PGE$_2$/EP$_4$ Signaling Controls the Transfer of the Mammary Stem Cell State by Lipid Rafts in Extracellular Vesicles

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Key Words. PGE$_2$ • Stem cell homeostasis • Extracellular vesicle • Lipid raft • Exosome • EP$_4$ receptor

ABSTRACT

Prostaglandin E$_2$ (PGE$_2$)-initiated signaling contributes to stem cell homeostasis and regeneration. However, it is unclear how PGE$_2$ signaling controls cell stemness. This study identifies a previously unknown mechanism by which PGE$_2$/prostaglandin E receptor 4 (EP$_4$) signaling regulates multiple signaling pathways (e.g., PI3K/Akt signaling, TGF$eta$ signaling, Wnt signaling, EGFR signaling) which maintain the basal mammary stem cell phenotype. A shift of basal mammary epithelial stem cells (MaSCs) from a non-basal-MaSC state occurs in response to prostaglandin E receptor 4 (EP$_4$) antagonism. EP$_4$ antagonists elicit release of signaling components, by controlling their trafficking into extracellular vesicles/exosomes in a lipid raft/caveolae-dependent manner. Consequently, EP$_4$ antagonism indirectly inactivates, through induced extracellular vesicle/exosome release, pathways required for mammary epithelial stem cell homeostasis, e.g. canonical/noncanonical Wnt, TGF$eta$ and PI3K/Akt pathways. EP$_4$ antagonism causes signaling receptors and signaling components to shift from non-lipid raft fractions to lipid raft fractions, and to then be released in EP$_4$ antagonist-induced extracellular vesicles/exosomes, resulting in the loss of the stem cell state by mammary epithelial stem cells. In contrast, luminal mammary epithelial cells can acquire basal stem cell properties following ingestion of EP$_4$ antagonist-induced stem cell extracellular vesicles/exosomes, and can then form mammary glands. These findings demonstrate that PGE$_2$/EP$_4$ signaling controls homeostasis of mammary epithelial stem cells through regulating extracellular vesicle/exosome release. Reprogramming of mammary epithelial cells can result from EP$_4$-mediated stem cell property transfer by extracellular vesicles/exosomes containing caveolae-associated proteins, between mammary basal and luminal epithelial cells. STEM CELLS 2017;35:425–444

SIGNIFICANCE STATEMENT

Recent studies have demonstrated that PGE$_2$-initiated signaling contributes to stem cell (SC) homeostasis and regeneration; but how? Our study identifies a previously unknown mechanism by which PGE$_2$/EP$_4$ signaling regulates both multiple signaling pathways (e.g., PI3K/Akt signaling, TGF$eta$ signaling, Wnt signaling, EGFR signaling), which maintain stem cell homeostasis. PGE$_2$/EP$_4$ signaling controls homeostasis of mammary epithelial stem cells through regulating extracellular vesicle/exosome release. Reprogramming of mammary epithelial cells can result from EP$_4$-mediated stem cell property transfer by extracellular vesicles/exosomes containing caveolae-associated proteins, between mammary basal and luminal epithelial cells.

INTRODUCTION

A number of observations have pointed to the key role of prostaglandin E$_2$ (PGE$_2$) in maintaining cells in the stem-cell (SC) state. In the bone marrow, PGE$_2$ signaling supports hematopoietic stem cells (HSCs) [1, 2]. Regeneration of the hematopoietic lineage is impaired in mice lacking cyclooxygenase-2 (COX-2), a key enzyme in PGE$_2$ biosynthesis [3]. However, the evidence that PGE$_2$ signaling contributes to SC function in tissues outside of the hematopoietic system has been elusive.

Extracellular vesicles (EVs) (i.e., exosomes) can mediate intercellular communication by transferring membrane and cytosolic proteins, lipids, and RNAs between cells [4]. These transferred molecules can function in recipient cells [5, 6]. Although exosome-mediated communication has been demonstrated to be involved in antigen presentation, development of tolerance [7] and tumor progression [8, 9],...
the functions and regulation of exosomes are still poorly understood.

We find that blocking prostaglandin E receptor 4 (EP4) mediated-signaling in basal mammary epithelial stem cells (MaSCs) causes a transition of the cells to a non-stem, more epithelial phenotype. The basal MaSCs lose SC properties (e.g., mesenchymal morphology, migration/invasion ability, and mammosphere forming ability) in response to EP4 antagonists. EP4 antagonist treatment causes signaling proteins that are essential for basal MaSCs to maintain stem cell properties to shift into lipid rafts and to then be released via EVs. In contrast, uptake of EVs which carry SC signaling components in caveolae/lipid rafts drives conversion of mammary luminal cells to basal cell-like MaSCs able to form mammary glands.

MATERIALS AND METHODS

Cell Culture

Human mammary epithelial cell lines (HMLE) were generated as described [10]. HMLE and the non-adherent mammary epithelial cell (NAMEC) were propagated in MEGM medium (Lonza, Allendale, NJ) according to standard protocols. See also “Supporting Information Materials and Methods”.

Isolation of Primary Mouse Basal/Luminal Mammary Epithelial Cells

Mouse mammary epithelial cells were isolated from 12-week-old virgin, female C57BL/6 cell, using procedures previously described by Mroue et al. [11] with some modifications. After 3-day culture, the cells were detached in accutase (eBioscience, San Diego, CA) for 20 min, and then neutralized with 5%FBS in PBS. Cell aggregates were further treated with Dispase (STEMCELL Technologies, Vancouver, Canada) for 20 min. The single cells were then stained with anti-CD49f and anti-EpCAM antibodies as described in the FACs section. See also “Supporting Information Materials and Methods”.

Boyden Chamber Migration Assay

NAMECs (2.5 × 10⁴ cells/well) were added to the upper chambers of BD Matrigel™ Chambers, 8.0 µm (BD biosciences, San Jose, CA). After 24 hours, residual cells were removed from the top of the membrane and the cells on the underside of the membrane were washed in PBS, fixed in 4% PFA, then stained with 0.1% crystal violet for 20 minutes. See also “Supporting Information Materials and Methods”.

Mammosphere Formation

Mammosphere-forming analysis was performed as described by Dontu et al. [12]. Cells were plated in 96 wells of ultra-low attachment plates (Corning Life Sciences, Corning, NY) at 1 × 10³ in MammoCult™ medium (STEMCELL Technologies, Vancouver, Canada) supplemented with MammoCult™ proliferation supplements (STEMCELL Technologies, Vancouver, Canada), 4 µg/ml heparin, 0.48 µg/ml hydrocortisone, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% methyl cellulose. After 3 weeks, the number of spheres (diameter > 100 µm) of each well was calculated. See also “Supporting Information Materials and Methods”.

Fat Pad Injection and Mammary Gland Whole Mount

All research involving animals complied with protocols approved by the NHRI Committee on Animal Care. The endogenous mammary epithelium in the 4th glands of 3-week-old female C57BL/6 pups was cleared and cells were injected into cleared fat pads. Mice were euthanized 8 weeks after surgery. The 4th glands were removed and fixed overnight in Kahle’s fix and then stained overnight in carmine alum (Sigma-Aldrich, St. Louis, MO). See also “Supporting Information Materials and Methods”.

EV/Exosome Isolation

Culture media were centrifuged at 300 g for 5 min to remove cells (P1), at 2,000 g for 20 min (P2), then at 10,000 g for 30 min (P3) all at 4°C. Finally, EVs/exosomes (P4) were separated from the supernatant by centrifugation at 110,000 g for 60 min. The EV/exosome pellet was washed once in PBS and then resuspended in PBS for further analysis. See also “Supporting Information Materials and Methods”.

Electron Microscopy of EVs/Exosomes

EM analysis of EVs/exosomes was performed as described previously by van Niel et al. [13]. Briefly, EVs/exosomes purified as described above were fixed by 4% paraformaldehyde (PFA) in PBS and dropped on formvar/carbon–coated copper grids (Poly-sciences, Inc., Warrington, PA). After washing, the samples were stained with 2% phosphotungstic acid and then viewed for transmission EM (TEM) using an electron microscope (HT7700, Hitachi, Tokyo, Japan). See also “Supporting Information Materials and Methods”.

Proteomic Analysis

EVs were collected and lysed with RIPA extraction buffer. Protein samples were trypsin-digested and treated with iodoacetamide for carboxamidomethyl modification. Proteomic experiments were performed using a LTQ-Orbitrap system (Thermo Fisher, Waltham, MA ) and MS/MS; protein identification was carried out using the Bioworks 3.3 (Thermo Fisher, Waltham, MA). Gene Ontology Analysis and GeneGo software were used for system biology analysis.

Micro-Western Array

Micro-western array analysis was performed at the NHRI Micro-Western Array core facility, using procedures described by Ciaccio et al. [14]. Briefly, the EV protein was extracted with RIPA lysis buffer, spotted on a micro-gel using a microarrayer, subjected to semidry electrophoresis, transferred to PVDF membranes, and probed with antibodies. The signals were scanned using the LI-COR Odyssey imager at 24 µm resolution.

Statistical Analysis

Data are presented as means ± SEM. Student’s t test was used to compare two groups. P < 0.05 was considered significant.

See “Supporting Information Materials and Methods” for more information.

RESULTS

EP4 Signaling in Mammary Basal Stem Cells and Their Epithelial Cell Counterparts

PGE₂ is the major prostanooid known to modulate stem cell development [1, 2]. In the mammary gland, cyclooxygenase 1 and cyclooxygenase 2 (COX-1 and COX-2) convert arachidonic
acid to PGH₂; Prostaglandin E Synthase-1 (mPGES-1) then catalyzes the isomerization of PGH₂ to PGE₂. To address the role of PGE₂ in regulating MaSC properties, we began by determining COX-2 and mPGES-1 expression in the human mammary gland. COX-2 expression was largely confined to basal cells of the mammary ducts, which are known to contain a basal MaSC subpopulation [15, 16] (Fig. 1A), whereas mPGES-1 was expressed in both basal and luminal cells.
EP₄ Receptor Antagonist Modulates the Morphology and Motility of Basal Stem NAMECs

GW treatment induced an epithelial morphology in NAMECs within 48 hours (Fig. 1H and Supporting Information Movie 1). The mesenchymal NAMECs (Fig. 1I), marked with colored dots; (figures excerpted from Supporting Information Movie 1) formed cobblestone-like, epithelial islands in the presence of GW. In contrast, NAMECs cultured in the absence of GW maintained their preexisting mesenchymal morphology (Supporting Information Movie 2). We conclude that blocking EP₄ signaling induced a mesenchymal-to-epithelial transition (MET) in NAMECs. The GW-treated NAMECs that had undergone a complete MET formed epithelial islands which became more resistant to trypsinization; while NAMECs were completely disassociated using 0.05% trypsin, ~80% of the GW-treated NAMECs were resistant to disassociation by 0.05% trypsin treatment (Fig. 1J).

NAMECs were pre-treated with the GW EP₄ antagonist for 4 days, then subjected to a migration assay, in the absence of GW, for 24 hours. The number of migrating cells was decreased ~60% by GW pre-treatment (Fig. 1K). Blocking EP₄-mediated signaling caused a rapid loss of both mesenchymal morphology and migratory ability of basal MaSCs.

EP₄-Mediated Signaling Modulates the Basal Stem Cell Surface Markers of NAMECs

GW treatment also caused decreases of the level of basal stem cell surface markers [22–24] on the basal stem cells. The 4-day GW treatment decreased the NAMEC surface levels of CD44, CD90, Integrin β1 [CD29], Integrin α3 [CD49c], and Integrin α6 [CD49f] (Fig. 1L). Long-term GW treatment for 19 days further decreased the surface CD44 level of NAMECs to the level of HMLE cells (Supporting Information Fig. 1C). Blocking EP₄-mediated signaling elicited NAMEC transit from the basal SC state to the non-basal SC state.

Figure 1. EP₄-mediated signaling is required to maintain mesenchymal/stem cells of the mammary epithelium (MaSCs). (A): The distribution of COX-2 (in green) and cytokeratin 8 (in red) in the mammary gland was analyzed by immunofluorescence. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, in blue). Scale bar, 50 μm. (B): Left: Bright-field microscopy: images of immortalized human mammary epithelial cells (HMLE) and NAMECs, the spontaneously basal/stem population derived from HMLE cells. Scale bar, 100 μm. Right: Numbers of mammospheres formed by HMLE cells and NAMECs. Data are means ± SEM (n = 5). ***p ≤ .001 (C): EpCAM marker profiles of HMLE cells and NAMECs were analyzed using flow cytometry. Blue line: HMLE cell. Red line: NAMEC. (D) COX-2 and EP₄ receptor protein expression in HMLE cells and NAMECs. Loading control, GAPDH. (E) Cell surface EP₄ expression in NAMECs and HMLE cells. Orange line: cells stained with anti-EP₄ antibody + Alexa 488-conjugated secondary antibody; Blue line: cells were stained with only Alexa 488-conjugated secondary antibody as control. Red line: unstained cells as negative control. (F) The distribution of EP₄ receptors (green) in HMLE cells and NAMECs was analyzed by immunofluorescence. The Golgi apparatus was stained with anti-Golgin-97 antibodies (red). Cell nuclei were stained with DAPI (blue). Scale bar, 20 μm. (G) Cell surface EP₄ receptor expression of NAMECs treated with vehicle (Crl), PGE₂ (1 μM) or GW627368X (GW) for 30 minutes. Blue line: cells stained with anti-EP₄ antibody + Alexa 488-conjugated secondary antibody; Red line: cells were stained with only Alexa 488-conjugated secondary antibody as control. (H): Bright-field images of NAMECs treated with vehicle (Crl) or GW627368X for 0 and 48 hours. (I): Bright-field images of GW-treated NAMECs were taken at 0, 3, 8.5, 10.5, 26, 40, 45, and 48 hour(s) after the beginning of the treatment. See Supporting Information Movie 1 for the time-lapse data. The images were excerpted from the movie. Colored dots track dividing cells. The cells labeled with the same color are the descendants of a single cell. Scale bar, 20 μm. (J): Percentage of vehicle-treated and GW627368X-treated NAMECs resistant to 0.05% trypsin. NAMECs were treated with vehicle (Crl), or GW627368X (1 μg/ml) for 4 days, then dissociated with 0.05% trypsin. Data are means ± SEM, (n = 3). *p ≤ .05 (K): Migration in Boyden chambers of NAMECs pre-treated with vehicle (Crl) or GW627368X for 4 days. The treatment was suspended during the migration assay. Data are means ± SEM (n = 4). ***p ≤ .001 (L): Cell surface CD44, CD90, integrin β1, integrin α3, and integrin α6 in NAMECs treated with vehicle (Crl) or GW627368X (GW, 1 μg/ml) for 4 days. Blue line: cells stained with specific primary antibody + Alexa 488-conjugated secondary antibody; Red line: cells stained only with Alexa 488-conjugated secondary antibody as control. (M): Numbers of mammospheres formed by NAMECs treated with vehicle (Crl) or GW627368X for 7 days. The treatment was suspended during sphere formation. Data are means ± SEM (n = 4). **p ≤ .01. Abbreviations: HMLE, Human mammary epithelial cell lines; NAMEC, non-adherent mammary epithelial cell.
EP₄ Receptor Antagonism Reduces NAMEC Mammosphere Formation

Sixty percentage of the mammosphere-forming ability of the NAMEC population was eliminated by inhibiting EP₄-mediated signaling with GW for a week prior to initiating the assays (Fig. 1M). Since drug treatment was suspended during the assays, the alteration caused by the transient treatment with the EP₄ antagonist was maintained after the drug...
treatment ceased. Interruption of EP4-mediated signaling resulted in a substantial loss of the mammosphere-forming basal SC phenotype. Blocking PGE2/EP4 signaling causes basal MaSCs to lose basal SC characteristics, including surface marker profiles, mesenchymal morphology, migratory ability, and mammosphere-forming ability.

**EP4 Antagonism Causes SCs to Release SC Surface Markers and Integrins via Extracellular Vesicles**

To determine whether EP4 antagonist-induced loss of the stem cell phenotype is the result of the loss of cellular components, we examined the conditioned media from EP4 antagonist-treated MaSCs. The conditioned media of HMLE cells and NAMECs treated with vehicle or with GW for 4 days were collected and analyzed for CD44 and GAPDH proteins. The conditioned media were subjected to differential centrifugation at 300g (P1), 1200g (P2), 10,000g (P3), and 110,000g (P4). Dead cells and cell debris were removed at 300g (P1) and 1200g (P2) [25]. Large amounts of CD44 and GAPDH protein were detected in the P4 medium fractions (CM-P4) of the GW-treated NAMECs (Supporting Information Fig. 1D). In contrast, little or no CD44 or GAPDH proteins were present in the CM-P4 fraction of untreated NAMECs and HMLE cells (Supporting Information Fig. 1D).

We used an avidin pull-down assay of biotinylated cell surface proteins to track whether SC markers detected in the CM-P4 fraction of EP4 antagonist-treated MaSCs were the markers disappearing from the cell surface following EP4 antagonist treatment. The avidin pull-down assay of GW-treated biotinylated NAMECs showed that the surface basal SC markers and integrins (e.g., CD44, CD90, integrin β1, and integrin α6) were released in the 4-day GW treatment (Fig. 2A). Collectively, these data suggest that cell-surface markers and integrins in basal MaSCs were released following EP4 antagonist treatment.

TEM analysis showed that the CM-P4 fraction of GW-treated NAMECs contained abundant membrane vesicles (Fig. 2B). The vesicles were further analyzed using both NanoSight Nanoparticle Tracking Analysis (NTA, Fig. 2C and Supporting Information Fig. 2A) and Dynamic Light Scattering Analyzer (DLS, Fig. 2D and Supporting Information Fig. 2B). These analyses showed that the EP4 antagonist triggered the release of membrane vesicles ranging from 80 to 120 nm (concentration: ~12 × 10^7 particle/m; mean diameter: 97 nm, Fig. 2C, 2D), while, NAMECs and PGE2-treated NAMECs released many fewer, larger vesicles (concentration: ~1 × 10^7 particle/m; mean diameter: 173 nm and 201 nm, Supporting Information Fig. 2A and 2B). The size of GW-induced vesicles released from NAMECs corresponded with that of exosomes; 50–150 nm [26]. Large amounts of the general exosome markers CD9, CD81, HSP70, Alix, TSG101, and GAPDH [26, 27] are present in the CM-P4 fractions of GW-treated NAMECs (Fig. 2E). The results suggest that the EP4 antagonist-induced release of the surface basal SC markers and mesenchymal markers occurs via the GW-induced extracellular vesicles (EVs) (i.e., exosomes).

GW blocking of EP4-mediated signaling induced a 10-fold increase in EV/exosome release, measured by the levels of the GAPDH EV/exosome marker in the CM-P4 fractions from the GW-treated NAMECs relative to untreated NAMECs (Fig. 2F, top panel). The levels of EV/exosome marker GAPDH corresponded to the actual numbers of EVs/exosomes in the CM-P4 fractions, measured by NTA (Fig. 2F, bottom table). Consequently, in our subsequent experiments, we used the levels of the EV/exosome marker GAPDH in the CM-P4 fractions to measure EV/exosome release.

**Figure 2.** Blocking EP4-mediated signaling in mammary epithelial stem cells causes release of stem cell surface markers and integrins via extracellular vesicles release. (A): Avidin pull-down of biotinylated surface proteins from the cell lysates and EV lysates of NAMECs treated with GW627368X. NAMECs, and NAMECs with biotinylated surface proteins were treated with GW627368X (1 µg/ml) for 3 days. The P4 medium fractions (CM-P4) and cell lysates (Cell) were subjected to avidin pull-down. CD44, CD90, CD81, integrin β1 and integrin α6 proteins were measured by western blotting in the pull-down and input EV and whole cell lysates. N: control NAMECs, NB: biotinylated NAMECs, (B): TEM image of the CM-P4 fraction of NAMECs treated with GW for 4 days. Scale bar, 1 µm. (C-D): Particle size and number analysis of GW-treated NAMEC P4 fractions. The CM-P4 of GW-treated NAMECs were collected and subjected to NanoSight Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering/Zeta Potential Analysis (DLS). NTA shows the particle size and absolute particle concentration in panel C. DLS shows the particle size, mean diameter and relative particle number in panel D. D: particle diameter. RN: relative number. (E): The CM-P4 marker and the GAPDH, CD81, CD9, Alix, TSG101, and Hsp70 exosome markers were measured in the indicated CM-P4 fractions and cell lysates (Cell). The culture media and cell lysates were collected from the same number of NAMECs treated with vehicle (Ctrl), GW627368X (GW), or PGE2 (1µM) for 4 days. Equal volumes of the lysates from the CM-P4 fractions collected from the same number of NAMECs are loaded in each lane of the SDS-PAGE gel. (F): Top panel: Quantification of EV release is shown as the ratio of [P4 GAPDH marker level]/[cell number]. Data are means ± SEM (n = 3). *p ≤ 0.05. **p ≤ 0.005. Bottom table: Quantification of EV release is shown as the ratio of [P4 particles/CM-P4 fractions released per cell]. The P4 particles were counted using nanoparticle tracking analysis. (G): GAPDH and CD81 EV/exosome markers and the CD44 marker were measured in the CM-P4 fraction of NAMECs or HMLE cells treated with vehicle (Ctrl) or GW for 2 days. 1X: P4 EV fraction released by 1 × 10⁶ cells; 2X: P4 EV fraction released by 2 × 10⁶ cells. The long exposure shows (i) slight GAPDH signals in HMLE samples and (ii) the GAPDH signals in HMLE cells did not increase under GW treatment. (H): The CM-P4 marker and the GAPDH and CD81 EV/exosome markers were measured in the indicated P4 condition medium fractions of NAMECs treated with vehicle (Ctrl), GW627368X (GW, 1 µg/ml), AZ2848 (EPα antagonist; 0.4, 2, 4 µg/ml), or AH6809 (EPβ1/2 antagonist; 0.8, 4, 8 µg/ml). (I): Top panel: The HSP70, GAPDH, and CD81 EV/exosome markers were measured in the indicated condition medium fractions of NAMECs treated with vehicle DMSO, GW627368X, or PGE2 for 24, 48, and 96 hours. Loading control, GAPDH. (K): Top panel: Rab27B and Rab35 were measured in the cell lysates (Cell) of GW-treated NAMECs transfected with the indicated siRNAs. Bottom panel: The GAPDH and CD81 EV/exosome markers were measured in the CM-P4 fraction of vehicle (Cir) or GW-treated (GW) NAMECs subjected to Rab27B/Rab35 knockdown. The CM-P4 fractions were collected from the same number of NAMECs, transfected with the indicated siRNAs, and treated with vehicle (Ctrl) or GW (1 µg/ml) for 2 days. NS siRNA: non-silencing control siRNA. Abbreviations: HMLE, Human mammary epithelial cell lines; NAMEC, non-adoherent mammary epithelial cell.
Extracellular Vesicles Are Released from Basal MaSCs by Blocking EP₄ Signaling or by Knocking down EP₄ Receptors

The EP₄ antagonist GW did not increase EV release from HMLE cells (Fig. 2G); GW EP₄ antagonist induced substantial EV release from basal MaSCs, but not from their epithelial cell counterparts. EV release from NAMECs was induced in a dose-dependent manner by AH23848, another EP₄ antagonist, but was not significantly induced by AH6809, an EP₁/EP₂/EP₃ antagonist (Fig. 2H). Corresponding with their effects on EV release from basal MaSCs, but not from their epithelial cell counterparts. EV release from NAMECs was induced in a dose-dependent manner by AH23848, another EP₄ antagonist, but was not significantly induced by AH6809, an EP₁/EP₂/EP₃ antagonist (Fig. 2H). Corresponding with their effects on EV release from basal MaSCs, but not from their epithelial cell counterparts. EV release from NAMECs was induced in a dose-dependent manner by AH23848, another EP₄ antagonist, but was not significantly induced by AH6809, an EP₁/EP₂/EP₃ antagonist (Fig. 2H). Corresponding with their effects on EV release from basal MaSCs, but not from their epithelial cell counterparts. EV release from NAMECs was induced in a dose-dependent manner by AH23848, another EP₄ antagonist, but was not significantly induced by AH6809, an EP₁/EP₂/EP₃ antagonist (Fig. 2H). Corresponding with their effects on EV release from basal MaSCs, but not from their epithelial cell counterparts. EV release from NAMECs was induced in a dose-dependent manner by AH23848, another EP₄ antagonist, but was not significantly induced by AH6809, an EP₁/EP₂/EP₃ antagonist (Fig. 2H). Corresponding with their effects on

Figure 3.
release, AH23848, but not AH6809, was able to cause a MET of NAMECs (Supporting Information Fig. 2C), as indicated by increased resistance of the AH23848-treated NAMECs to trypsinization (Supporting Information Fig. 2D). Both EV release and MET of NAMECs were induced by two EP4 antagonists (GW and AH23848), but not induced by an EP1/EP2/EP3 antagonist. The blockage of EP4 signaling on inducing EV release was verified using shRNAs against EP4. NAMECs expressing the doxycycline (dox)-inducible EP2 shRNAs NAMEC-shEP2–1 and NAMEC-shEP2–2 were treated with doxycycline to knock down EP2 protein expression (Fig. 2I). EVs were released by dox-treated NAMEC-shEP2–1 and dox-treated NAMEC-shEP2–2 during the 96-hour dox treatment, as demonstrated by the increased levels of the EV/exosome markers GAPDH, CD81, and HSP70 in the CM-P4 fractions (Fig. 2J). Blocking PGE2 signaling either by EP4 antagonists or by EP4 shRNAs can induce EV release from basal MaSCs.

Inhibiting COX2 Activity, Inhibiting mPGES-1 Activity, or Neutralizing PGE2 with anti-PGE2 Antibodies Elicits Extracellular Vesicle Release and Loss of Stemness from Basal MaSCs

PGE2 production is controlled by three enzymes; COX-1, COX-2, and PGE2 synthase (e.g., mPGES-1). To explore the physiological relevance of EP4 antagonism-induced modulation of MaSC phenotype, we investigated whether decreasing COX-2 expression, mPGES-1 expression or endogenous PGE2 production can induce MaSC EV release and loss of stemness. EV release from NAMECs was induced in a dose-dependent manner by both the COX-2 inhibitor NS-398 and the mPGES-1 inhibitor MF63, measured by NTA and confirmed by EV/exosome markers (e.g., CD9, CD63, CD81, GAPDH, TSG101, Alix) in CM-P4 fractions (Supporting Information Fig. 3A and 3B). NS-398 and MF63 were also able to reduce the cell surface levels of NAMEC SC markers (e.g., CD44 and Integrin α6; Supporting Information Fig. 3C). In addition, NAMEC cell migration was inhibited by NS-398 and MF63 by 95% and 40%, respectively (Supporting Information Fig. 3D). Inhibiting the enzymes required for PGE2 production induced EV release from MaSCs and a concomitant loss of stemness.

To directly link EP4 antagonism-induced effects on NAMECs and reduced extracellular PGE2 levels, we neutralized PGE2 with anti-PGE2 antibody and measured the resulting EV release and loss of stemness. Anti-PGE2 antibody induced EV release, demonstrated both by increased vesicle numbers in the CM-P4 fraction (Supporting Information Fig. 4A) and by EV/exosome markers (Supporting Information 4B) Anti-PGE2 antibodies also reduced cell surface CD44 and Integrin α6 (Supporting Information Fig. 4C). NameC migration and mammosphere formation were decreased 82% and 98% respectively following treatment with anti-PGE2 antibody (Supporting Information Fig. 4D and 4E). Both enzyme inhibition and PGE2 neutralization data demonstrate that PGE2 levels in the extracellular environment control EV release and stemness of basal mammary epithelial stem cells.

PGE2 Signaling Controls Extracellular Vesicle Release from Basal MaSCs by Regulating Rab GTPase Expression

EV/exosome biogenesis is controlled by Rab GTPase proteins [28, 29]. GW preferentially induced Rab27B and Rab35 protein expression while PGE2 preferentially induced Rab4B (Fig. 2J). Simultaneous Rab27B and Rab35 knockdown in GW-treated NAMECs (Fig. 2K, top panel) attenuated the release of EVs/exosomes induced by GW (Fig. 2K, bottom panel). Rab4B promotes fast-recycling of early endosomes, allowing endocytosed surface proteins to return to the cell surface [28]. In contrast, Rab27B and Rab35 promote the docking of multivesicular endosomes (MVEs) to plasma membrane, allowing exosomes to leave the cells [29, 30]. GW blocking of EP4-mediated signaling induced Rab proteins that promote EV/exosome release; in contrast, activating EP4-mediated signaling with PGE2 induced Rab proteins that promote fast endosome recycling.

EP4 Antagonist-Treated Basal MaSCs Lose SC Properties via Extracellular Vesicle Release

EV/exosome biogenesis begins with endosome formation via endocytosis [4, 28]. We attempted to interfere with EV/exosome biogenesis at the first step, endocytosis, using the
endocytosis-blocking agents Dynasore, Pitstop 2, and Chlorpromazine hydrochloride [31]. Among these agents, only Dynasore did not affect cell growth and/or cause cell death at the reported working concentrations (Supporting Information Fig. 5A). Accordingly, we analyzed the effects of interfering with EV/exosome biogenesis in response to EP4 antagonist-induced NAMEC transition using Dynasore.

Instead of transitioning to an epithelial morphology induced by the EP4 antagonist, NAMECs maintained their mesenchymal morphology when co-treated with the GW EP4 antagonist and
Dynasore (Supporting Information Fig. 5B). While ~80% GW-treated NAMECs were resistant to dissociation by 0.05% trypsin treatment, Dynasore reverted the effect of GW to decrease the resistant cells to 30% (Fig. 3A). The GW-induced increase of E-cadherin in NAMECs at day four was also attenuated by Dynasore (Fig. 3B). Using an avidin pull-down assay of biotinylated cell surface proteins, we found that interfering with EV/exosome biogenesis by Dynasore decreased the release of SC surface markers and integrins via EVs (Supporting Information Fig. 5C) and allowed the retention of the SC markers and integrins on the surface of GW + Dy-treated NAMECs (Fig. 3C; see details in Supporting Information 1). In addition, the decreases of migration ability and mammosphere-forming ability caused by the EP4 antagonist on NAMECs were also prevented by Dynasore (Fig. 3D, 3E). EP4 antagonism failed to induce changes associated with a basal-SC-to-non-basal-SC transition when EV/exosome biogenesis was blocked, suggesting that the EP4 antagonist caused the loss of the basal SC state as a consequence of EV formation and release from the cells.

We next investigated whether GW-induced EVs from NAMECs (GW-in-EVs) can revert the EP4 antagonist-induced MET of previously GW-treated NAMECs. GW-treated NAMECs, when cultured with GW-in-NAs EVs, regained their mesenchymal phenotype (Fig. 3F, 3G), migration ability (Fig. 3H) and mammosphere-forming ability (Fig. 3I). In contrast, GW-treated cells cultured in fresh medium without EVs maintained the GW-induced epithelial phenotype (Fig. 3F, arrows, and 3G), reduced migration ability (Fig. 3H) and reduced mammosphere-forming ability (Fig. 3I). Capturing released EVs allowed EP4 antagonist-treated NAMECs to reacquire their SC properties; that is, their mesenchymal phenotype, migration ability and mammosphere-forming ability.

**Figure 4.** EP4 antagonist-induced basal MaSC extracellular vesicles contain molecules that can transfer basal SC properties and mammary gland-forming ability. (A): EV uptake was measured in HMLE cells and NAMECs treated with vehicle (Crl) or GW627368X (GW) for 4 days. CFSE-labeled, GW-induced NAMEC EVs and negative control (see “Supporting Information Materials and Methods”) were added to the indicated cultures for 6 hours. The fluorescence intensities reflect the EV uptake. (B): EV uptake (green) by HMLE cells following vehicle (Crl) or GW EP4 antagonist treatment (see “Supporting Information Materials and Methods”). Drug treatment was maintained during EV uptake. Cells were treated with vehicle or with GW EP4 antagonist for 2 hours, then exposed to GW-in-NAs EVs. Plasma membrane was stained with CellMask Plasma Membrane Stain (red). Cell nuclei were stained with DAPI (blue). Scale bar, 20 µm. (C): Bright-field images of HMLE cells pre-treated with PBS or GW-induced NAMEC EVs. HMLE cells were treated with PBS (Crl) or 1 µg/ml GW627368X-induced NAMEC EVs. Fresh EVs (1 µg/ml) and medium were supplied every two days for 2 weeks. Cells were split every 4 days. The images (taken 14 days after initiation of PBS or EV treatment) are excerpted from the time-lapse movies in Supporting Information Movie 3 (EV-treated) and Movie 4 (PBS-treated). The treatment was suspended during imaging. Scale bar, 100 µm. (D, E): Migration tracks of HMLE cells pre-treated with PBS (Crl, left) or GW-induced (right) NAMEC EVs. NAMECs were treated as indicated in panel C. Cell migration was tracked for 24 hours using time-lapse imaging. The treatment was suspended after the assay. Panel D: The start positions of the tracked cells are plotted at (0, 0) in the X-Y plot and the final positions at the 24th hour are marked with red dots. Black lines show migration tracks of the cells (n = 20). Panel E: The average lengths of the migration tracks are plotted. Data are means ± SEM (n = 20). ***p < 0.001. (F): Boyden chamber migration of HMLE cells treated with PBS (Crl) or GW-induced NAMEC EVs. HMLE cells were treated as follows: HMLE + EV: 1 µg/ml EVs were added during the 72-hour migration assay. EV-HMLE: HMLE were pre-treated with EVs for 7 days and the drug treatment was suspended during the migration assay. Data are means ± SEM (n = 3). *p < 0.05. **p < 0.005. Scale bar, 100 µm. (G) Mammosphere formation of HMLE cells treated with PBS (Crl) or GW-induced NAMEC EVs (1 µg/ml) for 7 days. The treatment was suspended during the mammosphere assay. Data are means ± SEM (n = 5). *p < 0.05. Scale bar, 500 µm. (H): mammosphere formation of NAMECs (Crl), non-migrating GW-in-NA EV-treated NAMEC cells, and migrating GW-in-NAs EV-treated NAMEC cells. The non-migrating and migrating EV-treated NAMEC cells (EV-HMLE) were separated using Boyden chambers. EV treatment was suspended before the separation. Data are means ± SEM (n = 4). **p < 0.005. ***p < 0.001. Scale bar, 500 µm. (I): Overview of protocol to analyze the transfer of mammary gland-forming ability by EVs to mouse primary mammary epithelial cells. (J): Primary mouse mammary basal cells and luminal cells were isolated and sorted using surface EpCAM and integrin α6β1 profiles. Mouse mammary epithelial cells isolated from fat pads of 12-week-old mice were subjected to cell sorting. Mouse mammary luminal cells were enriched in the EpCAM+/integrin α6β1 population; basal cells were enriched in the EpCAM+/integrin α6β1 population. (K): Mammary gland formation by primary mouse mammary cells. The primary mouse EpCAM+/integrin α6β1 luminal cells were treated with PGE2-induced or GW-induced EVs from mouse primary mammary epithelial cells in cell culture for 10 days. After the treatment, the cells were centrifuged at the numbers per pad shown in the table, into the cleared fat-pads of 3-week-old mice. Mice were euthanized and necropsied after 8 weeks to analyze mammary gland formation. Grey spot: an injected pad with no gland formation. Black spot: an injected pad with completed gland formation. Partial black spot: an injected pad with partial gland formation. MRU frequencies were calculated, using ELDA, from the results in the figure. Scale bar, 0.75 cm.
EP4 Antagonist-Induced Basal SC Extracellular Vesicles Convert Mammary Epithelial Cells to Basal Stem Cells

Basal stem NAMECs possess the two characteristic basal SC traits—high cell motility and high mammosphere-forming ability. If GW-in-NA EVs can elicit the conversion of mammary epithelial cells to basal stem cells, the two traits—high cell motility and high mammosphere-forming ability—should be present in the same subpopulation of the GW-in-NA EV-treated HMLE cells. To verify this suggestion, the migrating cell population and non-migrating cell population of the GW-in-NA EV-treated HMLE cells were separated with Boyden chambers. Compared to PBS-treated HMLE cells, the non-migrating cell population of GW-in-NA EV-treated HMLE cells...
had a ~two-fold increase in mammosphere-forming ability and the migrating cell population had a ~7.5-fold increase in mammosphere-forming ability (Fig. 4H). The high migration ability and high mammosphere-forming ability were increased in the same population of the GW-in-NA EV-treated HMLE cells. Furthermore, HMLE cells proliferated much faster than NAMECs (Supporting Information Fig. 5E). GW-in-NA EV treatment suppressed the growth of both NAMECs and HMLE cells to the same extent (Supporting Information Fig. 3F). Thus, following EV treatment, the epithelial cells still grew faster than the basal stem cells. These data suggest that the increase of migrating cells and mammosphere-forming cells in GW-in-NA EV-treated HMLE cells cannot result from expanding the pre-existing basal stem cells in the population or by suppressing epithelial cells in the population. HMLE cells acquired basal SC properties from the NAMEC basal stem cells via the EP4 antagonist-induced EVs. The data suggest that the basal MaSC state can be transferred from basal MaSCs to their epithelial cell counterparts via EVs.

**EP4 Antagonist-Induced Extracellular Vesicles Transfer Mammary Gland-Forming Ability to Primary Mammary Luminal Cells**

Single-cell basal colonies derived from mice can re-populate mammary glands when transplanted in vivo but luminal cells cannot [15, 24, 32]. These cells able to form mammary glands are referred as mammary repopulating units (MRUs). To evaluating whether EP4 antagonist-induced EVs were able to convert luminal cells to MRUs, primary mouse mammary epithelial cells were isolated from fat pads to sort luminal cells and generate EP4 antagonist-induced EVs (Fig. 4I).

Mouse mammary basal and luminal cells were sorted using the differential expression of EpCAM and integrin α6 (CD49f) (Fig. 4I). In addition, PGE2-induced and GW-induced EVs were collected from mouse primary epithelial cells (Fig. 4I). MRU frequency of EpCAMα6/integrin α6+ luminal cells (gland-forming efficiency: 1/6, 0/6, <2/6; Fig. 4K) were low. The MRU frequency of EpCAMα6/integrin α6+ luminal cells (gland-forming efficiency: 5/6, 6/6, <4/6; Fig. 4K) was increased ~20-fold by GW-induced EV pre-treatment for 10-days. In contrast, PGE2-induced EV pre-treatment did not increase MRU frequency of EpCAMα6/integrin α6+ luminal cells (gland-forming efficiency: <2/6, <1/6, <2/6; Fig. 4K). These results suggest that EP4 antagonist-induced mammary epithelial cell EVs can transfer mammary repopulating ability to the luminal cells.

**EP4 Antagonist-Induced SC Extracellular Vesicles Contain Proteins Characteristic of Basal MaSCs**

To find the differences between GW-induced and PGE2-induced EVs responsible for their different abilities to transfer the mammary stem cell state, we further analyzed contents of GW-induced and PGE2-induced EVs. Proteins of the GW-in-NA EVs were subjected to liquid chromatography-tandem mass spectrometry (LCMSMS), system analysis (Fig. 5A) and micro-western arrays (Supporting Information Fig. 6A). The enrichment analysis [33] showed that the highest-scored pathways were involved in integrin-mediated cell adhesion and migration (Fig. 5A). The identified EV proteins were validated and compared in control, PGE2-elicited and GW-elicited EVs from NAMECs (Fig. 5B). EV proteins released by NAMECs treated with vehicle, GW, or PGE2 were compared on a per-cell basis (Fig. 5B and Supporting Information Fig. 6B, compare lanes 5, 6, and 7) and on a per-EV basis (Fig. 5B, compare lanes 4 and 7). The amount of total EV proteins released by GW-treated NAMECs was much greater than that of NAMECs treated with vehicle or with PGE2 (Fig. 5B and Supporting Information Fig. 6B, compare lanes 5, 6, and 7). In addition, the relative contents of various proteins in the GW-induced and PGE2-induced EVs were different (Fig. 5B, differences indicated in red and blue). The data demonstrate that PGE2/EP4 signaling regulates EV protein release both in quantity and in composition.

**EP4 Antagonist-Induced Extracellular Vesicles Contain Mesenchymal Markers and Basal SC Markers**

GW-induced EVs carried proteins (i.e., N-cadherin, fibronectin, CD146 and CD91) essential to maintain mesenchymal/stem-like properties (Fig. 5B and Supporting Information Fig. 6B). In contrast, PGE2-induced EVs carried the epithelial marker E-cadherin, which was not present in GW-induced EVs (Fig. 5B). The results suggest that PGE2/EP4 signaling pathway modulates basal MaSC morphology bidirectionally; the EP4 antagonist promoted MET by increasing the release of mesenchymal markers by EVs and by decreasing the release of E-cadherin by EVs.

Proteins contributing to cell migration (e.g., integrin β1, integrin α6, collagen, filamin A, CD91, CNP, talin, tropomyosin, galectin 3, Rap1, CD146), basal SC markers (e.g., CD44, CD90, integrin β1, integrin α6) and β-catenin were also released from the GW-treated NAMECs via EVs (Fig. 5B and Supporting Information Fig. 6B). Parsimony suggests that these EP4 agonist-induced, EV-mediated losses contribute to the changes in mesenchymal morphology, cell motility and SC identity of GW-treated basal MaSC NAMECs.

**EP4 Antagonist-Induced Extracellular Vesicle Release Affects PI3K/Akt, TGF-β, and Canonical/Noncanonical Wnt Signaling in Basal MaSCs**

EP4 antagonist treatment of NAMECs induced the release of receptors and signaling proteins via EVs. Signaling receptors (e.g., TGFβ1, TGFβ2, LRP6, FZD5, EGF, HER2, Met, and EP3) were detected in the GW-induced NAMEC EVs (Fig. 5B and Supporting Information Fig. 6A and 6B). The release of signaling receptors by blocking PGE2/EP4 signaling was reflected in the decreases of the cell-surface levels of these receptors in GW-treated NAMECs, measured by cell-surface protein biotinylation (Fig. 5C) and by flow cytometry (Fig. 5D).

In addition, signaling proteins involved in PI3K signaling (PI3K, PKD1, Akt, p-Akt), canonical Wnt signaling (β-catenin), EGF signaling (c-Src, p-Src), and MAPK/noncanonical Wnt signaling (SAPK/JNK) were also released from NAMECs via GW-induced EVs (Fig. 5B and Supporting Information Fig. 6A and 6B). By comparing the ratio of p-Akt-to-total Akt in the GW EP4 antagonist-treated cells and in the released EVs, we find that p-Akt was preferentially recruited into the GW-induced EVs relative to Akt (Fig. 5B). The 4-day GW treatment caused a substantial decrease of cellular p-Akt(S473) in GW-treated NAMECs (Fig. 5B, p-AktS473, lane 3), as a result of the significant release of p-Akt(S473) via GW-induced EVs (Fig. 5B, p-AktS473, lane 7 vs. lane 3). PGE2 treatment had little or no effect on distribution of either Akt or p-Akt(S473) in the
cellular or EV compartments of NAMEC cells (Fig. 5B, Akt/p-Akt(S473), lane 2).

To evaluate whether there is selective release of components of alternative signaling pathways, the activation and suppression of signaling pathways in GW-treated NAMECs was analyzed using RT2 Profiler PCR arrays. The signaling pathways most inhibited by GW are the PI3K/Akt, TGF-β, Wnt and androgen pathways (Fig. 5E). The PI3K/Akt, TGF-β and
canonical/noncanonical Wnt pathways are involved in maintaining the mesenchymal and SC states of human mammary epithelial cells [17, 34]. The role of the AR pathway in breast cancer is highly affected by the status of other receptor signaling pathways in the cells, for example, ER, PR, and HER2 [35], and its involvement in phenotype has been inconclusive. Inhibition of the PI3K/Akt, TGF-β and Wnt pathways by the GW EP4 antagonist was further confirmed by analyzing phosphorylation states of their signaling components; phosphorylation of AKT, GSK, and Smad2, key enzymes in these signaling pathways, was reduced in GW EP4 agonist-treated NAMECs, as expected for reduced signaling activity for each of these pathways (Fig. 5F; see details in Supporting Information 3).

The decrease of p-Akt in GW-treated NAMECs and the enrichment of p-Akt in GW-induced EVs are reflected in inactivation of PI3K/Akt-signaling (Fig. 5E). Thus, EP4 antagonists are likely to interfere with the Akt-dependent signaling pathway by decreasing cellular p-Akt via EV release. In addition, the decreases of cell surface receptors EGFR, HER2, c-Met, TGFBR1, TGFBR2, LRP6 and FZD5 in GW-induced EVs, are also reflected in inactivation of PI3K/Akt, TGF-β, and canonical/noncanonical Wnt signaling (Fig. 5E). These data suggest that blocking EP4 signaling can indirectly affect other cell-signaling pathways (Fig. 5E) as a consequence of EV release (Fig. 5B and Supporting Information Fig. 6B), by decreasing their surface receptors (Fig. 5C, 5D) and/or signaling components (Fig. 5F) and then induce the transition of a mesenchymal/SC state of MaSCs to an epithelial state.

**EP4 Antagonist Alters Protein Distribution in Basal MaSCs**

To investigate how EP4 antagonism triggered release of signaling receptors and signaling proteins via EVs, distribution of the signaling receptors and proteins in lipid raft and non-raft fractions in vehicle-, GW-, or PGE2-treated NAMECs was analyzed using density gradient fractionation [36] (Fig. 6A and Supporting Information Fig. 7A). Lipid rafts are specialized membrane microdomains organizing the trafficking of membrane proteins, signaling molecules, and receptors [37]. The lipid raft markers ganglioside GM1 and caveolin-1, which define the lipid raft fraction (LRF) [38], appeared in fractions 2, 3, 4, and 5. In contrast; GAPDH, which is a non-raft protein (non-LRF) [39], appeared in fractions 7, 8, and 9 (Fig. 6A). The basal stem cell markers CD44 and CD90 are present predominantly in the LRF of NAMECs (Fig. 6A and Supporting Information Fig. 7A). The receptors (e.g., EGFR, HER2, c-Met, and LRP6) and integrins (e.g., integrin β1 and integrin α6) appeared in both the LRF and non-LRF (Supporting Information Fig. 7A).

The distribution of many proteins in basal MaSCs was shifted into lipid rafts in response to EP4 antagonist treatment. While TGFBR1 and TGFBR2 largely presented in non-LRF of NAMECs and PGE2-treated NAMECs, both TGFβ receptors shifted into LRF in GW-treated NAMECs (Fig. 6A, red and blue squares). The essential signaling component of the PI3K/Akt pathway, p-Akt, also significantly shifted into LRF in GW-treated NAMECs (Fig. 6A, red square); while the distribution of total Akt remained the same in NAMECs, GW-treated NAMECs, and PGE2-treated NAMECs (Fig. 6A). Blocking PGE2/EP4 signaling caused redistribution of some proteins, including signaling receptors and an essential signaling component, from non-LRF into lipid rafts.

**EP4 Antagonist Triggers Release of Lipid Rafts from Basal MaSCs via Extracellular Vesicles**

Blocking PGE2 signaling with the EP4 antagonist decreased the level of cell surface GM1, imaged by total internal reflection fluorescence microscopy (Fig. 6B) and measured by flow cytometry (Fig. 6C). These results suggest that blocking EP4 signaling causes a decrease of lipid rafts on plasma membranes of NAMECs, perhaps as a result of endocytosis of the lipid rafts. To further track the lipid rafts disappearing from the cell surface of GW-treated NAMECs, the GM1 of lipid rafts on the plasma membranes were pre-labeled with Horseradish peroxidase-conjugated Cholera Toxin Subunit B (HRP-CTB) [40]. HRP-CTB was detected in the EV fraction of the NAMECs treated with GW for 2 days (Fig. 6D). In addition, comparing GM1 released by vehicle-, GW-, and PGE2-treated NAMECs on

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**Figure 6.** Blocking PGE2/EP4 signaling causes the relocation of signaling receptors and mediators into lipid rafts and then the release of proteins via extracellular vesicles. (A): Western blot analysis of TGFBR1, TGFBR2, p-Akt, and Akt, in cell membrane fractions enriched in lipid rafts (LRF, fractions 2–5) or non-lipid rafts (non-LRF, fractions 7–9). NAMECs were treated with vehicle (Crl), GW627368X (1 μg/ml), or PGE2 (1 μM) for 4 days. Proteins were detected in these membrane fractions by western blotting along with ganglioside GM1 (dot blotting) and caveolin-1 as markers of lipid rafts, and with GAPDH as a marker of non-lipid rafts. The blue squares indicate the disappearance of protein from non-LRF of GW-treated NAMECs; the red squares indicate the appearance of protein in LRF of GW-treated NAMECs, and PGE2-treated NAMECs (Fig. 6A). Blocking PGE2/EP4 signaling caused redistribution of some proteins, including signaling receptors and an essential signaling component, from non-LRF into lipid rafts.

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a per-cell basis (Supporting Information Fig. 7B) shows that the levels of released GM1 corresponded with the relative numbers of EVs released by the cells (Fig. 2F). These data suggest that the internalized lipid rafts from the plasma membranes of GW-treated NAMECs were released via EVs.

Blocking PGE2/EP4 signaling caused redistribution of some proteins from non-LRF into LRF and a decrease of lipid rafts on plasma membranes of GW-treated NAMECs. The decreases of the cell surface receptors (Fig. 5C, 5D), signaling components (Fig. 5F), and basal stem cell markers (Fig. 1L) were observed.

Figure 7.
correlated with the decrease of surface lipid rafts of GW EP₄ antagonist-treated NAMECs (Fig. 6C). Our findings show that EP₄ antagonism causes the cell surface receptors and signaling components to shift from non-lipid raft fractions to lipid raft fractions and to then be internalized along with the lipid rafts. These results suggest that the internalization of lipid rafts from the plasma membranes causes a decrease of basal stem cell markers and signaling receptors from EP₄ antagonist-treated basal NAMEC MaSCs.

Lipid Raft-Associated Proteins Are Selectively Released by Extracellular Vesicles from EP₄ Antagonist-Treated Basal MaSCs

Proteins (e.g., CD44 [41], CD90 [32], integrins [43], EGFR [44], c-Met [45], HER2 [46], LRP6 [47], Akt [48, 49], TGFβ [50]) can function differently when present in lipid rafts versus non-lipid raft portions of the membrane. The LRF-associated forms of the proteins on plasma membranes usually actively mediate signaling [42–44, 46, 48, 49, 51, 52]. NAMECs expressed different forms of integrin β1 (Hg-integrin β1 and Lg-integrin β1, integrin α6 (H-integrin α6 and L-integrin α6), and LRP6 (LRP6, pg-LRP6, g-LRP6 and Ng-LRP6) (Fig. 5B and Supporting Information Fig. 7A). These different forms result from different states of phosphorylation and glycosylation [51–53]. In NAMECs, the Hg-integrin β1, H-integrin α6, and pg-LRP appeared in the LRF while Lg-integrin β1, integrin α6, and Ng-LRP6 were only present in the non-LRF (Supporting Information Fig. 7A).

In the EV protein analysis (Fig. 5B) we observed that, for the proteins which have distinct LRF-associated and non-LRF-associated forms (e.g., integrin β1, integrin α6, LRP6 and Akt/p-Akt; Fig. 6A and Supporting Information Fig. 7A), their LRF-associated forms (e.g. Hg-integrin β1, H-integrin α6, pg-LRP6 and p-Akt) were preferentially sorted into the GW-induced EVs, compared to their non-LRF-associated forms (Fig. 5B). To verify whether the preferential sorting of LRF-associated forms of proteins into EVs occurs for additional EV proteins, proteins isolated from the cellular and EV compartments of GW EP₄ antagonist-treated NAMECs were further analyzed and compared using density gradient fractionation (Fig. 6E, comparing Supporting Information Fig. 7A and Supporting Information Fig. 7C). By comparing the protein distribution between LRF and non-LRF in the cellular and EV compartments of GW-treated NAMECs, we observed that LRF-associated forms of the proteins (e.g., integrin β1, integrin α6, HER2, c-Met, EGFR, LRP6, p-Akt, and Src) were enriched in the EV compartments (Fig. 6E, blue and red squares, comparing Supporting Information Fig. 7A and Supporting Information Fig. 7C). LRF-associated forms of many proteins were preferentially recruited into the GW-induced exosomes relative to non-LRF forms.

The presence of enriched lipid raft-associated proteins in the GW-induced NAMEC EVs suggested that proteins were sorted in a lipid raft-dependent mechanism in response to GW and Cav-1. Although the amount of lipid rafts in PGE₂-induced EVs, measured by the levels of the lipid raft markers GM1 and Cav-1, was only slightly lower than that of GW-induced EVs when compared on a per-EV basis (Supporting Information Fig. 7D), PGE₂-induced NAMEC EVs carried much smaller amounts of many proteins (Fig. 5B, lane 4 vs. 7, and Supporting Information Fig. 7D). Further analysis showed that, while the lipid rafts of GW-induced EVs carried abundant protein, the lipid rafts of PGE₂-induced EVs contained little protein (Fig. 6F). The lipid raft appears to be the determinant of EV protein differences between GW-induced and PGE₂-induced EVs. These data demonstrate that EP₄ antagonist-induced EVs selectively remove from basal MaSCs the LRF-associated forms of many proteins, the forms of these proteins functionally active in maintaining the basal MaSC phenotype [34, 54].

Caveolae/Lipid Rafts of Basal MaSCs Are Required for the Sorting of EV Protein

Caveolae are morphologically identifiable lipid raft structures whose formation and maintenance depend heavily on the protein caveolin [55]. Disruption of caveolae by knocking

Figure 7. EV proteins are sorted in EP₄ antagonist-induced extracellular vesicles in a caveolae/lipid-raft-dependent manner; the lipid-raft-associated factors are required to transfer basal MaSC properties. (A): Caveolin knock-down does not affect the level of EP₄ antagonist-induced EV release, measured by the levels of EV/exosome marker GAPDH and CD81. The GAPDH and CD81 EV/exosome markers were measured in the CM-P4 fraction of NAMECs (Ctrl), NAMEC-siCav1 knockdown cells (siCav1) and NAMEC-siCav1 + 2 knockdown cells (siCav1 + 2). The CM-P4 fractions were collected from the same number of NAMECs, transfected with the indicated siRNAs, and treated with vehicle (Ctrl) or GW (1 µg/ml) for 2 days. EV: extracellular vesicle. (B): The indicated proteins were measured in GW-induced EVs released from NAMECs, siCav1 NAMECs, or siCav1 + 2 NAMECs. Loading control: EV/exosome GAPDH and β-actin. (C): Disrupting cellular lipid rafts does not affect EP₄ antagonist-induced EV release, measured by the levels of EV/exosome markers GAPDH and CD81. The GAPDH and CD81 EV/exosome markers were measured in the CM-P4 fraction of NAMECs subjected to MjCDtreatment. The CM-P4 fractions were collected from the same number of GW-treated NAMECs, pre-treated with vehicle or MjCD for 2 days. (D): Caveolin-1 (Cav-1, top panel) and GM1 (bottom panel) were measured in GW-induced EVs released from NAMECs subjected to vehicle or MjCD treatment on a per-EV basis. Loading control: EV/exosome GAPDH. In the bar charts, data are normalized using the protein levels of the GAPDH EV/exosome marker, which corresponds to the number of EVs (see Fig. 2F, and corresponding text). n = 3, ***p < .001. (E): The indicated proteins were measured in GW-induced EVs released from NAMECs treated with vehicle or MjCD. Loading control: EV/exosome GAPDH, β-actin, and CD81. (F): Boyden chamber migration of HMLE cells pre-treated with PBS (Ctrl), GW-in-NA EV (1 µg/ml), PGE₂-in-NA EV (2 µg/ml), or GWMJ-in-NA EV (1 µg/ml) for 7 days (see “Supporting Information Materials and Methods”). The drug treatment was suspended during the migration assay. Data are means ± SEM (n = 3). *p < .05, ***p < .001. Scale bar, 100 µm. (G): Mammosphere formation of HMLE cells pre-treated with PBS (Ctrl), GW-in-NA EV (1 µg/ml), PGE₂-in-NA EV (2 µg/ml), or GWMJ-in-NA EV (1 µg/ml) for 7 days. The treatment was suspended during the mammosphere assay. Data are means ± SEM (n = 5). *p < .05. (H): Mammary gland formation by primary mouse mammary cells. Primary mouse EpCAM⁺/integrin α6⁺ luminal cells were treated with PBS, GW-in-NA EV (1 µg/ml), PGE₂-in-NA EV (2 µg/ml), or GWMJ-in-NA EV (1 µg/ml) in vitro for 10 days. After the treatment the cells were implanted, at the numbers per pad shown in the table, into the cleared fat-pads of the 3-week-old mice. Mice were euthanized and necropsied after 8 weeks to analyze mammary gland formation. Grey spot: an injected pad with no gland formation. Black spot: an injected pad with completed gland formation. Partial black spot: an injected pad with partial gland formation. MRU frequencies were calculated, using ELDA, from the results in the table. Scale bar, 0.75 cm.

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down caveolin-1 and caveolin-2 did not affect overall EV release induced by the GW EP4 antagonist, as measured by the amounts of GAPDH and CD81 EV/exosome markers (Fig. 7A). However, the GW-induced EVs released from caveolin knock-down NAMECs largely lost lipid-raft associated proteins (e.g., CD44, CD90, Integrin α6, EGFR, HER2, Met, LRP6, TGFβ-R1, TGFβ-R2, p-Akt, Akt; Fig. 7B), suggesting caveolae contribute to protein sorting into GW EP4 antagonist-induced EVs. Cholesterol is an important component of lipid rafts and depletion of cell membrane-associated cholesterol can disrupt lipid rafts [56]. Extraction of cellular cholesterol with methyl-β-cyclodextrin (MβCD) did not affect the amount of GW-induced EV release from NAMECs, measured by the levels of EV/exosome markers GAPDH and CD81 (Fig. 7C) and measured by the numbers of vesicles using NTA (Supporting Information Fig. 8A). Comparing the GW-induced EVs from NAMECs (GW-in-NA EVs) and from MβCD-treated NAMECS (GWMMβ-in-NA EVs), disruption of cellular lipid rafts by cholesterol extraction decreased the EV caveolin-1 and lipid raft marker GM1 in the GW-induced NAMEC EVs (Fig. 7D). In addition, lipid raft-associated proteins largely decreased in GWMMβ-in-NA EVs; while levels of non-lipid raft membrane marker CD71/Trf R [57] and cytosolic proteins (e.g., β-Actin, GAPDH) in EVs were not altered by MβCD treatment (Fig. 7E). The results suggest that cellular lipid rafts are required for the GW-elicted sorting of proteins into EVs.

**EP4 Antagonist-Induced Basal MaSCs Extracellular Vesicles Transfer Basal MaSC Properties via Lipid Raft-Associated Factors**

To verify whether the lipid raft-associated factors were responsible for the ability of EP4 antagonist-induced basal MaSC EVs to transfer basal MaSC properties, HMLE cells were cultured with the same numbers of GW-, PGE2-, or GW + MβCD-induced NAMEC EVs (GW-in-NA EV, PGE2-in-NA EV, or GWMMβ-in-NA EV), determined by EV GAPDH levels (Supporting Information Fig. 8B, also see “Supporting Information Materials and Methods”). The number of migrating cells of HMLE cells was increased ~10-fold by GW-in-NA EV pre-treatment, while PGE2-in-NA EVs only increased the number of migrating cells to 3-fold (Fig. 7F). In addition, the effect of GWMMβ-in-NA EVs on increasing migrating cells was significantly decreased, compared to GW-in-NA EVs (Fig. 7F). These data suggest that the decreased effect of GWMMβ-in-NA EVs on increasing cell migration is the result of loss of lipid raft-associated proteins (Fig. 7E). In contrast, the marginal/robust effects of PGE2-in-NA EVs and GWMMβ-in-NA EVs on increasing cell migration may result from some non-lipid raft associated proteins involved in cell migration (e.g., CNP [58], filamin-A [59], galectin-3 [60]; Supporting Information Fig. 6B) present in these EVs. Similarly, the number of mammospheres formed by the HMLE cells was increased approximately fourfold by the GW-in-NA EV pre-treatment for 7 days, while both PGE2-in-NA EVs and GWMMβ-in-NA EVs had no an effect on mammosphere formation (Fig. 7G). The results suggest that uptake of EP4 antagonist-induced basal MaSC EVs can increase migration ability and mammosphere-forming ability of mammary epithelial cells largely via lipid raft-associated factors in the EVs.

**Discussion**

The evidence presented here suggests that PGE2/EP4 signaling is essential for mammary epithelium homeostasis (Fig. 1). While PGE2 signaling is known to support hematopoietic stem cells by modulating the Wnt pathway [1], the definitive mechanisms by which PGE2/EP4 mediates its effects on other signaling pathways are not well understood. We describe here a previously unrecognized mechanism by which PGE2/EP4 signaling regulates multiple pathways in basal/stem mammary epithelial cells, modulating recycling or release of the various signaling components by controlling trafficking of these molecules into extracellular vesicles (EVs) in a lipid raft/caveolae-dependent manner.

The PI3K/Akt, TGFβ and canonical/noncanonical Wnt pathways have been identified as essential to maintain MaSC homeostasis [17, 34]. The distribution of many components of these pathways (e.g., p-Akt, LRP6, FZD5, TGFβR1, TGFβR2, EGFR, HER2, c-Met) are regulated by PGE2 signaling (Fig. 5C, 5D and 6A). Blocking PGE2/EP4 signaling induced the relocation of those signaling components into lipid rafts/caveolae (Fig. 6A). Removal of the lipid rafts/caveolae (Fig. 6B, 6C) from plasma membrane results in a decrease of the signaling receptors from the cell surface (Fig. 5C, 5D). In addition to these relocated receptors, basal stem cell markers CD44, CD90, integrin β1, and integrin α6, which largely exist in lipid rafts/caveolae (Supporting Information Fig. 7A), also decrease from the cell surface (Fig. 1L) in response to EP4 antagonism. Blocking PGE2/EP4 signaling promotes the trafficking of signaling components, SC markers, and integrins into lipid rafts/caveolae.
caveolae and the endocytosis of these components together with lipid rafts/caveolae.

In addition to the mechanism described here, PGE2 signaling has also been reported to modulate stemness in multiple ways. Studies, including our own, demonstrated that PGE2 signaling also affects the distribution of β-catenin between the cell nucleus and cytoplasm [61, 62], in addition to the observation we report here that EP4 antagonism promotes β-catenin release via EVs (Supporting Information Fig. 6B). Blocking PGE2 signaling decreases expression of mRNAs for the EMT-inducing transcription factors Zeb1 and Snail. In contrast, PGE2 induces Zeb1 and Snail mRNA expression, leading to suppression of E-cadherin mRNA expression [63]. In addition to PGE2 reduction of E-cadherin expression [63], we show here that PGE2 also promotes E-cadherin export via EVs (Fig. 5B).

The results described here suggest that EV release induced by blocking PGE2 signaling may be a trigger for the clearance of selected cell proteins (e.g., cell surface receptors, p-Akt, and β-catenin; Fig. 5B and Supporting Information Fig. 6B). Loss of these proteins may then disturb/disrupt cell signaling events that initiate, stabilize, finalize and maintain the MET by subsequent changes in gene transcription (e.g., the decrease of nuclear β-catenin in EP4 antagonist-treated cells [61, 62]; the decrease of Zeb1/Snail mRNA in COX-2-inhibited cells [63]).

Blocking EP4-mediated signaling induces the Rab27B and Rab35 proteins (Fig. 2) responsible for docking MVEs to the plasma membranes in the SCs and, in turn, induces EV/release from basal MaSCs (Fig. 2K). We suggest that the Rab27B/Rab35-mediated EV/exosome release induced by EP4 antagonism causes the subsequent cellular loss of EV contents. EP4 antagonist-induced EVs remove from basal MaSCs both surface receptors that initiate intracellular signaling and pathway proteins that mediate signaling, mesenchymal morphology, cell motility, and cell stemness (Fig. 5A-5F). The data provided here demonstrate that antagonizing PGE2/EP4 signaling modulates the SC phenotype by inducing EV-selective removal of specifically targeted receptors and signaling proteins. In contrast, agonist activation of PGE2/EP4 signaling induced Rab4B, responsible for early endosome recycling [28]. The early endosome recycling promoted by the activation of PGE2/EP4 signaling allows endosome contents to return to the cell surface. Hence, PGE2/EP4 signaling can indirectly sustain multiple signaling pathways by replenishing the relevant receptors on the cell surface.

The combined effect of protein trafficking into lipid rafts/caveolae and docking MVEs to the plasma membranes causes the decrease of signaling pathway mediators and surface receptors via the EP4 antagonist-induced EV release. The loss of a subset of these signaling mediators and receptors via EP4 antagonist-induced EVs is undeniably causal for the transition of basal MaSCs into non-basal MaSca in response to EP4 antagonism (Fig. 3). For example, p-Akt was preferentially recruited into the induced EVs relative to Akt (Fig. 5B), suggesting that EP4 antagonist-induced EV release results in attenuation of PI3K-Akt signaling. Both blocking caveolin-1-dependent endocytosis by caveolin-1 knockdown (Fig. 7A and 7B) and disrupting lipid rafts by MβCD (Fig. 7C-7E) in basal MaSCs greatly decrease the proteins in EP4 antagonist-induced EVs. These data demonstrate that lipid rafts/caveolae in basal MaSCs are required for EV content trafficking into EP4-antagonist-induced EVs.

Our data also provide evidence to demonstrate a function not previously considered for EVs—transferring the basal SC state from basal MaSCs to other epithelial cell counterparts. EP4 antagonist-induced basal-SC-to-non-basal-SC transition was attenuated either by interfering with EV/exosome biogenesis (Fig. 3A-3E) or by re-uptake of SC EVs (Fig. 3F-3I). Furthermore, EV uptake by luminal mammary epithelial cells caused the cells to acquire basal MaSC properties, for example, migration, mammosphere formation, mammary gland formation (Fig. 4). Taken together, these data suggest that inhibition of EP4-mediated signaling elicits transition of a mesenchymal/SC state to an epithelial state, doing so by affecting multiple signaling loops known to act in concert to maintain the basal SC state of basal MaSCs via EVs.

The lipid raft/caveolae-associated proteins in EP4 antagonist-induced EVs are required to transfer the basal SC properties to luminal mammary epithelial cells. EP4 antagonist-induced EVs, which contain lipid rafts/caveolae-associated proteins, can transfer basal SC properties to luminal cells (Fig. 7F-7H). In contrast, PGE2-induced EVs from basal cells and EP4 antagonist-induced EVs from cholesterol-depleted basal cells, which contain little lipid rafts/caveolae-associated proteins, cannot change the properties of the luminal cells (Fig. 7F-7H). EV-mediated transfer of basal MaSC properties requires lipid rafts/caveolae that contain proteins known to be essential to maintain SC properties (Fig. 5B, 6F, 7E). Our data suggest that the lipid raft/caveolae-associated proteins are functionally different from those in non-LRF. EP4 antagonist-induced removal of lipid raft/caveolae and their associated factors from the cell plasma membrane significantly altered the behavior of basal MaSCs. Also, decreasing the lipid raft/caveolae and their associated factors from EP4 antagonist-induced EVs attenuated the basal-SC-property-transferring ability of the EVs.

CONCLUSION

Supporting Information Figure 9 describes the proposed regulation of PGE2/EP4 signaling in basal MaSCs. While PGE2 does not change distribution of the proteins (Supporting Information Fig. 9A, panel i), blocking PGE2/EP4 signaling with EP4 antagonists causes the relocation of signaling receptors, mediators, and integrins into lipid rafts/caveolae (Supporting Information Fig. 9B, panel i). Following endocytosis (Supporting Information Fig. 9A and 9B, panel ii), PGE2/EP4 signaling can, by inducing Rab4B, which is responsible for early endosome recycling (Supporting Information Fig. 9A, panel iii), sustain multiple signaling pathways by replenishing the relevant receptors, signaling mediators, and surface markers. In contrast, EP4 antagonists induce Rab27B and Rab35, which promote the docking of MVEs at the plasma membrane and the subsequent release of EVs (Supporting Information Fig. 9A, panel iii). Eliciting EV release by blocking PGE2/EP4 signaling with an EP4 antagonist decreases basal MaSC lipid raft-associated surface receptors, stem cell surface markers, and signaling mediators; as a result, EP4 antagonists interfere with multiple signaling pathways in basal MaSCs (Supporting Information Fig. 9B, panel iv). In contrast, ingestion by mammary luminal cells of EVs containing these abundant lipid raft-associated factors can elicit basal MaSC properties (Supporting Information Fig. 9B, panel iv).

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AUTHOR CONTRIBUTIONS

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