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A novel role of EMMPRIN/CD147 in transformation of quiescent fibroblasts to cancer-associated fibroblasts by breast cancer cells

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Abstract

We tested the novel hypothesis that EMMPRIN/CD147, a transmembrane glycoprotein overexpressed in breast cancer cells, has a previously unknown role in transforming fibroblasts to cancer-associated fibroblasts, and that cancer-associated fibroblasts in turn induce epithelial-to-mesenchymal transition of breast cancer cells. Co-culture of fibroblasts with breast cancer cells or treatment of fibroblasts with breast cancer cell conditioned culture medium or recombinant EMMPRIN/CD147 induced expression of α -SMA in the fibroblasts in an EMMPRIN/CD147-dependent manner and promoted epithelial-to-mesenchymal transition of breast cancer cells and enhanced cell migration potential. These findings support a novel role of EMMPRIN/CD147 in regulating the interaction between cancer and stroma.

Keywords

EMMPRIN/CD147; Cancer-associated fibroblasts; Epithelial-to-mesenchymal transition; α -SMA; Cancer stroma

1. Introduction

The classic oncogene- and tumor suppressor gene-centric dogma of cancer initiation and progression has been adjusted in recent years to take into account our realization that the tumor microenvironment is a co-conspirator in tumor progression [1]. Cancer stromal cells co-evolve with cancer cells and constitute the major components of the microenvironment, which is essential for cancer cell growth, survival, invasion, and metastasis [1–5]. Cancer-

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Conflict of Interest

None declared.

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associated fibroblasts (CAFs) are the most abundant cells in cancer stroma and contribute to the malignant phenotype of cancers [4,5]. Compared with normal quiescent fibroblasts, CAFs acquire distinctive phenotypes, such as myofibroblast differentiation characterized by expression of alpha-smooth muscle actin (α -SMA) [5–8]. That CAFs contribute to the supportive microenvironment of cancer cells by secreting several growth factors and matrix-degrading enzymes has long been recognized [2–5,9]. It has been shown that CAFs can also induce epithelial-to-mesenchymal transition (EMT) of breast cancer cells through secretion of cellular factors that have yet to be identified [10,11].

Although the important role of CAFs in cancer initiation and progression is being increasingly recognized, the molecular mechanisms underlying how CAFs are formed or transformed are still largely elusive. Transforming growth factor-beta (TGF- β), which has both pro- and anti-tumor functions, is one of a few well-recognized inducers of transformation of normal fibroblasts to CAFs [5,12,13]. Additional molecules involved in activation of CAFs via cancer-stroma interaction, especially molecules with the potential to be drug targets, need to be identified.

Extracellular matrix metalloproteinase inducer (EMMPRIN), termed basigin in mice and also known as CD147, is a type I transmembrane glycoprotein enriched in various types of cancer tissues [14]. It is a cell adhesion molecule belonging to the immunoglobulin superfamily [15,16]. EMMPRIN/CD147 has an extracellular portion, a transmembrane domain, and a short intracellular domain; each domain exerts different functions [17]. The best-known function of EMMPRIN/CD147 expressed on cancer cells is to induce the production of various matrix metalloproteinases (MMPs) in cancer cells and fibroblasts following epithelial-stromal interaction, leading to degradation of extracellular matrix, which facilitates cancer cell invasion and metastasis [15]. Overexpression of EMMPRIN/CD147 in cancer tissues is correlated with poor prognosis of patients with several types of solid tumors, including breast cancer [18–20]. Overexpression of EMMPRIN/CD147 is found in 50–80% of breast cancer specimens [14,19,20] and contributes to the malignant phenotype of breast cancer through promoting cancer cell invasion and metastasis, inducing angiogenesis, preventing anoikis, regulating metabolism, and conferring resistance to chemotherapeutic drugs [21–26]. EMMPRIN/CD147 has also been reported to be able to activate downstream signaling pathways such as phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK that are important pathways for cancer cell proliferation, survival, invasion, and metastasis; however, the exact mechanisms underlying these diverse functions of EMMPRIN/CD147 remain to be elucidated [23,27–29].

The aforementioned characteristics of EMMPRIN/CD147 make it a promising candidate drug target for cancer treatment. Furthermore, the function of EMMPRIN/CD147 in regulating cancer-stroma interaction, such as through inducing fibroblasts to secrete MMPs, makes it a potential target for drug development not only to inhibit cancer cells but also to interfere with the interaction between cancer and cancer stroma, which is drawing increasing attention because stromal cells are genetically more stable than cancer cells. Currently, it is largely unknown how quiescent fibroblasts are transformed to CAFs in cancer stroma. In this study, we tested our hypothesis that EMMPRIN/CD147 has a previously unrecognized function of activating quiescent fibroblasts and transforming them to CAFs in breast cancer and that CAFs in turn induce EMT of breast cancer cells and promote cancer progression and metastasis. Our findings support a novel role of EMMPRIN/CD147 in regulating the interaction between cancer and stroma.

2. Materials and methods

2.1. Materials

The sources of antibodies used for Western blot analysis and immunofluorescent staining are as follows: vimentin and β -actin mouse monoclonal antibody (mAb) (Sigma-Aldrich, St. Louis, MO); α -SMA rabbit mAb (Epitomics, Inc., Burlingame, CA); fibronectin mouse mAb (BD Biosciences, San Jose, CA); N-cadherin and Twist rabbit polyclonal Ab and E-cadherin mouse mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Mouse EMMPRIN/CD147 mAb and recombinant EMMPRIN/CD147 protein were prepared by the Cell Engineering Research Center (Xi'an, China). The glycosylated recombinant EMMPRIN/CD147 protein was constructed in FLP-IN/FRT-pcDNA5 vector and expressed in FLP-IN-CHO cells. Recombinant human TGF- β 1 was purchased from R&D Systems, Inc. (Minneapolis, MN). All other materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Cell lines and culture

All breast cancer cell lines were maintained in Dulbecco's minimal essential medium containing high glucose levels and 10% fetal bovine serum (FBS). Human 1068SK normal breast fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and maintained in minimal essential medium containing 10% FBS. The growth medium was replaced with serum-free medium when cell conditioned medium was collected. The cell conditioned medium was concentrated with Amicon ultra-4 (Millipore Corp., Billerica, MA) according to the manufacturer's instructions.

2.3. Western blotting

After desired treatments, cultured cells were harvested with a rubber scraper and washed twice with cold phosphate-buffered saline (PBS). Cell pellets were then lysed with a buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% Igepal CA-630, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 25 μ g/ml leupeptin, and 25 μ g/ml aprotinin) and set on ice for at least 10 minutes prior to clearance by centrifugation (14,000 g for 30 minutes at 4°C). Protein concentrations of cell lysates were determined using the Bradford Coomassie blue method (Pierce Chemical, Rockford, IL). The samples were analyzed by separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer of the proteins onto nitrocellulose membrane using Western blotting and probing of the membrane with various primary antibodies and horseradish peroxidase-labeled secondary antibodies. The signals were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ).

2.4. Immunofluorescent double staining of cells

For fluorescence microscopic analysis, cells grown on sterile glass coverslips were briefly washed with PBS, fixed with cold 100% methanol for 5 minutes, and then air dried. The slides were first incubated with 10% species-specific serum for blocking for 30 minutes, then with two primary antibodies or nonspecific isotype antibodies on ice overnight. After incubation with the primary antibodies, the slides were washed again twice with PBS, then incubated with fluorescence-labeled secondary antibodies (FITC-labeled goat-anti-mouse IgG [1:300] and Rhodamine Red-X-labeled goat-anti-rabbit IgG [1:300]) (Life Technologies, Carlsbad, CA) in a dark chamber for 45 minutes. Cell nuclei were counterstained with DAPI (1:50,000 dilutions in PBS). The glass coverslips were mounted with coverslips with 50% glycerol (in PBS, pH 9.6) and examined under a fluorescence microscope with appropriate filters. To permit proper recognition of cytoplasmic markers by antibodies, the cells were permeabilized with 0.1% Triton X100 in PBS buffer at room

temperature for 5 minutes followed by 3 washes with PBS prior to blocking of nonspecific binding with serum.

2.5. RNA interference

Predesigned siRNA oligonucleotide duplexes against EMMPRIN/CD147 (sense strand: 5'-GGUUCUUCGUGAGUCCUCtt-3', anti-sense strand: 5'-GAGGAACUCACGAAGAACCtg-3'), which were verified in our previous study [30], were ordered from Ambion, Inc. (Grand Island, NY). Nontargeting siRNA was ordered from Dharmacon, Inc. (Lafayette, CO). Cells were seeded at 6×10^5 cells/well in six-well plates and were cultured overnight prior to transfection. EMMPRIN/CD147 siRNA or nontargeting siRNA (250 pmol) was mixed with 5 μ l of FuGENE 6 in 300 μ l of serum-free medium for 20 minutes, and then the siRNA/FuGENE 6 mixture was added into 2.2 ml of the culture medium overnight. The final concentration of siRNA was 100 nM. After siRNA transfection, the cells were cultured for an additional 48 hours to allow knockdown of EMMPRIN/CD147 expression.

2.6. Cell migration assay

Cell migration assay was performed using Boyden chambers with polycarbonate membrane (pore size, 8 μ m) (Corning, Inc., Corning, NY). After rehydration of the filter membrane with serum-free culture medium, the lower chamber of the transwell was filled with 0.4 ml of 5% FBS medium supplemented with various additions for treatment, and to the upper chamber were added 6×10^4 cells suspended in 0.2 ml of 0.5% FBS medium. The chambers were placed in a 37°C incubator for the desired time periods. After incubation, the upper-chamber medium was removed, and any residual cells in the chamber were gently wiped out with a cotton-tipped swab. The filter membranes in the chambers were rinsed with PBS and stained with HEMA3 solution according to the manufacturer's instructions (Fisher Scientific, Pittsburgh, PA). The cells penetrated into the membrane were counted under a microscope. Cells in 10 different microscope fields of each filter were counted. Each treatment group was set in triplicate inserts/wells. Representative areas for each treatment were photographed.

2.7. Statistical analysis

Student's *t* test was used to compare two groups, and one-way analysis of variance was used for multiple comparisons. The data were expressed as means $\pm$ standard deviation. All statistical tests were two-sided, and *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Overexpression of EMMPRIN/CD147 is common in breast cancer cells

We examined the expression of EMMPRIN/CD147 in a panel of 13 established breast cancer cell lines. EMMPRIN/CD147, which showed a classic underglycosylated band (approximately 37 kD) and a highly glycosylated band (approximately 55–65 kD), was detected in all cell lines examined (Fig. 1). The cell lines MDA157, SKBR3, MCF7, BT20, and HS578T expressed high levels of highly glycosylated EMMPRIN/CD147. Except for SUM190, BT474, and T47D, which expressed relatively low levels of EMMPRIN/CD147, 10 of 13 cell lines expressed variable levels of underglycosylated or highly glycosylated EMMPRIN/CD147.

3.2. EMMPRIN/CD147 overexpressed by breast cancer cells transforms fibroblasts to CAFs

We next tested our hypothesis that the EMMPRIN/CD147 in breast cancer cells transforms normal fibroblasts to CAFs by detecting expression of α -SMA, a well-recognized marker of

CAFs [5,6], in 1068SK breast fibroblasts after co-culture individually with two types of EMMPRIN/CD147-overexpressing breast cancer cell lines, SKBR3 and MCF7. Expression of α -SMA was increased in the lysates of co-cultured cells but not in the mixed lysates of individually cultured cancer cells and fibroblasts (Fig. 2A). Further, the increase in α -SMA expression was specifically in the fibroblasts as indicated by double immunofluorescent staining of co-cultured cells with antibodies direct against α -SMA and vimentin (a marker of fibroblasts) (Fig. 2B). The levels of expression of α -SMA and vimentin were low in 1068SK breast fibroblasts but were markedly increased after co-culture with SKBR3 or MCF7 breast cancer cells, suggesting transformation of fibroblasts to CAFs by breast cancer cells after co-culture. It is noteworthy that SKBR3 breast cancer cells expressed no detectable level of vimentin or α -SMA, whereas MCF7 breast cancer cells expressed a detectable level of α -SMA even when cultured alone, a so-called myoepithelial phenotype that was previously described [31,32].

To determine whether EMMPRIN/CD147 expressed by the breast cancer cells played a role in transforming the fibroblasts, we silenced the expression of EMMPRIN/CD147 in SKBR3 and MCF7 breast cancer cells using validated specific siRNA or treated the cells with control siRNA. Compared with the results after co-culture of 1068SK breast fibroblasts with control siRNA-treated SKBR3 or MCF7 cells, knockdown of EMMPRIN/CD147 expression in SKBR3 and MCF7 cells abolished the co-culture-induced expression of α -SMA in the fibroblasts (Fig. 2C). These findings indicate an important role of EMMPRIN/CD147 in the transformation of fibroblasts to CAFs.

To determine if EMMPRIN/CD147 was present in the conditioned medium of breast cancer cells, which would provide useful information about whether direct interaction between cancer cells and fibroblasts is necessary for EMMPRIN/CD147-induced transformation of fibroblasts to CAFs, we used Western blotting to detect the presence of EMMPRIN/CD147 in the conditioned medium from SKBR3 and MCF7 breast cancer cells. EMMPRIN/CD147, mainly in the glycosylated form, was detected in concentrated conditioned medium from both SKBR3 and MCF7 cells but not in fresh medium (control medium) (Fig. 3A). When the concentrated conditioned medium was added into culture of fresh 1068SK breast fibroblasts, an increase in the level of α -SMA in the fibroblasts was detected (Fig. 3B), which was similar to the dose-dependent increase in the level of α -SMA expression in the fibroblasts upon treatment with recombinant EMMPRIN/CD147 or TGF- β 1, a positive control (Fig. 3C). Immunofluorescent staining of the cells with α -SMA antibody showed increased expression of α -SMA in the fibroblasts (Fig. 3D), supporting the findings of Western blotting (Fig. 3C). Taken together, these findings indicate a previously unknown function of EMMPRIN/CD147 overexpressed in breast cancer cells in inducing the transformation of fibroblasts to CAFs, which may occur via tumor-stroma direct interaction or indirectly via a paracrine mechanism.

3.3. EMMPRIN/CD147 mediates CAF-induced EMT of breast cancer cells

CAFs have been reported to induce the EMT-like changes of cancer cells [10, 11]. In the present study, we found that co-culture of MCF7 or SKBR3 breast cancer cells with 1068SK breast fibroblasts led to clear decrease in the level of E-cadherin in MCF7 cells (note: the expression of E-cadherin was not detectable in SKBR3 cells due to a homozygous deletion of a large portion of the E-cadherin gene in the cells [33–35]), and marked increase in the levels of several well-characterized mesenchymal markers, including fibronectin, N-cadherin, and Twist (Fig. 4A), along with morphologic changes characteristic of EMT in both MCF7 and SKBR3 cells (Fig. 4B). Double immunofluorescent staining showed that the level of E-cadherin was decreased and the level of N-cadherin was increased in MCF7 cells co-cultured with fibroblasts (Fig. 4C). To further confirm a role of EMMPRIN/CD147 in transforming fibroblasts to CAFs and in inducing EMT of breast cancer cells, we knocked

down the expression of EMMPRIN/CD147 in MCF7 cells and SKBR3 cells and found that the increased expression of the mesenchymal markers induced by co-culture was markedly decreased, although the levels of the markers were still slightly higher than that in the lysate mixture of fibroblasts and EMMPRIN-knocked down cells due to incomplete knockdown of EMMPRIN/CD147 in the cells, particularly in SKBR3 cells (Fig. 4D).

To determine whether the change in EMT markers after co-culture of breast cancer cells with fibroblasts was linked to altered function of the breast cancer cells, we measured the migration potential of SKBR3 and MCF7 cells after co-culture with fibroblasts or not. The migration potential without co-culture with fibroblasts was low in both SKBR3 and MCF7 cells (Fig. 5A). Knockdown of EMMPRIN/CD147 resulted in further decrease in cell migration of the cells. Co-culture of the cells with 1068SK breast fibroblasts led to marked increase in migration potential but the increase was substantially mitigated when the level of EMMPRIN/CD147 in SKBR3 and MCF7 cells was knocked down. We also measured the migration potential of SKBR3 and MCF7 cells after treatment with the concentrated conditioned medium from co-culture of the cancer cells (SKBR3 and MCF7) and the fibroblasts. Similar to the findings obtained from cell co-culture, the conditioned medium led to an increase in the migration potential of SKBR3 and MCF7 breast cancer cells measured with Boyden chamber assays (Fig. 5B). This increase was significantly less when SKBR3 and MCF7 cells were treated with conditioned medium from co-culture of fibroblasts and SKBR3 and MCF7 cells in which the expression of EMMPRIN/CD147 was knocked down. Together, these findings indicate a direct role of EMMPRIN/CD147 in CAF-induced EMT of breast cancer cells.

4. Discussion

In the present study, we report our novel finding that EMMPRIN/CD147, a highly glycosylated cell adhesion molecule overexpressed on the surface of various types of cancer cells, has a previously unknown function of transforming normal fibroblasts to CAFs. We showed that co-culture of EMMPRIN/CD147-overexpressing breast cancer cells with normal fibroblasts could induce the expression of α -SMA, a CAF marker, in the fibroblasts, and that knockdown of EMMPRIN/CD147 expression in breast cancer cells largely abolished the induction of α -SMA in the fibroblasts. We further demonstrated that CAFs could induce EMT of breast cancer cells in an EMMPRIN/CD147 expression-dependent manner and enhance the migration potential of breast cancer cells, which is functionally important for the invasion and metastasis of cancer cells [36].

Solid tumors consist of not only cancer cells but also several other components, including stromal cells, extracellular matrix, and vasculature, which collectively create the so-called tumor microenvironment. Ample evidence from basic and clinical research has shown that the stromal microenvironment contributes substantially to cancer development and progression [37]. CAFs are the most abundant type of stromal cells in the tumor microenvironment. CAFs, sometimes referred to as “activated fibroblasts,” have a myofibroblast phenotype characterized by α -SMA expression and possess biological characteristics distinct from those of normal fibroblasts [5,6]. The abundant myofibroblasts in the stroma of invasive human tumors contribute to tumor growth, angiogenesis, and resistance to anticancer therapies through secreting high levels of cytokines such as stromal cell-derived factor 1, platelet-derived growth factor, and others [4,5,38]. However, the source of CAFs remains elusive. It has been proposed that CAFs are mainly derived from quiescent normal fibroblasts and are transformed to CAFs by cancer cell-originated signals; however, the exact underlying mechanisms remain poorly understood [4,5]. TGF- β and platelet-derived growth factor have been proposed to be involved in the transformation of normal fibroblasts to CAFs [5,12,13], but additional molecular candidates need to be

identified. In the present study, we demonstrated that EMMPRIN/CD147 is commonly expressed at high levels in a panel of established breast cancer cell lines and presented, to our knowledge, the first evidence that EMMPRIN/CD147 may be such a molecule that can transform fibroblasts to CAFs.

The well-known function of EMMPRIN/CD147 is to induce the secretion by cancer cells or fibroblasts of various MMPs, which degrade the extracellular matrix to promote the invasion and metastasis of cancer cells [15]. Cancer-associated EMMPRIN/CD147 can also induce the secretion of vascular endothelial growth factor in fibroblasts to promote angiogenesis [23]. EMMPRIN/CD147 is expressed highly in cancer tissues but at a very low level or not at all in normal tissues [20]. Overexpression of EMMPRIN/CD147 in cancer tissues correlated with poor prognosis of cancer patients [39–41]. We previously reported that EMMPRIN/CD147 is overexpressed in various cancer tissues and can serve as a prognostic factor for hepatocellular carcinoma and breast cancer [20,42,43]. We reported EMMPRIN expression in 65% of cancer tissues from breast cancer patients [20], which is a higher rate of expression than the rate of expression of HER2 (~25%), a well-characterized prognostic biomarker and drug target in breast cancer [44]. Our previous studies showed that the MMPs secreted by fibroblasts in response to EMMPRIN/CD147 expressed on cancer cells play a more critical role in cancer cell invasion than those secreted by cancer cells themselves [30]. Our new observations in the present study that EMMPRIN/CD147 is an important cancer-associated molecule involved in cancer-stroma interaction through transforming fibroblasts to CAFs and that the CAFs can in turn promote EMT of breast cancer cells expand our knowledge of the roles of EMMPRIN/CD147 in tumorigenesis.

Soluble EMMPRIN/CD147 has been found in the serum or urine of cancer patients [45–48]. Our current study revealed EMMPRIN/CD147 in the conditioned medium of MCF7 and SKBR3 breast cancer cells, which supports a paracrine model through which the soluble form of EMMPRIN secreted by cancer cells can modulate the functions of stromal cells in the tumor microenvironment. This finding provides a possible mechanism underlying the transformation of normal fibroblasts to CAFs in breast cancer.

EMT has been linked to cancer metastasis as the EMT phenotype gives cancer cells enhanced migration potential [36]. CAFs have been reported to be able to induce EMT of cancer cells, but the molecular mechanism is largely unknown [10, 11]. We previously reported a positive correlation between EMMPRIN/CD147 expression in cancer tissues and likelihood of cancer metastasis in patients with hepatocellular carcinoma and breast cancer [20,42]. Our current data support a role of EMMPRIN/CD147 in CAF-mediated EMT of breast cancer cells.

In summary, our study has revealed evidence at the cellular level that supports a novel role of EMMPRIN/CD147 in the transformation of normal fibroblasts to CAFs through cancer-stroma interaction. Future study is warranted to validate that targeting EMMPRIN/CD147 inhibits transformation of fibroblasts to CAFs, which might subsequently inhibit CAF-induced EMT of cancer cells. Novel therapeutic strategies developed to interfere with the function of EMMPRIN/CD147 or to lower the protein level of EMMPRIN/CD147 may not only directly inhibit cancer cells but also interfere with the interaction between cancer cells and stromal cells, which holds new promise for improving the outcome of cancer treatments.

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Figure 1. Expression of EMMPRIN/CD147 in breast cancer cell lines

Expression of EMMPRIN/CD147 in the indicated breast cancer cell lines was detected by Western blotting. HG, highly glycosylated; LG, underglycosylated.

Figure 2. Role of EMMPRIN/CD147 in transformation of fibroblasts to CAFs by breast cancer cells by co-culture

(A) Lysates of SKBR3 and MCF7 cells after their respective co-culture with 1068SK fibroblasts for 24 hours (cancer cells: fibroblasts ratio = 2:1) and the mixed lysates of individually cultured SKBR3 or MCF7 cells and 1068SK fibroblasts (at the same ratio as for co-cultured cells) were subjected to Western blot analysis with the antibodies shown. (B) SKBR3 and MCF7 cells were co-cultured with 1068SK fibroblasts respectively for 24 hours (cancer cells: fibroblasts ratio = 2:1). Cell samples from individual culture and co-culture were subjected to double immunofluorescent staining with antibodies directed against vimentin (mouse antibody) and α-SMA (rabbit antibody), followed by incubation with mixed Rhodamine Red-X-labeled goat-anti-rabbit (red fluorescence) and FITC-labeled goat-anti-mouse IgG antibodies. Representative areas under a fluorescent microscope are shown. Arrows, fibroblasts; bar, 50 μm. Neg Ctrl: negative control. (C) SKBR3 cells and MCF7 cells with and without knockdown of EMMPRIN/CD147 were co-cultured with 1068SK fibroblasts for 24 hours. Cell samples from individual culture and co-culture were subjected to double immunofluorescent staining as described in (B). Representative areas under a fluorescent microscope are shown. Arrows, fibroblasts; bar, 50 μm.

Figure 3. Transformation of fibroblasts to CAFs by soluble EMMPRIN/CD147

(A) Samples of 50x concentrated conditioned medium from 24-hour SKBR3 and MCF7 cell culture (20 μl) were subjected to Western blotting for detection of EMMPRIN/CD147, which was compared to the EMMPRIN detected in the lysate of SKBR3 cells. kDa, kiloDalton; Ctrl: control (50x concentrated fresh medium). (B) 1068SK fibroblasts were cultured for 24 hours with 24-hour conditioned medium from cell-free culture (control) or from SKBR3 or MCF7 cell culture. Cell lysates were subjected to Western blotting with the antibodies shown. (C) 1068SK fibroblasts were treated with a recombinant EMMPRIN/CD147 (rEP) or TGF-β1 as indicated for 6 hours. TGF-β1 was used as a positive control to induce α-SMA expression in the fibroblasts. Cell lysates were prepared for detection of α-SMA by Western blotting. In B and C, the intensity of α-SMA in relative to that of β-actin was quantified by ImageJ software (NIH). (D) 1068SK fibroblasts were untreated or treated with 2.5 μg/ml rEP or 5 ng/ml TGF-β1 for 6 hours. Cell samples were prepared for detection of α-SMA by immunofluorescent staining. Representative areas under a fluorescent microscope are shown (the red fluorescence indicates α-SMA); bar, 50 μm.

Figure 4. Role of EMMPRIN/CD147 in CAF-induced EMT of breast cancer cells
 (A) MCF7 and SKBR3 cells were individually cultured and co-cultured with 1068SK fibroblasts for 24 hours. Cell lysates were prepared and subjected to Western blotting with the antibodies shown. Mixed lysates of cancer cells and fibroblasts (at the same ratio and same quantity as for co-cultured cells) were used as a reference for addition of lysates without co-culture. (B) MCF7 and SKBR3 cells were individually cultured and co-cultured with 1068SK fibroblasts for 24 hours prior to observation under a phase contrast microscope. Arrows, fibroblasts; bar, 25 μ m. (C) MCF7 cells were individually cultured and co-cultured with 1068SK fibroblasts for 24 hours. Cell samples were then subjected to double immunofluorescent staining with antibodies directed against E-cadherin (mouse antibody) and N-cadherin (rabbit antibody), followed by incubation with mixed Rhodamine Red-X-labeled goat-anti-rabbit (red fluorescence) and FITC-labeled goat-anti-mouse IgG antibodies. Representative areas under a fluorescent microscope are shown. Arrows, fibroblasts; bar, 25 μ m. (D) MCF7 and SKBR3 cells with and without knockdown of EMMPRIN/CD147 were cultured with fibroblasts for 24 hours. Lysates of co-cultured cells and mixed cells were subjected to Western blotting with the antibodies shown.

Figure 5. Inhibition of CAF-induced potentiation of cell migration potential after knockdown of EMMPRIN/CD147

(A) SKBR3 and MCF7 cells with and without knockdown of EMMPRIN/CD147 were cultured individually and co-cultured with 1068SK fibroblasts for 24 hours. Cells were harvested and subjected to Boyden chamber assays as described in Materials and Methods. Representative areas of the chamber filters are shown after staining with HEMA3 solution. * $p < 0.05$; bar, 100 μm . (B) SKBR3 and MCF7 cells were cultured for 24 h after addition of concentrated conditioned medium (CM). Cells were then subjected to Boyden chamber assay as in (A). Representative areas of the chamber filters are shown after staining with HEMA3 solution. * $p < 0.05$; bar, 100 μm .