-free) image. The displacement field between a pair of images was then obtained by identifying the coordinates of the peak of the cross-correlation function.[25] From the displacement field and the known elastic properties of the gel (Young's Modulus, 1 kPa to 20 kPa and Poisson's ratio 0.48), the traction field was computed using both constrained and unconstrained Fourier transform traction cytometry as described previously.[25] The computed traction field was used to obtain net contractile moment, which is a scalar measure of the cell's contractile strength expressed in units of pico-Newton meters (pNm).

Magnetic Twisting Cytometry (MTC). To quantify material properties of the adherent human breast cancer cells, we used MTC as described by us in detail elsewhere.[31-33] In brief, an RGD-coated ferrimagnetic microbead (4.5 μm in diameter) anchored to the CSK through cell surface integrin receptors was magnetized horizontally and then twisted in a vertically aligned homogenous magnetic field that varied sinusoidally in time; measurements were performed at a single frequency of 0.75 Hz or oscillatory frequencies between 10^{-1} and 10^{3} Hz. The sinusoidal twisting field causes both a rotation and a pivoting displacement of the bead (**Supplementary Fig. 6**). As the bead moves, the cell develops internal stresses which in turn resist bead motions.[51] Lateral bead displacements in response to the resulting oscillatory torque were detected via a CCD camera (Orca II-ER, Hamamatsu, Japan), and with an accuracy of 5 nm using an intensity-weighted center-of-mass algorithm.[51] The ratio of specific applied torque to lateral bead displacements was computed and expressed as the cell stiffness in units of Pascals (Pa) per nanometer.

Detection of COX-2 activity. COX-2 activity in human breast cancer cells was assessed by measurement of secreted PGE_2. For this study, we plated 1×10^6 cells per elastic gel block with varying rigidity (1 kPa to 20 kPa) for 24 h. The PGE_2 concentration in the supernatants was determined using a commercial EIA kit (DetectX Prostaglandin E2 High Sensitivity Immunoassay Kit, Arbor Assays, Ann Arbor, MI) following the manufacturer's instructions. The absorbance in the samples was measured at 490 and 540 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The concentration of PGE_2 was calculated from a standard curve derived using recombinant proteins. All samples were assayed in triplicate.

Fluorescence-activated cell-sorting (FACS) analysis. For the adherent cells on the respective elastic gel block (as above in COX-2 assay), we also quantified the cell surface expression levels of integrin β1 and β3 using FACS. In brief, cells were detached using cell dissociation buffer (Gibco Life Technologies, Grand Island, NY) and suspended in staining buffer (RPMI 1640 media, 2% FBS). Equal amount of cells in 100 μl volume was transferred into 12 × 75 mm polypropylene FACS tubes and incubated with 1 μg/ml of FcR blocking antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 10 min at room temperature. After blocking, cells were incubated with FITC-conjugated antibody against CD29 for integrin β1 (Millipore, San Diego, CA) and CD61 for integrin β3 (Millipore) for 30 min at room temperature in the dark. Cells were then washed twice with staining buffer and resuspended in 500 μl staining buffer for analysis. The antibody staining was quantified with a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) and data from 10,000 events were collected for further analysis. The three independent data from each group were analyzed using Cell Quest Pro (BD Biosciences Immunocytometry Systems) to determine the mean fluorescence intensity and the standard deviation.

Statistical analysis. Unless otherwise stated, we used Student's *t*-test and the Analysis of Variance (ANOVA) with adjusting for multiple comparisons by applying the Bonferroni's methods. All analyses were performed in SAS V.9.2 (SAS Institute Inc., Cary, NC), and the 2-sided *P*-values less than 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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