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Regulation of CD44 expression and focal adhesion by Golgi phosphatidylinositol 4-phosphate in breast cancer

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Key words
Breast cancer, CD44, focal adhesion, phosphatidylinositol-4-monophosphate, SAC1

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Breast cancer is one of the most common cancers worldwide and its metastatic invasion is the primary cause of breast cancer mortality. It is classified by the expression of various molecular markers, including estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER-2). CD44 is a cell surface receptor for the components of the ECM and plays various roles in cancer invasion, metastasis, and tumor initiation. The gene encoding CD44 is subjected to alternative splicing. The smallest CD44 (CD44s or standard CD44) is ubiquitously expressed, whereas the variant CD44 (CD44v) is mainly expressed in proliferating cells and cancer cells. In human metastatic and claudin-low type breast cancers, CD44v is expressed at a high level, with CD44v6 expressed at an extremely high level. CD44 expression is associated with an invasive phenotype. Growth factors and primary cytokines induce the upregulation of CD44 variants and enhance adhesion. Human breast cancer cell lines with high CD44+/CD24− populations, such as MDA-MB-231, are basal/mesenchymal in nature and are more invasive than other breast cancer cell lines, favoring bone and lung metastasis. In contrast, non-aggressive epithelial, predominantly luminal MCF7 and SK-BR-3 cells contain a small CD44+/CD24− subpopulation.

CD44, a transmembrane receptor, is expressed in the standard or variant form and plays a critical role in tumor progression and metastasis. This protein regulates cell adhesion and migration in breast cancer cells. We previously reported that phosphatidylinositol-4-phosphate (PI(4)P) at the Golgi regulates cell migration and invasion in breast cancer cell lines. In this study, we showed that an increase in PI(4)P levels at the Golgi by knockdown of PI(4)P phosphatase SAC1 increased the expression of standard CD44, variant CD44, and ezrin/radixin phosphorylation and enhanced the formation of focal adhesions mediated by CD44 and ezrin/radixin in MCF7 and SK-BR-3 cells. In contrast, knockdown of PI 4-kinase IIIβ in highly invasive MDA-MB-231 cells decreased these factors. These results suggest that SAC1 expression and PI(4)P at the Golgi are important in tumor progression and metastasis and are potential prognostic markers of breast cancers.
Fig. 1. Knockdown of SAC1 increased CD44 expression in luminal breast cancer cell lines. (a) Analysis of SAC1 expression in CD44⁺ and CD44⁻ fractions of MCF7 cells. Western blot analysis of the lysates obtained from the CD44⁺ and CD44⁻ fractions of MCF7 cells. The relative amounts of SAC1 expression in each fraction compared to unfractionated MCF7 cells are shown in the graph. The levels were quantified by densitometry, which were further normalized to β-tubulin levels. The results are presented as the mean ± SEM of three independent experiments. **P < 0.01. (b) Semiquantitative RT-PCR analysis of CD44s, CD44v, and Gapdh in MCF7 cells transfected with control or SAC1-targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of Gapdh mRNA. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. (c) Semiquantitative RT-PCR analysis of CD44s, CD44v, and β-actin in SK-BR-3 cells transfected with control or SAC1-targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of Gapdh mRNA. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05; ***P < 0.001. (d) Changes in the expression of CD44 and CD44 variant 6 (CD44v6) proteins, induced by knockdown of SAC1 in MCF7 cells. MCF7 cells were transfected with control or SAC1-targeted siRNAs for 72 h before lysate collection. The relative amounts of SAC1, standard CD44 (CD44s), and CD44v6 expression were quantified by densitometry, which were further normalized to the amount of GAPDH. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05; **P < 0.01. (e) Changes in expression of CD44 and CD44v6 proteins, induced by knockdown of SAC1 in SK-BR-3 cells. The relative amount of SAC1 expression was quantified by densitometry, which was further normalized to the amount of GAPDH. The results are presented as the mean ± SEM of three independent experiments. *P < 0.05.
motility. In the lamellipodia of migrating cells, nascent adhesions, consisting of integrin, focal adhesion kinase, and vinculin are formed. These structures are short-lived and undergo rapid turnover. Thus, a number of focal adhesions is involved in cancer cell migration.

Our recent finding suggested a strong correlation between phosphatidylinositol-4-phosphate (PI(4)P) at the Golgi and the invasive phenotype of breast cancer cell lines.\(^{(19)}\) Phosphatidylinositol-4-phosphate is enriched at the trans-Golgi and is required for trafficking from the Golgi to the plasma membrane.\(^{(20)}\) In highly invasive breast cancer cell lines (MDA-MB-231 and Hs578t), Golgi PI(4)P levels are significantly higher than those in weakly invasive (MCF7 and T-47D) cell lines. Moreover, the expression of PI 4-kinase III\(\beta\) (PI4KIII\(\beta\)), which generates PI(4)P at the Golgi, is higher in late-stage metastatic human breast cancer tissues (stages III and IV) than in early-stage tissues (non-metastatic, stages I and IIa). In contrast, SAC1 expression is decreased in human metastatic breast cancer tissues at stages III and IV.\(^{(19)}\) In this study, we identified the mechanism underlying these changes in the invasive phenotype. Phosphatidylinositol-4-phosphate generation at the Golgi and SAC1 expression are co-related to an invasive phenotype.\(^{(19)}\) In this study, we identified the mechanism underlying these changes in the invasive phenotype. Phosphatidylinositol-4-phosphate generation at the Golgi resulted in altered expression of breast cancer stem cell markers, including CD44, and increased focal adhesion formation.

**Materials and Methods**

**Cell lines.** MCF-7, MDA-MB-231, and Hs578t cells were cultured as described previously.\(^{(19)}\) SK-BR-3 cells were cultured in DMEM supplemented with 10% FBS.

**Materials.** Mouse anti-PI4KIII\(\beta\) mAb was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Mouse anti-vinculin mAb was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD44s pan mouse mAb (clone #2C5) and CD44v6 mouse mAb (clone #2F10) were purchased from R&D Systems (Minneapolis, MN, USA). Anti-phospho ezrin (Thr-558) and anti-radixin (Thr-564)–tubulin rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA). Anti-ezrin rabbit polyclonal, anti-radixin rabbit monoclonal, and anti-SAC1 rabbit polyclonal antibodies

### Table 1. Effect of SAC1 knockdown on standard CD44 (CD44s) and variant CD44 (CD44v) mRNA levels in MCF7 and SK-BR-3 breast cancer cells

|                 | CD44s ΔCq (mean ± SEM) | Fold change in CD44s mRNA expression \(2^{ΔCq}\) (mean ± SEM) | P-value (t-test) | CD44v ΔCq (mean ± SEM) | Fold change in CD44v mRNA expression \(2^{ΔCq}\) (mean ± SEM) | P-value (t-test) |
|-----------------|------------------------|-------------------------------------------------------------|-----------------|------------------------|-------------------------------------------------------------|-----------------|
| **MCF7**        |                        |                                                             |                 |                        |                                                             |                 |
| Control siRNA   | 8.94 ± 0.18            | 1.00 ± 0.11                                                 |                 | 13.26 ± 0.36           | 1.00 ± 0.15                                                 |                 |
| SAC1 siRNA #1   | 8.10 ± 0.13            | 1.73 ± 0.15                                                 | 3.88 × 10⁻⁴     | 12.69 ± 0.48           | 1.48 ± 0.36                                                 | 0.018           |
| SAC1 siRNA #2   | 8.07 ± 0.26            | 1.83 ± 0.52                                                 | 0.0160          | 12.06 ± 0.39           | 2.28 ± 0.64                                                 | 0.022           |
| **SK-BR-3**     |                        |                                                             |                 |                        |                                                             |                 |
| Control siRNA   | 10.16 ± 0.20           | 1.00 ± 0.16                                                 |                 | 14.27 ± 0.64           | 1.00 ± 0.25                                                 |                 |
| SAC1 siRNA #1   | 8.24 ± 0.22            | 3.79 ± 0.70                                                 | 0.0150          | 12.25 ± 0.54           | 3.52 ± 0.72                                                 | 0.037           |
| SAC1 siRNA #2   | 7.64 ± 0.096           | 5.75 ± 0.41                                                 | 0.0033          | 11.81 ± 0.25           | 5.48 ± 0.84                                                 | 0.028           |

Each reaction contained cDNA derived from 10 ng total RNA. The results are presented as the mean ± SEM of 11 independent experiments for MCF7 cells and of five independent experiments for SK-BR-3 cells, respectively. ΔCq, Cq (CD44s or CD44v) - Cq (Gapdh).

\[ΔCq = Cq_{CD44s} - Cq_{Gapdh}\]

\[ΔCq = Cq_{CD44v} - Cq_{Gapdh}\]

**Fig. 2.** Immunofluorescence analysis of CD44 in normal or SAC1-silenced MCF7 and SK-BR-3 cells. (a) Immunofluorescence analysis of endogenous CD44 in MCF7 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Scale bar = 20 μm. (b) Immunofluorescence analysis of endogenous CD44 in SK-BR-3 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Scale bar = 20 μm.
were purchased from Abcam (Cambridge, UK), and Proteintech (Chicago, IL, USA), respectively. All fluorescent-conjugated secondary antibodies (Alexa Fluor 647–phalloidin, and Alexa Fluor 488- and 568-conjugated goat anti-rabbit or anti-mouse secondary antibodies) were purchased from Life Technologies (Carlsbad, CA, USA).

Cell culture and transfection of siRNA. For the knockdown of SAC1 and PI4KIIIβ, Stealth siRNAs were purchased from Life Technologies.

Fig. 3. Knockdown of phosphoinositide 4-kinase IIIβ (PI4KIIIβ) attenuated the expression of CD44 variants (CD44v) in MDA-MB-231 cells. (a) Semiquantitative RT-PCR analysis of CD44s, CD44v, and Gapdh in MDA-MB-231 cells transfected with control or PI4KIIIβ-targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of Gapdh mRNA. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05; **P < 0.01. (b) Semiquantitative RT-PCR analysis of CD44s, CD44v, and β-actin in HS578t cells transfected with control or PI4KIIIβ-targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of β-actin mRNA. The results are presented as the mean ± SEM of three independent experiments. *P < 0.05; **P < 0.01. (c) Changes in the expression of CD44 and CD44v6 proteins induced by the knockdown of PI4KIIIβ in MDA-MB-231 cells. MDA-MB-231 cells were transfected with control or PI4KIIIβ siRNAs for 72 h before lysate collection. The relative amounts of PI4KIIIβ, CD44, and CD44v6 expression were quantified by densitometry. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05. (d) Changes in the expression of CD44 and CD44v6 proteins induced by the knockdown of PI4KIIIβ in HS578t cells. MDA-MB-231 cells were transfected with control or PI4KIIIβ siRNAs for 72 h before lysate collection. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05.
Technologies.(19) Twenty nanomoles of Stealth siRNAs were transfected into each cell line using Lipofectamine RNAiMAX (Life Technologies); experiments were carried out 72 h after transfection.

**Separation of CD44** and **CD44**-**MCF7** cells. MCF7 cells were incubated with labeled CD44 microbeads at 4°C for 15 min. After brief washing with washing buffer containing PBS (pH 7.2), 1% BSA, and 2 mM EDTA, the cells were separated using a MultiMACS Cell Separator Plus (Miltenyi Biotec, Bergisch Gladbach, Germany) with CD44 microbeads (Miltenyi Biotec).

**Semiquantitative RT-PCR and real time RT-PCR.** The one-step RT-PCR kit from Life Technologies was used to synthesize cDNA from the RNA purified from MCF7, SK-BR-3, MDA-MB-231, and Hs578t. Semiquantitative RT-PCR was undertaken using the following primer sets: β-actin forward, 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse, 5'-ATATCCTGTGTCGTGATCC-3' (product size 611 bp); GAPDH forward, 5'-CTCTGTGAAATGGATATGG-3' and reverse, 5'-CTGCCCTGCAAATGAG-3'; CD44s forward, 5'-CAGAAGGAATCCTGAAGAC-3' and reverse, 5'-GTGCTCTGGTCAAGTACG-3' (product size 198 bp); and CD44v forward, 5'-CAGAACGAATCCTGAAAGAC-3' and reverse, 5'-GTGCTCTGGTCAAGTACG-3' (product size 198 bp); and CD44v reverse, 5'-GTGCTCTGGTCAAGTACG-3'.

For quantitative real-time PCR, the Dynamo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used with a PikoReal Real-Time PCR System (Thermo Fisher Scientific) and analyzed using PikoReal 2.0 software. The reaction conditions were as follows: incubated at 95°C for 1 min, 40 cycles at 95°C for 15 s, 60°C for 1 min, followed by a final extension step at 72°C for 10 min. 

**Immunofluorescence analysis.** Cells were fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.2% Triton X-100 in PBS. They were blocked with 1% BSA and then incubated with primary antibodies in blocking buffer. After five washes with PBS, the cells were incubated with the appropriate secondary antibodies in blocking buffer. After brief washing with PBS, coverslips were mounted onto slides using PermaFluor Mounting Medium (Thermo Fisher Scientific) and observed using a Fluoview 1000-D confocal microscope (IX81; Olympus, Tokyo, Japan) equipped with 473-, 568-, and 633-nm diode lasers through an objective lens (60x oil immersion objective, NA 1.35; Olympus) using Fluoview software. Acquired images were processed using Photoshop (Adobe, San Jose, CA, USA).

**Quantification of focal adhesions.** Focal adhesions were quantified as described previously.(21) Cells were fixed and stained with anti-vinculin antibody and Alexa Fluor 647-phalloidin. Images were obtained by confocal microscopy. The focal adhesions were quantified by counting vinculin plaques. Forty cells obtained from three independent experiments were analyzed. All image processing was carried out using ImageJ software (NIH, Bethesda, MD, USA).

**Results**

Knockdown of SAC1 increased the expression of CD44 in luminal breast cancer cells. MCF7 cells were originally suggested to show the luminal–epithelial phenotype, some portion of which express CD44 but does not show the CD44+/CD24- cell phenotype.(5,6) To obtain CD44+/low cells from MCF7 cells, we undertook cell separation using an antibody that recognized both CD44s and CD44v. Western blot analysis showed that SAC1 expression was lower in the CD44+ fraction than in the CD44+/low fraction (Fig. 1a). Although MCF7 cells predominantly express CD44v, CD44 variants, including CD44v6-10, v2-10, and v3-10, are also expressed.(23) Among these variants, the expression of CD44v6-10 is much higher than in the CD44v6-10, v2-10, and v3-10, are also expressed.(23) Among these variants, the expression of CD44v6-10 is much higher than in the CD44v6-10, v2-10, and v3-10, are also expressed.

**Table 2. Effect of phosphoinositide 4-kinase IIIβ (PI4KIIIβ) knockdown on standard CD44 (CD44s) and variant CD44 (CD44v) mRNA levels in MDA-MB-231 and Hs578t breast cancer cells**

|                  | CD44s ΔCq (mean ± SEM) | Fold change in CD44s mRNA expression [2^ΔCqΔCt] (mean ± SEM) | P-value (t-test) | CD44v ΔCq (mean ± SEM) | Fold change in CD44v mRNA expression [2^ΔCqΔCt] (mean ± SEM) | P-value (t-test) |
|------------------|------------------------|---------------------------------------------------------------|-----------------|------------------------|---------------------------------------------------------------|-----------------|
| MDA-MB-231       |                        |                                                               |                 |                        |                                                               |                 |
| Control siRNA    | 3.21 ± 0.13            | 1.00 ± 0.086                                                  |                 | 1.00 ± 0.086           | 7.36 ± 0.29                                                    | 1.00 ± 0.21     |
| PI4KIIIβ siRNA #1| 4.00 ± 0.22            | 1.08 ± 0.079                                                  | 0.0460          | 8.92 ± 0.59            | 0.34 ± 0.14                                                   | 0.020           |
| PI4KIIIβsiRNA #2 | 3.90 ± 0.87            | 1.02 ± 0.039                                                  | 0.0062          | 8.10 ± 0.28            | 0.60 ± 0.13                                                   | 0.034           |
| Hs578t           |                        |                                                               |                 |                        |                                                               |                 |
| Control siRNA    | 1.31 ± 0.13            | 1.00 ± 0.085                                                  |                 | 1.00 ± 0.085           | 5.77 ± 0.14                                                   | 1.00 ± 0.093    |
| PI4KIIIβsiRNA #1 | 2.55 ± 0.27            | 1.08 ± 0.085                                                  | 0.0037          | 6.92 ± 0.47            | 0.45 ± 0.13                                                   | 0.0498          |
| PI4KIIIβsiRNA #2 | 3.07 ± 0.35            | 1.06 ± 0.074                                                  | 0.0106          | 7.76 ± 0.49            | 0.25 ± 0.082                                                   | 0.0097          |

Each reaction contained cDNA derived from 10 ng total RNA. The results are presented as the mean ± SEM of five independent experiments. ΔCq, Cq (CD44s or CD44v) - Cq (Gadph).
and CD44v mRNA was increased by SAC1 knockdown in MCF7 (Fig. 1b) and SK-BR-3, another luminal-type, HER-2-enriched breast cancer cell (Fig. 1c). Similar results were obtained using quantitative real-time PCR (Table 1). We confirmed that the amplified RT-PCR products for CD44 variants were CD44v6-10 by sequencing. The levels of CD44s and CD44v6 proteins were increased when SAC1 was knocked down in MCF7 cells (Fig. 1d). The expression of these proteins following knockdown of SAC1 in SK-BR-3 cells was also examined. Expression of SAC1 protein was decreased by 75% by the knockdown of SAC1 compared to control siRNA-transfected cells (Fig. 1e). However, these proteins were very weakly detected by Western blot analysis, which might be caused by the very low CD44 expression in these cells (Fig. 1e). SAC1 depletion reduces cell-cell adhesion. Although non-specific immunoreactivities in the nuclei were observed, immunofluorescence analysis showed that CD44s were localized at the cell surface by SAC1 knockdown in MCF7 (Fig. 2a) and SK-BR-3 cells (Fig. 2b).

Knockdown of PI4KIIIβ decreased CD44 expression in basal breast cancer cells. Decreased PI(4)P at the Golgi mediates the suppression of migration and invasion of basal-mesenchymal MDA-MB-231 and Hs578t breast cancer cells. These cells originally abundantly expressed CD44s and most cells showed the CD44+/CD24− phenotype. The mRNA expression of CD44s and CD44v6 was markedly decreased by the knockdown of PI4KIIIβ in these cells (Fig. 3a,b, Table 2). Protein expression of CD44s and CD44v6 was also decreased by PI4KIIIβ knockdown (Fig. 3c,d). Knockdown of PI4KIIIβ promoted an increase in cell–cell adhesion in MDA-MB-231 cells. Immunofluorescence analysis of these cells revealed that the expression of cell surface CD44v6 was also decreased by PI4KIIIβ knockdown (Fig. 4). These results indicate that Golgi PI(4)P mediates the expression of CD44 isoforms such as CD44s and CD44v6.

Golgi PI(4)P regulated focal adhesion formation. The ERM family proteins act as linkers between the actin cytoskeleton and CD44 transmembrane receptor. Among them, ezrin is reportedly involved in changes in cell motility. Phosphorylation of ezrin at Thr-567 by Rho-kinase triggers its association with CD44 at the plasma membrane, which subsequently enhances the formation of focal adhesions. Our previous results showed that Golgi PI(4)P regulates the activity of a small GTPase Rho. Knockdown of SAC1 in MCF7 and SK-BR-3 cells caused a slight increase in ERM phosphorylation at Thr-567 and radixin phosphorylation at Thr-564 (Fig. 5a,b). In contrast, these phosphorylations were decreased by PI4KIIIβ knockdown in MDA-MB-231 and Hs578t cells (Fig. 5c,d). Next, we evaluated the effect of SAC1 knockdown on focal adhesion formation because phosphorylated proteins from the ERM family regulate the formation of focal adhesions by cross-linking CD44 and the actin cytoskeleton. MCF7 and SK-BR-3 cells normally form a small number of vinculin-positive focal adhesions; this number was increased by SAC1 knockdown (Fig. 6a–d). Highly invasive MDA-MB-231 cells form vinculin-positive focal adhesions. However, their number was decreased by PI4KIIIβ knockdown in MDA-MB-231 and Hs578t cells (Fig. 6e–h). These results suggest that Golgi PI(4)P regulates the formation of focal adhesions in breast cancer cell lines.

Discussion

Phosphatidylinositol-4-phosphate at the Golgi is required for lipid and protein sorting. It is under the control of PI4KIIIβ and SAC1 to coordinate secretion with cell growth. We
recently proposed a model for breast cancer cell progression in which the Golgi PI(4)P apparatus affects cell motility and invasion in breast cancer cell lines.\(^{(19)}\) Increased PI(4)P at the Golgi by knockdown on the phosphorylation of ezrin-radixin-moesin (ERM) protein in MCF7 (A) and SK-BR-3 (B) breast cancer cells. Values were normalized to total ezrin protein content. (c) Effect of phosphoinositide 4-kinase IIIβ (PI4KIIIβ) silencing on the phosphorylation of ERM in MDA-MB-231 (C) and Hs578t (D) cells. Values were normalized to total ezrin protein content. All results are presented as the mean ± SEM of four independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. p, phosphorylated.

PI4KIIIβ in MDA-MB-231 cells decreased them (Fig. 7). Although the mechanisms underlying CD44 expression are poorly understood, nuclear factor-kB is known to be involved in the regulation of CD44 expression in breast cancer cell lines.\(^{(30)}\) Golgi phosphoprotein 3 (GOLPH3), defined as an oncoprotein, is a PI(4)P effector at the Golgi. An increasing number of studies have implicated GOLPH3 in several cancers, including hepatocellular carcinoma,\(^{(31)}\) glioblastoma,\(^{(32,33)}\) and breast cancer.\(^{(34)}\) The PI(4)P-binding ability of GOLPH3 is essential for breast cancer cell metastasis \textit{in vitro} and \textit{in vivo}.\(^{(35)}\) Given that the expression of GOLPH3 in hepatocellular carcinoma cell lines promotes the activation of nuclear factor-kB,\(^{(35)}\) it is not surprising that PI(4)P at the Golgi regulates CD44 expression in breast cancer cells. Phosphatidylinositol-4-phosphate is abundant at the trans-Golgi compartment and a small portion was detected at the plasma membrane and nuclear envelope.\(^{(36)}\) The molecular function of PI(4)P is the regulation of COPII vesicles fusion with the Golgi and trafficking proteins from the endoplasmic
reticulum to the plasma membrane. Immunofluorescence analysis of breast cancer cell lines showed that SAC1 and PI4KIIβ regulate the localization of CD44s and CD44v6 at the plasma membrane. Golgi phosphoprotein 3 has pivotal roles in vesicle trafficking from the Golgi, and thus PI(4)P at the Golgi may affect the membrane trafficking of CD44s and

Fig. 6. Role of phosphatidylinositol-4-phosphate (PI(4)P) in the formation of focal adhesions in breast cancer cells. (a) Immunofluorescence analysis of vinculin in MCF7 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Scale bar = 20 μm. (b) Vinculin-positive focal adhesion in MCF7 cells transfected with control or SAC1-targeted siRNA. The number of adhesions was determined using the images captured in 40 cells by confocal microscopy. (c) Immunofluorescence analysis of vinculin in SK-BR-3 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Enlarged images of boxed areas are shown in the lower panels. Arrowheads indicate focal adhesions. Scale bar = 20 μm. (d) Vinculin-positive focal adhesion in SK-BR-3 cells transfected with control or SAC1-targeted siRNA. The number of adhesions was determined using the images captured in 40 cells by confocal microscopy. (e) Immunofluorescence analysis of endogenous vinculin in MDA-MB-231 cells transfected with control or phosphoinositide 4-kinase IIIβ (PI4KIIβ)-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Focal adhesions were analyzed in more than 40 cells. (g) Immunofluorescence analysis of endogenous vinculin in Hs578t cells transfected with control or PI4KIIβ-depleted MDA-MB-231 cells. Focal adhesions were analyzed in 40 cells. All data are presented as box and whisker plots with boxes representing 25th-75th percentile range and whiskers representing 10th-90th percentile range. *P < 0.05; **P < 0.01.
Luminal–epithelial-like breast cancer (MCF7, SK-BR-3)

Basal–mesenchymal breast cancer (MDA-MB-231, Hs578T)

SAC1

Golgi PI(4)P

Pi4KIIIβ

Golgi GOLPH3

Cell invasion

Cell–cell adhesion

CD44 expression

Focal adhesion

Fig. 7. Schematic illustration of regulatory cross-talk occurring between Golgi phosphatidylinositol-4-phosphate (PI(4)P), CD44 expression, and cell adhesion in breast cancer cell invasion and motility. PI(4)P at the Golgi plays a critical role in cancer progression. Changes in the Golgi PI(4)P levels altered the expression and localization of CD44. Focal adhesion formation, cell–cell adhesion formation, cell migration/invasion, and metastasis in breast cancer cell lines. Regulation of the localization of the PI(4)P effector Golgi phosphoprotein 3 (GOLPH3) at the Golgi was predicted to cause these phenotypes. The involvement of PI(4)P levels in molecular subclassification of breast cancer cells is shown.

CD44v. A recent study showed that the interactions among GOLPH3, PI(4)P, and sialyltransferases efficiently regulate sialylation on several target proteins expressed on the cell surface, including integrins and some receptor tyrosine kinases, and the resulting glycoproteins cooperatively enhanced integrin-mediated cell migration and activated the PI 3-kinase signaling pathway. Glycosylation of CD44 has been implicated in CD44-mediated cell adhesion to hyaluronan. Taken together, our results suggest that PI(4)P at the Golgi regulates the expression, localization, and glycosylation status of CD44, and determines cancer cell characteristics (Fig. 7).

The ERM protein family plays a role in cytoskeletal organization in the plasma membrane. Among them, ezrin plays an important role in morphogenesis, cell migration, tumor metastasis, and cell adhesion. CD44, particularly CD44v6, recruits ezrin, which is phosphorylated at Thr-567 and becomes activated. Activated ezrin triggers the activation of focal adhesion kinase and the subsequent formation of focal adhesions. When SAC1 was knocked down in MCF7 and SK-BR-3 cells, which are luminal-type breast cancer cells, ezrin phosphorylation and the number of focal adhesions were increased, whereas knockdown of Pi4KIIIβ in MDA-MB-231 cells decreased these phenomena. Given that the formation of focal adhesions is a critical step for developing the invasive–metastatic characteristics of breast cancer cells, PI(4)P at the Golgi likely mediates these signaling pathways. Immunofluorescence analysis revealed the accumulation of cell surface CD44s by SAC1 knockdown in MCF7 cells. Among the phosphoinositides, (PI(4,5)P2) has been reported to bind to ERM family proteins and increase the binding affinity of ERM family proteins to CD44. This increase promotes a link between filamentous actin and the receptors. Together with our previous findings that PI(4)P promotes an increase in RhoA activity, we suggest that PI(4)P at the Golgi modulates the expression of CD44 (CD44s and CD44v) and promotes the formation of vinculin-positive focal adhesions; thus, it regulates the invasive phenotype of breast cancer cell lines. CD44 signaling acts on the Hippo-YAP/TAZ signaling pathway, which mediates cancer progression, cell survival, cell survival, metastasis, and stem cell maintenance of stem cell properties. Hippo pathway mutations give rise to tumors, and deregulation of this pathway is frequently observed in human cancers. Downregulation of CD44 or knockdown of RhoA decreases YAP expression, which promotes cell apoptosis and inhibits cell proliferation. Phosphatidylinositol-4-phosphate at the Golgi may modulate this signaling by regulating CD44 expression and its localization at the cell surface.

Increased CD44 expression is one of the characteristics of cancer stem cells. The presence of cancer stem cells has been observed in a variety of cancers, including leukemia, colon cancer, ovarian cancer, glioma, and breast cancer. Cancer stem cells are more resistant to chemotherapy and radiotherapy than other cancer cells within the same tumors. In addition, they are responsible for tumor recurrence, invasion, and metastasis. Breast cancer stem cells constitute only a small population of breast tumor cells, with the characteristic CD44+/CD44−/low phenotype. Recently, the expression of another phosphoinositide phosphatase, SHIP2, was reported to be correlated with the size of the CD44+/CD44−/low subpopulation. Although the precise mechanisms underlying the changes in breast cancer phenotype remain unclear, our results showed that PI(4)P affects the molecular subtypes of breast cancer cell lines. Understanding these mechanisms will provide further insight into the acquisition of invasive potential by cancer cells and provide a foundation for therapeutic intervention.

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Disclosure Statement

The authors have no conflict of interest.
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