

Prostaglandin E2 receptor EP4 as the common target on cancer cells and macrophages to abolish angiogenesis, lymphangiogenesis, metastasis, and stem-like cell functions

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Cyclooxygenase-2, an inflammation-inducible enzyme, is associated with progression of epithelial cancers.^(1–3) Cyclooxygenase-2 overexpression, noted in nearly half of all breast cancer patients, marks poor prognosis.⁽⁴⁾ High COX-2 activity resulting in elevated prostaglandin (PG) E2 levels in the tumor milieu promotes breast cancer progression and metastasis through multiple events: inactivation of host immune cells;⁽⁵⁾ stimulation of tumor cell migration/invasiveness;^(6–8) and tumor-associated angiogenesis^(6,9) and lymphangiogenesis.⁽¹⁰⁾ Expression of vascular endothelial growth factor (VEGF)-C/D in human breast cancer *in situ* is strongly correlated with lymphangiogenesis, lymphovascular invasion, and lymphatic metastasis.^(11–14) Cyclooxygenase-2 is a major stimulator of VEGF-C production in human⁽¹¹⁾ and VEGF-C/D production in murine⁽¹⁰⁾ breast cancer models. In addition

We previously established that COX-2 overexpression promotes breast cancer progression and metastasis. As long-term use of COX-2 inhibitors (COX-2i) can promote thrombo-embolic events, we tested an alternative target, prostaglandin E2 receptor EP4 subtype (EP4), downstream of COX-2. Here we used the highly metastatic syngeneic murine C3L5 breast cancer model to test the role of EP4-expressing macrophages in vascular endothelial growth factor (VEGF)-C/D production, angiogenesis, and lymphangiogenesis *in situ*, the role of EP4 in stem-like cell (SLC) functions of tumor cells, and therapeutic effects of an EP4 antagonist RQ-15986 (EP4A). C3L5 cells expressed all EP receptors, produced VEGF-C/D, and showed high clonogenic tumorsphere forming ability *in vitro*, functions inhibited with COX-2i or EP4A. Treating murine macrophage RAW 264.7 cell line with COX-2i celecoxib and EP4A significantly reduced VEGF-A/C/D production *in vitro*, measured with quantitative PCR and Western blots. Orthotopic implants of C3L5 cells in C3H/HeJ mice showed rapid tumor growth, angiogenesis, lymphangiogenesis (CD31/LYVE-1 and CD31/PROX1 immunostaining), and metastasis to lymph nodes and lungs. Tumors revealed high incidence of EP4-expressing, VEGF-C/D producing macrophages identified with dual immunostaining of F4/80 and EP4 or VEGF-C/D. Celecoxib or EP4A therapy at non-toxic doses abrogated tumor growth, lymphangiogenesis, and metastasis to lymph nodes and lungs. Residual tumors in treated mice revealed markedly reduced VEGF-A/C/D and phosphorylated Akt/ERK proteins, VEGF-C/D positive macrophage infiltration, and proliferative/apoptotic cell ratios. Knocking down COX-2 or EP4 in C3L5 cells or treating cells *in vitro* with celecoxib or EP4A and treating tumor-bearing mice *in vivo* with the same drug reduced SLC properties of tumor cells including preferential co-expression of COX-2 and SLC markers ALDH1A, CD44, OCT-3/4, β -catenin, and SOX-2. Thus, EP4 is an excellent therapeutic target to block stem-like properties, angiogenesis, and lymphangiogenesis induced by VEGF-A/C/D secreted by cancer cells and tumor infiltrating macrophages.

to its lymphangiogenic role, COX-2-upregulated VEGF-C directly promoted breast cancer cell motility, a phenotype for metastasis, by binding to a diverse group of VEGF-C receptors.⁽¹⁵⁾

Although the above evidence makes COX-2 a reasonable therapeutic target, increased risks of thrombo-embolic effects of long-term use of high-dose COX-2 inhibitors^(16,17) suggest the need for identifying alternative target(s) downstream of COX-2 that may spare the risks. The vaso-protective role of COX-2 was attributed to IP receptors interacting with PGI₂.⁽¹⁸⁾ Thus, targeting one or more of the PGE (EP) receptors should retain IP actions. They are G protein-coupled receptors with differential signaling abilities: EP1 is coupled with Gq, stimulating (Ca⁺⁺); EP2 and EP4 are coupled with Gs, stimulating the adenylate cyclase/PKA pathway; whereas most EP3

isoforms are coupled with G_i , thus inhibiting adenylate cyclase.⁽¹⁹⁾

Unlike EP2, EP4 can additionally stimulate phosphatidylinositol 3-kinase (PI3K)/Akt-mediated cell survival pathway as well as the pro-migratory ERK pathway.⁽²⁰⁾ Most of the COX-2 mediated events in breast cancer, such as cancer cell migration/invasiveness,^(7,8) VEGF-C or -D upregulation in cancer cells^(10,11) and inactivation of natural killer cells⁽²¹⁾ were shown to follow activation of EP4 on these cells, making it an excellent therapeutic target, without crippling the vaso-protective arm of COX-2. This target was validated by preclinical studies in syngeneic murine breast cancer models with a number of EP4 antagonists.^(10,22)

Tumor progression, metastasis, and recurrence after therapy-initiated remission are all believed to result from a tumor cell subpopulation known as “stem-like cells” (SLC).^(23,24) Interestingly, PGE-2 was shown to stimulate hematopoietic stem cells⁽²⁵⁾ and EP4 activation was reported to be essential for hematopoietic stem cell expansion.⁽²⁶⁾ Recently, EP4 has been implicated in promotion of the SLC phenotype in breast cancer cells.⁽²⁷⁾

Although tumor-associated macrophages (TAMs) can play a complex role in both halting and promoting tumor progression, there is compelling evidence for the latter in established solid tumors.⁽²⁸⁾ Tumor-associated macrophages can facilitate many key processes in breast cancer progression such as immune suppression, production of proteases, and promotion of angiogenesis.^(29,30) Indeed, macrophage infiltration in the tumor stroma is an independent indicator of poor prognosis in human breast cancer.⁽³¹⁾ The capacity of macrophages to produce both VEGF-A⁽³²⁾ and VEGF-C/D⁽³³⁾ explains their stimulatory roles in angiogenesis and lymphangiogenesis. It is presently unclear whether VEGF-A/C/D production by TAMs in breast cancer is COX-2- or EP4-dependent.

In view of the above, the present study was designed in our COX-2 expressing syngeneic breast cancer model⁽¹⁰⁾ to explore: (i) whether VEGF-C or -D production by TAMs is an additional driver of lymphangiogenesis *in situ* and, if so, whether it is COX-2- or EP4-dependent; (ii) the role of EP4 in stem-like tumor cell functions; and (iii) the potential therapeutic effects of a COX-2 inhibitor celecoxib and an EP4 antagonist RQ-15986 on these events, including tumor growth and spontaneous metastasis to the lungs and lymph nodes. Effects of these drugs on angiogenesis and lymphangiogenesis were tested with VEGF-A/C/D expression in residual tumors and *in situ* immunostaining of tumor vasculature for LYVE-1/CD31 and PROX1/CD31. In addition, *in vitro* effects of the drugs were tested on VEGF-A/C/D production by a murine macrophage cell line. Results revealed that EP4 is an excellent therapeutic target to block stem-like properties in cancer cells and tumor-associated angiogenesis and lymphangiogenesis induced by VEGF-A/C/D production by cancer cells as well as TAMs.

Materials and Methods

Cell line. C3L5 is a highly metastatic derivative of a spontaneous mammary adenocarcinoma in C3H/HeJ mice,⁽³⁴⁾ which expresses high levels of COX-2, the PGE-2 secreting ability primarily attributed to COX-2.^(6,8) Mouse macrophage cell line RAW 264.7 were purchased from ATCC (Manassas, VA, USA). Cells were maintained in high glucose DMEM (Gibco, Invitrogen, ON, Canada), 10% FBS, 100 U/mL penicillin G, and 100 µg/mL streptomycin.

Mice. Six-week-old female C3H/HeJ mice from Jackson Laboratories (Bar Harbor, ME, USA) were allowed to acclimatize for 2–3 weeks, maintained on standard mouse chow and tap

water (unless otherwise indicated) on a 12:12 h light:dark cycle, and treated in accordance with the guidelines set by the Canadian Council on Animal Care.

Drugs and reagents. NS-398 (COX-2 inhibitor) was from Cayman Chemical, Ann Arbor, MI, USA. Selective COX-2 inhibitor celecoxib and EP4 antagonist CJ-042794 (renamed as RQ-15986) were respective gifts of Pfizer, Groton, CT, USA and RaQualia Pharma, Nagoya, Japan. The structure, binding affinity, high selectivity, and pharmacokinetic properties of RQ-15986 (henceforth called EP4A) has been characterized.^(35,36) For *in vitro* treatments and treating tumor-transplanted mice, the details of all drugs and vehicles used to dissolve or administer them are presented in “Materials and Methods” and “Results”. For *in vitro* or *in vivo* treatments, respective vehicles served as controls.

Proliferation, migration, and VEGF-C/D production by C3L5 cells. Proliferation and migration at 24 h were measured as reported.⁽¹⁴⁾ C3L5 cells had been shown to express all the EP receptors.⁽¹⁰⁾ The effects of treating C3L5 and cells with EP4A was measured with real-time quantitative PCR (for VEGF-C and -D mRNA), ELISA (for VEGF-C), and Western blot analysis (for VEGF-D) as reported.⁽¹⁰⁾

Vascular endothelial growth factor-A/C/D production by RAW 264.7 cells. Cells were treated for 24 h with DMSO, celecoxib, and EP4A before RNA and protein extraction to carry out *Taq*-Man real-time PCR (VEGF-A, cat # 4453320; VEGF-C, cat # 4453320; VEGF-D, cat # 444889; and *Gabpb1*, cat # 4448892) and Western blots as described later in Western blot analysis.⁽¹⁰⁾

Detection of SLC *in vitro* by clonogenic spheroid formation assay. Cells were serum-starved for 12 h and serially diluted at 1000, 100, 10, and 1 cell(s)/100 µL in HUMEK medium (Gibco) supplemented with 20 ng/mL basic fibroblast growth factor (Invitrogen, ON, Canada), 20 ng/mL epidermal growth factor (Invitrogen), and B-27 supplement (1:50 dilution; Life Technologies, Burlington, ON, Canada). They were cultured on ultra-low attachment plates (Corning, MA, USA) to observe the temporal kinetics for spheroid (tumorsphere) formation. In some experiments, to explore dependence of spheroid formation on COX-2 or EP4 activity, cells were pretreated with either 0.003% DMSO or 2 µM celecoxib or EP4A for 5 days before plating and the same drug concentrations continued during spheroid formation. Spheroid formation was also evaluated after stable knockdown of COX-2 with shRNA plasmid (sc-29278-SH) and transient knockdown of EP4 with siRNA (sc-40174), both obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The number of spheroids (at least 60 µm in diameter) and their perimeters were measured with ImageJ (<http://imagej.nih.gov/ij/>) at different time points and spheroids were dissociated and recultured to assess their spheroid-forming ability at successive generations.

Detection of SLC or embryonic stem cell-associated markers with immunofluorescence. Five micrometer-thick frozen sections of C3L5 cell *in vitro* spheroids and 12-day-old tumors (see later in measurements of angiogenesis and lymphangiogenesis section) were subjected to dual immunostaining for COX-2 (Abcam, Toronto, ON, Canada) and breast cancer stem cell markers aldehyde dehydrogenase (ALDH) and CD44 or embryonic stem (ES) cell markers OCT-3/4 and SOX-2 or hematopoietic stem cell marker β -catenin, with antibodies from BD Biosciences.

Tumor implantation and treatment regimen. Briefly, 5×10^4 C3L5 cells, suspended in diluted growth factor-reduced Matrigel were implanted s.c. in both inguinal mammary regions. Animals were divided into five different groups. Group I (six mice)

received implants of Matrigel alone and no other treatment to serve as negative controls for angiogenesis or lymphangiogenesis. The remaining four groups (groups II–V, 24 mice per group) were implanted with tumor cells suspended in Matrigel. Group II received celecoxib (250 mg/kg/day dissolved in 0.5% methylcellulose; Sigma, Oakville, ON, Canada) by oral gavage twice daily. Group III received EP4A (10 mg/kg/day dissolved in 0.003 N NaOH by oral gavage twice daily). Groups IV and V received vehicles alone serving as respective controls for celecoxib and EP4A. The dosage and vehicles for these drugs were guided by earlier reports for celecoxib⁽³⁷⁾ and EP4A.^(35,36) Mice (two from group I and eight from each of the other groups) were killed on days 8, 12, and 16. After retrieval of the implants (two per mouse), their weights and diameters (maximum and minimum) were recorded and gross morphology photographed. Tumors were then sliced in half, each half fixed or frozen for further histological or immunohistological analysis. Tumor-draining inguinal and distant axillary lymph nodes were removed before fixing them for histopathology.

Evaluation of drug-mediated toxicity in treated mice. All animals were monitored for daily water intakes, and examined at autopsy for any visible bleeding in the gastrointestinal tract.

Scoring metastases. Metastatic lung colonies were scored microscopically in both lungs in H&E stained coronal sections

through mid-trachea (providing the maximal surface area of both lungs) in fixed tissues. Mean and median scores were based on eight mice/group, with each score acquired from counts of all lobes. Metastases to tumor-draining inguinal and distant axillary lymph nodes were identified histologically in H&E stain as reported.⁽¹⁰⁾ Both pulmonary and lymphatic metastases were scored by two independent observers with less than 8% variation.

Measurements of angiogenesis and lymphangiogenesis. Eight micrometer-thick frozen sections were dual immunostained for LYVE-1/CD31 and PROX1/CD31.^(10,38) Adjacent sections of fixed tissues stained with Masson's trichrome, examined under bright field, provided a map to locate vessels within the peritumoral stroma and tumor parenchyma. With this method, capillaries devoid of red cells could not be adequately discriminated between blood or lymphatic capillaries. Blood microvessel density and lymphatic vessel density were assessed in dual immunostained sections (CD31, red; LYVE-1, PROX1, green) as reported.^(10,38) Three hotspots per tumor (48/group/day) were examined at $\times 400$ magnification, and mean scores for blood microvessel density and lymphatic vessel density were obtained after background correction, using ImageJ.

Western blot analysis of proteins in tumor tissues. As reported, two vehicle-treated tumor extracts were pooled.⁽¹⁰⁾

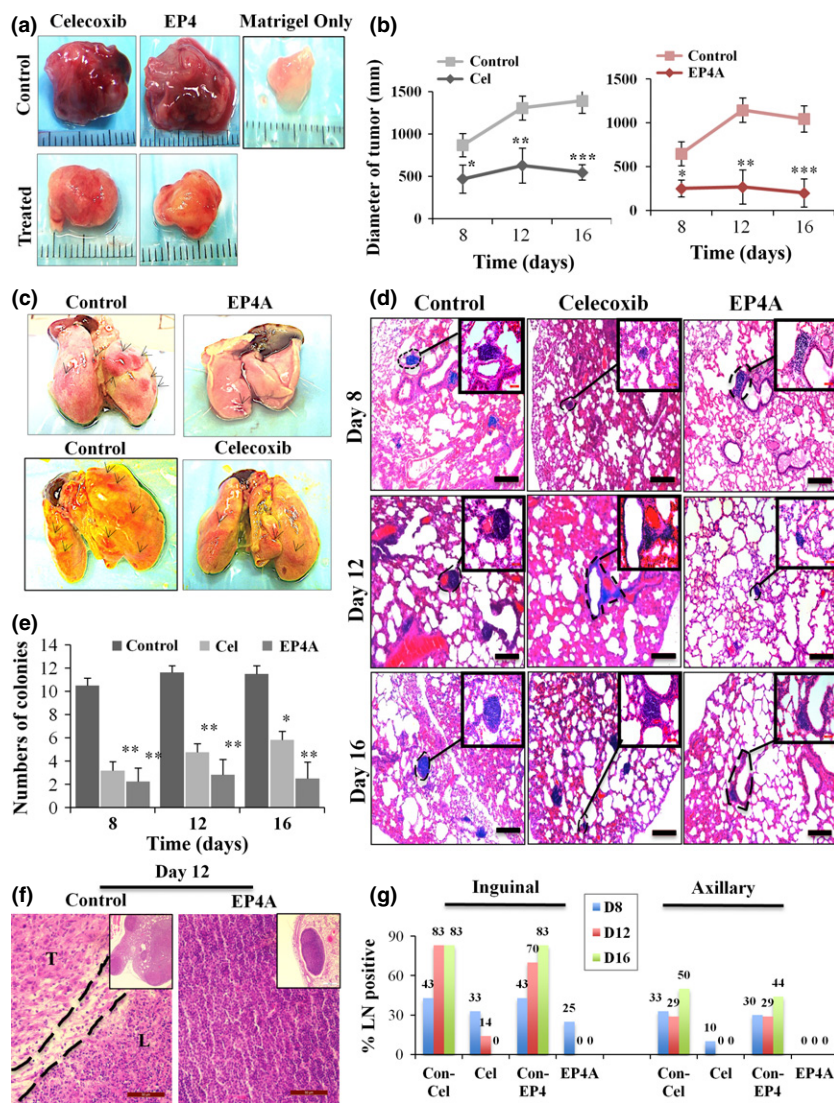


Fig. 1. Tumor growth (a, b), lung colonies (c–e), and lymph metastasis (f, g) in a murine C3L5 breast cancer model. (a) Representative images of tumor-inclusive Matrigel or Matrigel only implants retrieved on day 16 (a scale in mm shown in the background). (b) Mean diameters of tumors were reduced significantly in mice treated with celecoxib or RQ-15986 (EP4A), compared to respective vehicle-treated controls. This reduction was significant at all time points for both therapies. Data represent means ($n = 16$ /group/day) \pm SE. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. (c) Digital camera photographs of surface lung colonies at day 12 (black arrows) on left. (d) Representative images of micrometastases in H&E stained lung sections on days 8, 12, and 16. Scale bar = 200 μ m. (e) Charts showing mean numbers of metastatic lung colonies were reduced with celecoxib (Cel) and EP4A therapies at all time points after tumor transplantation. Data represent mean \pm SE ($n = 8$ /group/day). * $P < 0.05$; ** $P < 0.01$. (f) Histological pictures (H&E stained) of representative inguinal lymph nodes in control (vehicle-treated) and EP4A-treated mice on day 12, including low power views in the inset. Control lymph node (L) is nearly completely replaced by tumor cells (T). Lymph node treated with EP4A is tumor-free. Scale bar = 50 μ m. (g) Column graphs show percent of lymph nodes (LN) with metastasis in control (Con) and treated mice at days 8, 12, and 16.

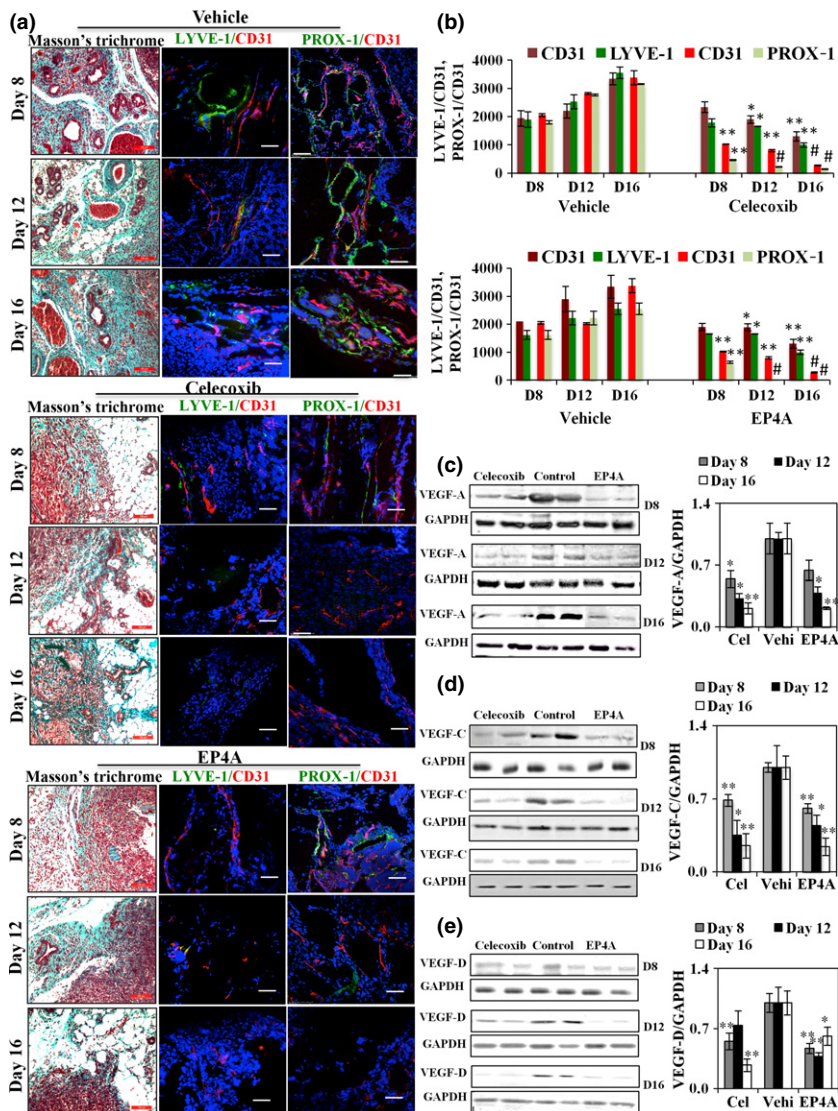


Fig. 2. (a, b) Angiogenesis and lymphangiogenesis in a murine C3L5 breast cancer model. (a) Bright field images of Masson's trichrome stained sections of tumor showing peritumoral stroma inclusive of vessels (scale bar = 200 μ m), and fluorescence images of corresponding tumors representing "hot spots" within the tumors. Tumor-associated angiogenesis (red, CD31 immuno-staining) and lymphangiogenesis (green, LYVE-1 and PROX1 immunostaining) are shown as representative fluorescent merged images. (b) Quantification of the hot spots of vascularity per unit area ($n = 16$, using the mean of three hot spots from each of the 16 tumors per group) \pm SE. * $P < 0.05$; ** $P < 0.005$; # $P < 0.0005$. Therapy with celecoxib and RQ-15986 (EP4A) reduced the levels of both angiogenesis and lymphangiogenesis. (c–e) Immunoblots for vascular endothelial growth factor (VEGF)-A/C/D. Tumor lysate proteins ($n = 16$, pooled from 16 tumors/group, triplicate measurements, shown in duplicate) were subjected to Western blots for VEGF-A (c), VEGF-C (d), VEGF-D (e), and GAPDH. Both celecoxib (Cel) and EP4A therapies reduced the levels of VEGF-A, -C, and -D proteins in residual tumors as revealed with densitometry, relative to GAPDH (normalized to vehicle (Vehi)-treated controls). Data represent mean \pm SE. * $P < 0.05$; ** $P < 0.001$. D, day.

We used the following primary antibodies: polyclonal rabbit anti-mouse VEGF-A (1:200), polyclonal goat anti-mouse VEGF-C (1:200), monoclonal rabbit anti-mouse VEGF-D (1:200), monoclonal anti-mouse GAPDH (Santa Cruz Biotechnology), polyclonal rabbit anti mouse Akt (cat #9272), mouse monoclonal phospho-Akt (Ser 473) (cat #4051S), and polyclonal rabbit anti-mouse antibodies to detect total ERK1/2 and phospho-ERK1/2 proteins (1:1000 dilution) (Cell Signaling Technology, Danvers, MA, USA). IRDye polyclonal secondary antibodies from LI-COR (Lincoln, NE, USA), either donkey anti-goat or goat anti-rabbit and donkey anti-mouse were used. Dilution for all VEGFs was 1:5000; for GAPDH, it was 1:20 000. Membranes were scanned on an Odyssey infrared imaging system (LI-COR).

Proliferative/apoptotic cell ratios. Proliferative/apoptotic cell ratios were measured in tumors from drug- and vehicle-treated mice by dual immune staining for Ki-67 (proliferation marker) and TUNEL (apoptosis marker).⁽¹⁰⁾

Detection of EP4, F4/80, and VEGF-C/D in cells. EP4 was localized in tumor cells *in vitro* and tumor tissues *in vivo* by immunostaining with a highly specific antibody (sc-20677; Santa Cruz Biotechnology), replacing the primary antibody with

immune IgG in negative controls. EP4 expression in tumor infiltrating macrophages was identified with dual immunostaining of EP4 and F4/80 (sc-71085; Santa Cruz Biotechnology) in frozen sections. Macrophages within tumors expressing VEGF-C/D were identified by dual immunostaining with F4/80 and VEGF-C/D antibodies (sc-9047 and sc-13085 respectively; Santa Cruz Biotechnology).

Statistical analysis. Data were analyzed using GraphPad Prism, GraphPad Software, La Jolla, CA, USA. All parametric data were compared with one-way ANOVA followed by Tukey–Kramer or Dunnett post hoc comparisons; two datasets were compared with Student's *t*-test. Statistically significant differences were accepted at $P < 0.05$.

Results

Proliferation, migration, and VEGF-C and -D production inhibited by EP4A in C3L5 cells *in vitro*. The COX-1/COX-2 inhibitor indomethacin, COX-2 inhibitor NS-398 (both at 20 μ M), as well as EP4A (10–5000 nM), all inhibited C3L5 cell proliferation and migration (Fig. S1, respectively). Production of VEGF-C and -D by C3L5 cells (data not shown) after treatment with

EP4A (50–5000 nM) was inhibited at a level similar to that reported with another EP4 antagonist (ONO AE3-208, 2 μ M).⁽¹⁰⁾

Celecoxib and EP4A treatments reduced tumor growth and spontaneous lung metastasis. Implants of Matrigel alone appeared as clear avascular jelly beans (Fig. 1a, shown for day 16) at all time points with no evidence of angiogenesis or lymphangiogenesis, as previously reported.^(10,38) Tumor growth was significantly blocked (at all time points) with both EP4A (as early as day 8) and celecoxib treatments (Fig. 1b). Spontaneous lung metastases are shown in Figure 1(c), as surface colonies and microscopic colonies (Fig. 1d) on day 12. The incidence of microscopic colonies was high in vehicle-treated mice on day 8, with a further small increase on days 12 and 16. There was a significant reduction of lung colonies at all time points with celecoxib and EP4A treatments (Fig. 1e).

Celecoxib and EP4A therapies abrogated metastasis to lymph nodes. Histopathological analysis of H&E stained sections of all lymph nodes collected from vehicle and drug-treated groups was used to compile the data presented in Figure 1(g). Representative H&E images of day 12 lymph node sections of control and EPA-treated mice are shown

on Figure 1(f). As metastasis at the single cell or small cell cluster level was not identifiable reliably on histological grounds, many putative lymph nodes showing only tumor cells and no lymph node architecture were excluded from the analysis. Thus, the above incidence based on scoring 6–10 nodes in each group can be considered as minimal in all groups.

Celecoxib and EP4A therapies inhibited tumor-associated angiogenesis and lymphangiogenesis. Masson's trichrome staining of tumor sections adjacent to those used for fluorescence microscopy showed blood vessels containing red blood cells, most abundant in the peritumoral stroma (Fig. 2a). High levels of angiogenesis (identified with CD31) and lymphangiogenesis (identified with LYVE-1 and PROX1 immunostaining) of "hot spots", as shown in merged pictures, were evident in all vehicle-treated mice at all time points (days 8, 12, and 16) with very little temporal change (Fig. 2a). Treatments with EP4A as well as celecoxib significantly blocked both events as early as day 8 (Fig. 2b).

Therapy with celecoxib and EP4A reduced levels of VEGF-C and -D proteins in residual tumors. As both EP4A and celecoxib significantly reduced angiogenesis and lymphangiogenesis, we examined the expression levels of VEGF-A/C/D proteins in

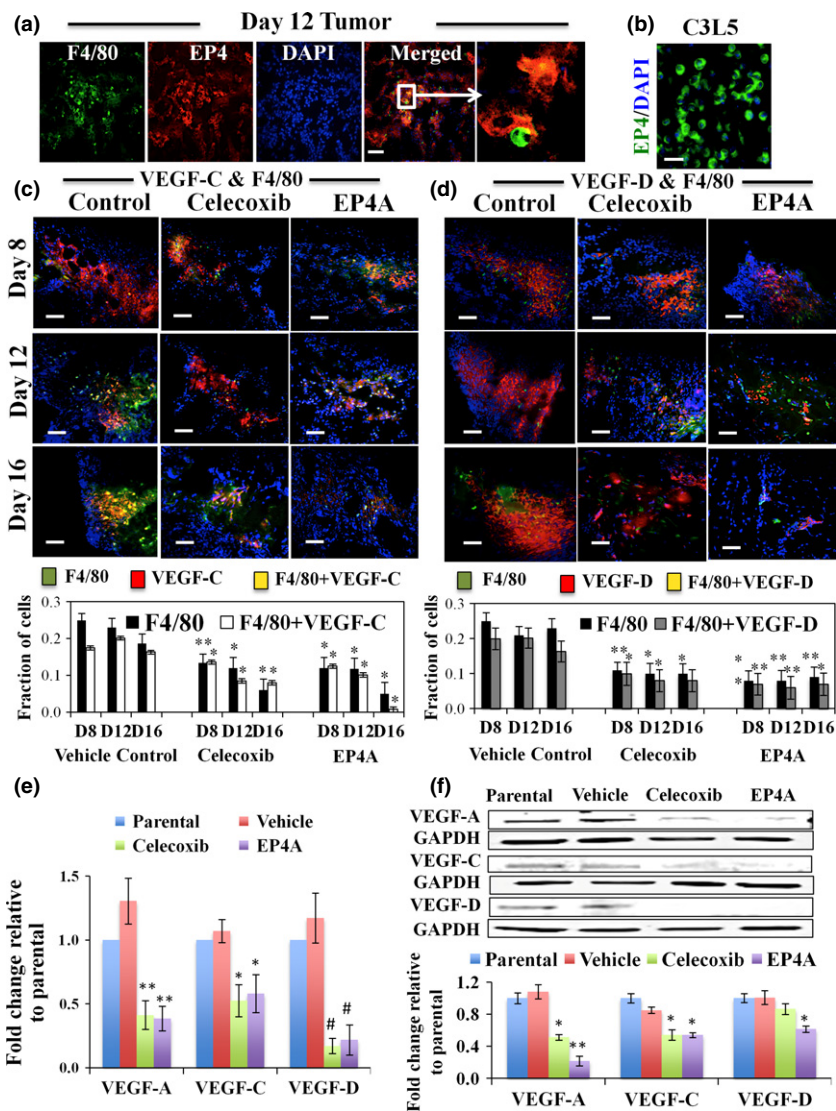


Fig. 3. Tumor infiltrating macrophages express EP4 in a murine C3L5 breast cancer model. (a) Day 12 vehicle-treated tumor sections showing F4/80-positive cells (green), expressing EP4 (red) in single channels and yellow in merged channel. The inset is shown enlarged in the next panel. Every nucleated cell (nuclei stained blue with DAPI) inclusive of tumor cells was EP4-positive. (b) Cytoplasmic staining of EP4 in C3L5 cells in culture. (c, d) Treatments with celecoxib and RQ-15986 (EP4A) reduce the incidence of macrophages and vascular endothelial growth factor (VEGF)-C/D-producing macrophages. Merged immunofluorescence images of tumor sections in vehicle-treated control or drug (celecoxib or EP4A)-treated mice showing staining for F4/80 (green), (c) VEGF-C (red, left panel), or (d) VEGF-D (red, right panel) and nuclei (blue for DAPI). Double-stained cells appear yellow. As the vehicle controls for both drugs were similar, a single control is shown. Bottom charts showing incidence of macrophages expressing VEGF-C (bottom left) or VEGF-D (bottom right). Scale bar = 50 μ m. ($n = 16$, using the mean of three hot spots from each of the 16 tumors per group) \pm SE. * $P < 0.005$; ** $P < 0.0005$. (e, f) Treatments with celecoxib and EP4A reduce the VEGF-A/C/D production of macrophages. RAW 264.7 cells treated for 24 h with celecoxib and EP4A significantly reduced VEGF-A/C/D production tested with (e) quantitative PCR and (f) Western blots. Data represents mean fold change ($n = 3$) \pm SE. * $P < 0.05$; ** $P < 0.005$; # $P < 0.0005$.

residual tumors. Both therapies significantly reduced the levels of VEGF-A, -C, and -D proteins (Fig. 2c–e, respectively) in residual tumors.

Celecoxib and EP4A treatments reduced the incidence of TAMs expressing EP4, VEGF-C/D *in situ*. Nearly every nucleated cell (DAPI stained) inclusive of tumor cells and macrophages (F4/80 stained) within tumors expressed EP4 (Fig. 3a), as did C3L5 cells in culture (Fig. 3b). As shown in Figure 3(c,d), control tumors in vehicle-treated mice showed an appreciable incidence (25–30%) of F4/80 positive macrophages within the tumor parenchyma. Approximately 75–90% of these macrophages produced VEGF-C/D *in situ* in control mice, shown by dual immunostaining for F4/80 and VEGF-C or -D (Fig. 3c,d). Residual tumors collected from treated mice revealed markedly reduced incidence of total as well as VEGF-C/D positive macrophages amongst nucleated cell population, indicating that the cyto-reductive effects of EP4A were higher on the macrophages than on tumor cells. There was no significant change in the incidence of VEGF-C or VEGF-D-bearing macrophages (75–90%) between control and treated mice. Macrophage cells (RAW 264.7) treated *in vitro* with COX-2 inhibitor and EP4A, showed significantly reduced VEGF-A/C/D production (Fig. 3e,f).

Therapy with celecoxib and EP4A reduced phosphorylated Akt and ERK in residual tumors, concomitant with an increase in apoptotic/proliferative cell ratios. As EP4, unlike EP2, is known to stimulate the PI3K/Akt and Ras/MAPK pathways,⁽²⁰⁾ we examined the activation levels of both Akt and ERK in residual tumors in treated mice. Treatment with EP4A as well as celecoxib effectively reduced the levels of phosphorylation of both ERK and Akt measured with Western blots (Fig. 4) and there was a concomitant increase in apoptotic/proliferative cell ratios (Fig. S2).

Assessment of drug-related toxicity. We found no significant differences in daily water intake between vehicle-treated and drug-treated mice (data not presented). Furthermore, during autopsy on day 16, none of the mice revealed visible bleeding in the gastrointestinal tract, indicating that at the current dosage the drugs were non-toxic.

Stem-like cell-associated functions and phenotype of C3L5 cells *in vitro* and *in vivo* are dependent on COX-2 and EP4 activity. Tumorsphere forming ability was used as an *in vitro* surrogate of SLC function.⁽³⁹⁾ C3L5 cells quickly formed spheroids for successive generations with the lowest dilution of 1 cell/well (Fig. 5a). Tumorsphere formation (both size and number) *in vitro* was significantly reduced by treatment with celecoxib or EP4A at non-toxic concentrations for successive generations (Fig. 5a) or by knockdown of COX-2 or EP4 in C3L5 cells (Fig. 5b). Figure S3 shows COX-2 and EP4 mRNA levels after knockdown.

We tested co-expression of COX-2 and breast cancer stem cell markers CD44 and ALDH1A, ES cell markers SOX-2 and OCT-3/4, and hematopoietic stem cell marker β -catenin in frozen sections of both tumorspheres formed *in vitro* (Fig. 6a) and day 12 tumors *in vivo* (Fig. 6b). Knockdown of COX-2 resulted in a significant reduction of marker-bearing cells within the spheroids (image in Fig. 6a, upper; quantitated in Fig. 6a, bottom). Similarly, treatments of tumor-bearing mice with celecoxib and EP4A significantly reduced the incidence of marker-bearing cells in residual tumor sections, as compared to those in mock or vehicle-treated control mice (image in Fig. 6b, upper; quantitated in Fig. 6b, bottom). A dramatic reduction (to <20%) in spheroid forming efficiency as well as spheroid sizes after EP4 knockdown precluded immunostaining of spheroids for SLC or ES cell markers.

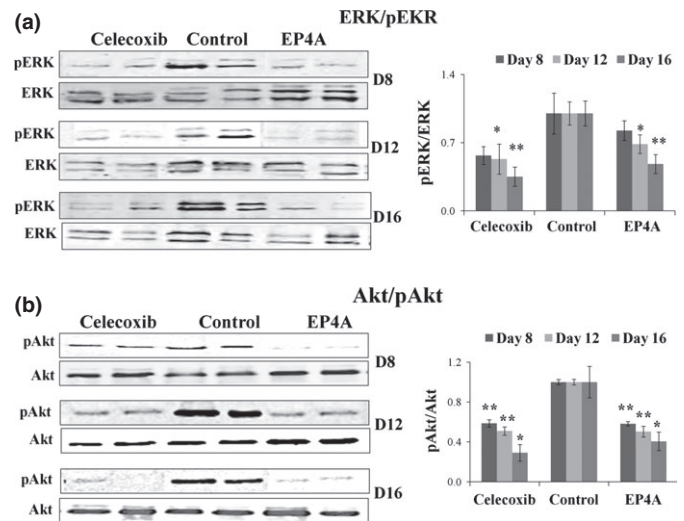


Fig. 4. Therapy with celecoxib and RQ-15986 (EP4A) reduced the levels of phosphorylation (p) of both ERK and Akt proteins in residual breast tumors at all time intervals. Total tumor lysate proteins (pooled from 16 tumors per group and experiments run in triplicate, but only duplicate shown) were subjected to Western blot analysis for (a) pERK and total ERK or (b) pAkt and total Akt proteins. Densitometric measurements of phosphorylated *versus* total ERK (upper right) or Akt (bottom right) proteins relative to vehicle controls (mean of 16 ± 5 SE). * $P < 0.005$; ** $P < 0.001$.

Discussion

In earlier studies, using high COX-2-expressing C3L5 mammary adenocarcinoma cells we showed that primarily EP4, and to a minor extent EP1, activity promoted cellular migration.⁽⁷⁾ Furthermore, a COX-2 inhibitor celecoxib and an EP4 antagonist ONO AE3-208, but not an EP1 antagonist ONO-8713, reduced VEGF-C and -D production by the tumor cells *in vitro*, and inhibited tumor growth, tumor-associated angiogenesis, and lymphangiogenesis, as well as metastasis to the lungs and lymph nodes *in vivo*.⁽¹⁰⁾ In that study, residual tumors in treated mice showed reduced VEGF-C and -D proteins, Akt phosphorylation, and increased apoptotic/proliferative cell ratios. The present study confirmed all these results in the same tumor model with another EP4 antagonist, RQ-15986. In addition, we show here that: (i) tumorsphere formation and growth were reduced with a COX-2 inhibitor, COX-2 or EP4 knockdown, and also with EP4A, indicating their dependence on COX-2 and EP4 expression or activity; (ii) macrophages produced VEGF-A/C/D *in vitro*; (iii) tumor-infiltrating macrophages expressed EP4 and further contributed to VEGF-C and -D production *in situ* in promoting lymphangiogenesis; (iv) VEGF-C/D production *in situ* was curtailed with EP4A; and (iv) there was reduced ERK phosphorylation in the treated residual tumors. Taken together, these findings show the key role of EP4 receptor on tumor cells as well as TAMs in multiple cellular events required for tumor progression and metastasis, which can be effectively blocked with currently available EP4 antagonists.

Among four EP receptors, EP2 and EP4 are both Gs linked and thus their activation leads to an increase in intracellular cAMP followed by activation of PKA.⁽⁴⁰⁾ In addition, EP4 activation can also stimulate ERK⁽⁴¹⁾ and PI3K/Akt⁽⁴²⁾ pathways, promoting PGE-2 dependent migration⁽⁴³⁾ and cell survival, respectively.⁽⁴²⁾ In the present study, residual tumors in mice treated with celecoxib or EP4A showed a parallel reduction of

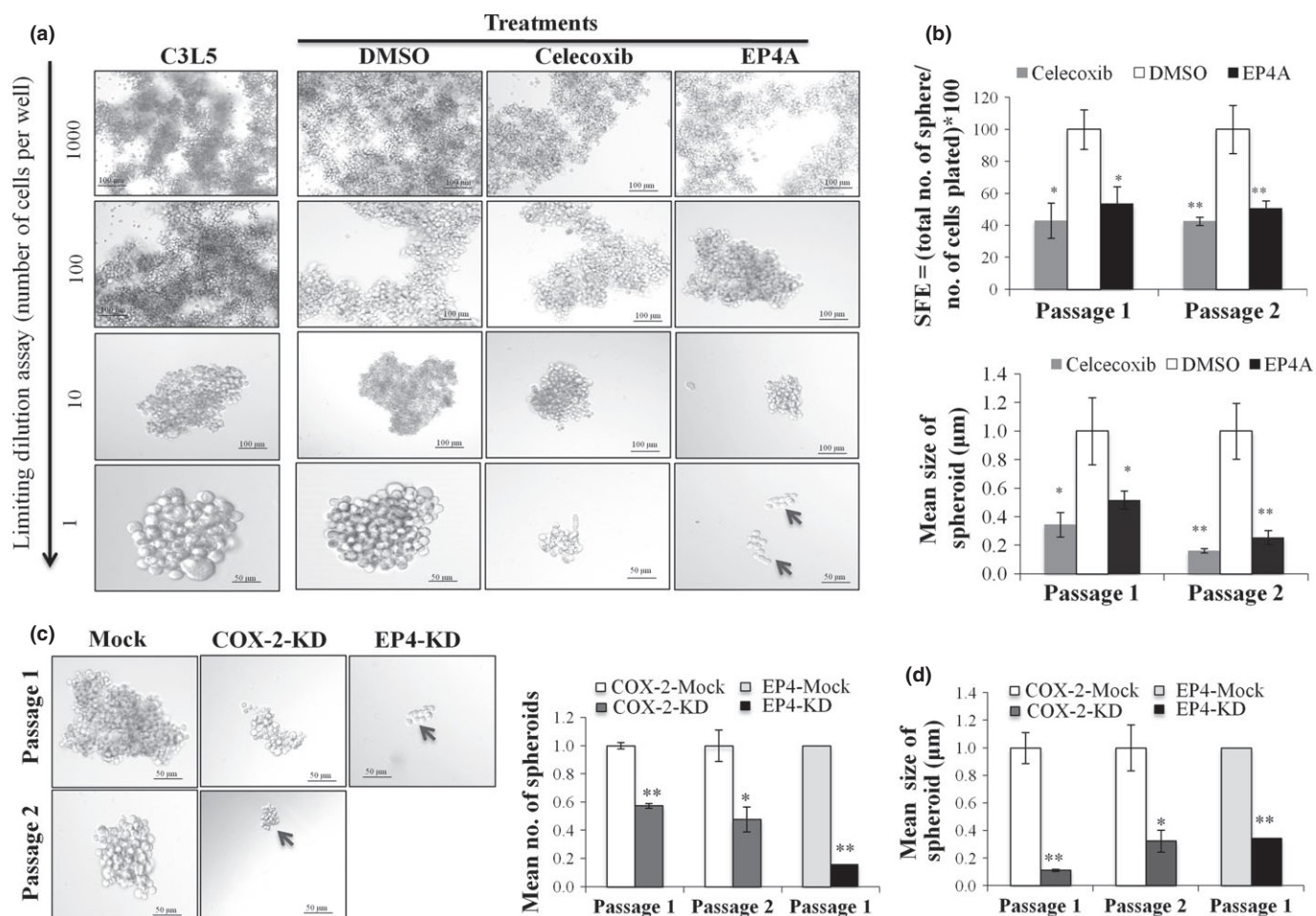


Fig. 5. (a) Treatment with celecoxib or RQ-15986 (EP4A) significantly blocked tumorsphere-forming ability of C3L5 breast cancer cells. With limiting cell dilutions (1000, 100, 10, or 1 cell/100 μ L), C3L5 cells rapidly formed spheroids for successive generations (without overcrowding at 1 cell/well). Spheroid formation was inhibited with celecoxib (2 μ M) and EP4A (2 μ M) at successive passages. (b) Both spheroid forming efficiency (SFE) and sizes (representing spheroid perimeters), shown for serial passages, were significantly reduced with treatments. (c) Knockdown of COX-2 (COX-2-KD) (with shRNA plasmid) and EP4 (EP4-KD) (with siRNA) in C3L5 cells significantly reduced spheroid formation. (d) Quantitative measurement presented as numbers and sizes of spheroids showing that COX-2 knockdown can reduce spheroid formation for successive generations and with EP4 transient knockdown for one generation. Data represents mean of three experiments \pm SE. * P < 0.005; ** P < 0.001.

Akt and ERK phosphorylation, concomitant with an increase in apoptotic/proliferative cell ratios. Our *in vitro* studies with C3L5 cells (Xin X and Lala PK, 2014, unpublished data) have validated the need for PI3K/Akt for cell survival and ERK1/2 for migratory functions. Taken together, the present results indicate that a blockade in the above EP4 signaling pathways contributed to antitumor and antimetastatic effects of the drug. However, a contribution of PKA, shared by EP2/EP4 cannot be excluded.

Prostaglandin E2 produced by tumor cells has been shown to contribute to the immunosuppressive function of TAMs. For example, tumor-derived PGE-2 was shown to inhibit the production of T-cell stimulating chemokine CCL5 by LPS-activated macrophages, which could be restored with COX-2 inhibitor or EP2/EP4 antagonists.⁽⁴⁴⁾ Peritumoral lymphangiogenesis is a well-known phenomenon in human breast cancer that promotes lymphatic invasion and metastasis.^(13,34)

In the present study, we show that TAMs expressed EP4 and produced VEGF-C/D. Furthermore, therapy with EP4A reduced the incidence of VEGF-C/D-producing macrophages *in situ*. This could be an indirect effect of the blockade of PGE-2 production by EP4-bearing tumor cells, leading to

reduced migration of VEGF-C/D producing macrophages into the tumor site, or a direct effect on macrophages to block VEGF-C/D production by these cells, or both. As there were no major differences in VEGF-C or -D expression (75–90%) of macrophages between vehicle-treated *versus* drug-treated tumors, we could not clearly distinguish between these possibilities. However, as previously reported,^(33,34) our *in vitro* results revealed that macrophages produced VEGF-A/C/D, and their production was inhibited with both celecoxib and EP4A. Thus, reduced macrophage migration as well as VEGF-C/D production by macrophages *in situ* in EP4A-treated mice would contribute to reduced lymphangiogenesis. We have further evidence for EP4 activity on lymphatic endothelial cells contributing to tumor-induced lymphangiogenesis.⁽⁴⁵⁾ These findings, taken together, may explain the role of host EP4 in metastasis promotion.⁽⁴⁶⁾

Stem-like cells constitute a minor subpopulation of tumor cells, which can perpetuate tumors and escape traditional therapies.^(24,25) The tumorsphere assay is an *in vitro* surrogate of SLC function *in vivo*.⁽³⁹⁾ We show here that C3L5 cells formed tumorspheres at the single cell level for successive generations, and both COX-2 inhibitor and EP4A inhibited this

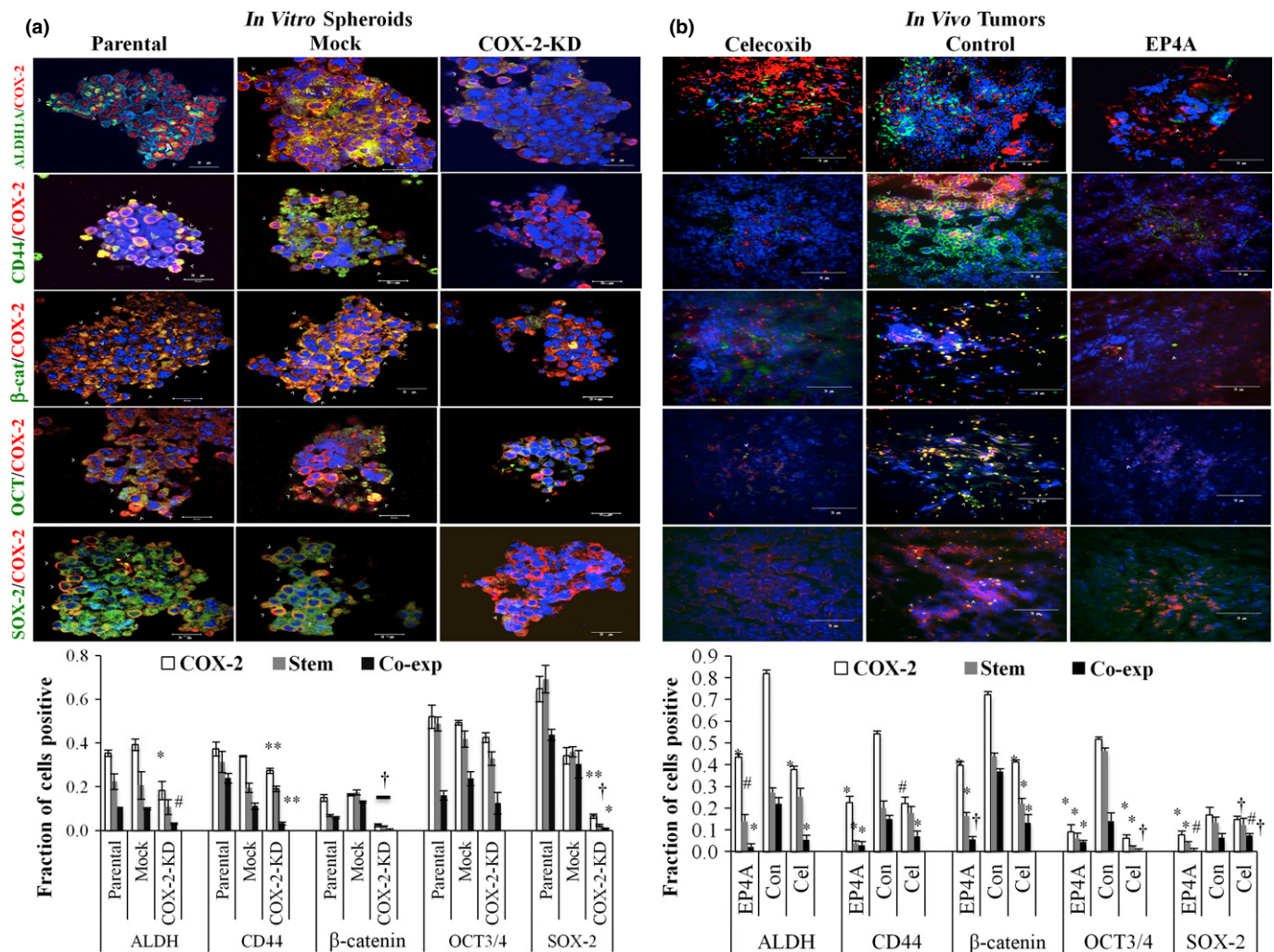


Fig. 6. Co-expression (Co-exp) of COX-2 and several stem cell markers in frozen sections of C3L5 breast carcinoma tumorspheres (parental, mock, COX-2 knockdown [KD]) *in vitro* and in day 12 tumors in control and treated (with celecoxib and RQ-15986 [EP4A]) mice. (a) COX-2 knockdown in C3L5 cells reduced the incidence of stem cell marker-bearing cells and cells co-expressing COX-2 and any of the stem cell markers in tumorspheres (image, top left; quantification, bottom left). Data represent mean ($n = 3$) \pm SE. * $P < 0.0001$; # $P < 0.004$; † $P < 0.05$. (b) *In vivo* treatments with celecoxib (Cel) or EP4A also reduced the incidence of marker bearing cells in residual tumors, day 12 tumor sections (image, top right; quantification, bottom right). Data presented as ($n = 16$, using the mean of three hot spots from each of the 16 tumors per group) \pm SE. ALDH, aldehyde dehydrogenase; Con, control.

ability. Similarly, COX-2 and EP4 knockdown produced profound inhibitory effects. Numerous SLC-associated markers such as CD44⁽⁴⁷⁾ and ALDH1⁽⁴⁸⁾ have been reported in breast cancer cells. OCT-4 was implicated as an SLC marker within MCF-7 breast cancer cell-derived mammospheres.⁽⁴⁹⁾ Embryonic stem cell markers OCT-3/4 and SOX-2⁽⁵⁰⁾ and hematopoietic stem cell marker β -catenin have also been reported to be associated with SLCs in cancer cells.⁽⁵¹⁾ Although COX-2 is not considered as an SLC marker, we found co-expression of CD44, ALDH, β -catenin, OCT 3/4, and SOX-2 in a subset of COX-2-positive cells within the tumorspheres as well in tumors *in vivo*, reinforcing their SLC phenotype. Although very little is known about the molecular pathways responsible for SLC induction, it is likely that multiple pathways can converge on the phenotypes that represent SLCs. Tumor-associated macrophages can contribute to an SLC-stimulating tumor microenvironment⁽⁵²⁾ and conversely, SLCs can recruit myeloid-derived tumor-promoting TAMs.⁽⁵³⁾ We suggest that COX-2 and the PGE-2/EP4 signaling axis are partners in SLC induction in breast cancer. This suggestion is supported by: (i)

a reduction in tumorsphere formation with COX-2 inhibition or EP4 antagonism or gene knockdown of COX2/EP4 *in vitro*; and (ii) a reduction of the marker-bearing cells in residual tumorspheres after COX-2 knockdown *in vitro* or residual tumors *in situ* in EP4A-treated mice. A recent report on the role of EP4 in promoting the SLC phenotype in breast cancer cells concurs with our results.⁽²⁷⁾

Long-term use of COX-2 inhibitors at higher doses has raised concerns about increased thrombo-embolic incidents.^(16,17) By IP gene disruption, combined with the use of IP agonists, IP receptors interacting with PGI-2 were shown to be obligatory for the vaso-protective roles of COX-2.⁽¹⁸⁾ Since EP4 antagonists do not inhibit PGI production, they should be safer than COX-2 inhibitors, a view that can only be tested with human trials. As shown earlier for ONO-AE3-208,⁽¹⁰⁾ presently used EP4A was also found to be non-toxic in mice at the current dosage regimen. Currently, unpublished results of a closely-related, well-characterized EP4 antagonist AAT-007 (CJ-023423),⁽⁵⁴⁾ used in several phase I/II human trials on osteoarthritis patients ($n > 800$) have validated its safety and tolerance at therapeutic

doses (Dr Yukinori Take, AskAt Inc./RaQualia Pharma Inc., Japan, personal communication presented with permission). Based on the evidence of multiple roles of EP4 on tumor and host cell-mediated mechanisms in breast cancer progression, we suggest that testing the use of EP4 antagonists as an adjuvant in human breast cancer is timely.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. We tested the effect of different COX-2 inhibitors (indomethacin, NS-398) and EP4 antagonists (CJ-0427994, GW627368X) on proliferation and migration of C3L5 cells.

Fig. S2. Therapy with RQ-15986 (EP4A) caused a reduction in proliferative cells and an increase in apoptotic cells.

Fig S3. Cyclooxygenase-2 and EP4 knockdown.