CD146, an epithelial-mesenchymal transition inducer, is associated with triple-negative breast cancer

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*This Direct Submission article had a prearranged editor.

Author contributions: Q.Z. and X.Y. designed research; Q.Z., W.L., D.L., Z.W., and H.D. performed research; J.F. and D.Y. contributed new reagents/analytic tools; Q.Z., W.L., Y.L., L.F., and X.Y. analyzed data; and Q.Z. and X.Y. wrote the paper.

The authors declare no conflict of interest.

Edited* by Zhu Chen, State Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, RuiJin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China, and approved October 28, 2011 (received for review July 10, 2011)

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increased formation of central stress fibers by F-actin rearrangement, indicating that the intermediate filaments in A5 cells had reformed to a mesenchymal format.

The characteristic features of cells that have undergone an EMT are their dramatically increased migratory and invasive behaviors. As shown in Fig. 1 E and F, increased expression of CD146, especially in A5 cells, significantly induced a higher level of migration and invasion through Matrigel, whereas little migration or invasion was observed in Mock cells.

To verify whether these changes associated with EMTs were specifically induced by CD146, we down-regulated CD146 expression in A5 cells using siRNAs. As shown in Fig. S2A, CD146 expression in A5 cells transfected with siRNAs targeting CD146 was markedly decreased to one-quarter of that of the control transfected with siRNAs targeting GFP. We further observed that CD146 silencing decreased the level of mesenchymal markers vimentin and fibronectin and increased the level of the epithelial marker E-cadherin. Consistent with these changes in EMT markers, some A5 cells reverted to grow into tight cell clusters after CD146 silencing; cell migration and invasion were also significantly inhibited (Fig. S2B-D). Taken together, changes in morphology, EMT marker, and cell migration and invasion after CD146 silencing demonstrate that CD146 underlies the EMTs in A5 cells.

To determine whether CD146-induced EMTs were cell type-specific or not, we expressed CD146 in the CD146− Madin-Darby canine kidney (MDCK) cells, a cell model for EMT study. Similar to the results obtained in MCF-7 cells, changes in morphology, EMT markers, and cell migration and invasion were also significantly inhibited (Fig. S2B-D). Taken together, changes in morphology, EMT marker, and cell migration and invasion after CD146 silencing demonstrate that CD146 underlies the EMTs in A5 cells.

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**CD146-Induced EMTs Generate Breast CSC-Like Cells.** It is reported that mammary epithelial cells that have undergone EMTs increase the CD44high/CD24low population and the capabilities of mammosphere formation, which are characteristic features of normal

**Fig. 1.** CD146 induces EMTs in human breast cancer cells. (A) FACS analysis of CD146 expression in vector control MCF-7-Mock (Mock) and CD146 clones MCF-7-B10 (B10) and MCF-7-A5 (A5). (B) Morphology of Mock, B10, and A5 cells. Magnification, 200×. (C and D) Expression of CD146 and EMT markers analyzed by immunoblotting and immunofluorescence. Nuclei are shown with DAPI staining. (Scale bars, 20 μm.) (E and F) Migration and invasion assays of Mock, B10, and A5 cells. Data were collected from three wells, *P < 0.05, ***P < 0.001, compared with Mock cells. Representative images of migrated or invaded cells are also shown.

**Fig. 2.** CD146-induced EMTs generate breast CSC-like cells. (A) FACS analysis of cell-surface markers CD44 and CD24 in Mock, B10, and A5 cells. (B and C) Morphology and quantification of mammospheres formed by Mock, B10, and A5 cells. (Scale bars, 100 μm.) *P < 0.05, compared with Mock cells.
mammary stem cells and breast CSCs (3). To determine whether CD146-induced EMTs generate CSC-like cells, we performed FACS to analyze the CD44+CD24− population in MCF-7 clones. As shown in Fig. 2A, almost all of the A5 cells acquired a CD44+CD24− expression phenotype with higher CD44 expression, but most of the B10 cells acquired this phenotype with lower CD44 expression; this shift was not observed in Mock cells, which mainly maintained the CD44+CD24− phenotype. These results imply that CD146 expression leads to gradual down-regulation of CD24, whereas its impact on CD44 expression seems more complex. Mammosphere formation assay showed an increase in both the size and number of mammospheres in A5 (P < 0.05) and B10 cells compared with Mock cells (Fig. 2B and C). These observations indicate that CD146 triggers the expression of cell-surface markers and functional characteristics associated with CSCs, an important feature recently defined for inducers of EMTs.

RhoA Activation and Slug Up-Regulation Mediate CD146-Induced EMTs. Because we observed significant alterations of F-actin cytoskeleton in A5 cells, we further examined the activity of Rho GTPases, including RhoA, Rac1, and Cdc42, in our cell lines. These GTPases have been found to play important roles in mediating cytoskeleton rearrangements (17). We found that the active form of RhoA (GTP-RhoA) was dramatically increased in A5 cells compared with Mock and B10 cells (Fig. 3A, Upper), whereas the active forms of Rac1 (GTP-Rac1) and Cdc42 (GTP-Cdc42) were unchanged (Fig. S4A). CD146 silencing efficiently decreased the level of GTP-RhoA in A5 cells (Fig. 3A, Lower), suggesting that RhoA acts downstream of signal pathways induced by CD146 overexpression in A5 cells.

To further determine whether RhoA directly mediates CD146-induced EMTs, we used exoenzyme C3 transferase, a specific inhibitor of Rho, to inhibit RhoA activity in A5 cells. We observed that inhibition of RhoA activity in A5 cells increased expression of E-cadherin and decreased expression of vimentin and fibronectin, although it had no effect on CD146 expression (Fig. 3B). Consistent with the changes in EMT markers, A5 cells became less fibroblastic and less dispersed, their migratory and invasive behaviors were also significantly inhibited (Fig. 3C and D), suggesting that RhoA activation is responsible for CD146-induced EMTs.

Loss of E-cadherin expression is the key event leading to the disruption of tight cell-cell contacts and the triggering of EMTs (18). A number of signal pathways associated with EMTs converge on transcription factors SIP1, Snail, Slug, and Twist to inhibit E-cadherin transcription. Because E-cadherin transcripts were decreased in B10 and A5 cells (Fig. S1), we wondered which transcription factors contributed to these changes. RT-PCR and immunoblotting (Fig. 3E) showed that Slug was gradually increased in Mock, B10, and A5 cells, and expressed at the highest level in A5 cells that showed the highest CD146 expression and the lowest E-cadherin expression. However, SIP1 was unchanged, Snail was undetected in A5 cells, and Twist was slightly increased compared with Mock cells, whose expressions were all not correlated with CD146 expression in the three cells (Fig. S4B). More importantly, CD146 silencing or RhoA inhibition by exoenzyme C3 transferase in A5 cells significantly decreased the level of Slug (Fig. 3F), suggesting that Slug is downstream of RhoA in CD146-induced EMTs and is positively regulated by CD146 expression and RhoA activation.

Taken together, these results demonstrate that CD146 overexpression contributes to the activation of RhoA, which induces F-actin cytoskeleton rearrangements and Slug expression. Slug subsequently inhibits E-cadherin transcription, resulting in decreased expression of E-cadherin and disruption of tight cell-cell contacts, and eventually leads to EMTs in MCF-7 cells.

Down-Regulation of CD146 in Mesenchymal Breast Cancer Cells Suppresses the Mesenchymal Phenotype. Having shown that CD146 is an EMT inducer in epithelial breast cancer cells, we further investigated the function of CD146 in two invasive mesenchymal...
breast cancer cell lines, MDA-MB-231 and Hs578T (19). As anticipated, CD146 expression was much higher in these two cells than in MCF-7 cells (Fig. 4A). They also expressed mesenchymal markers vimentin, fibronectin, and Slug, but the epithelial marker E-cadherin was lower or could not be detected.

We next down-regulated CD146 expression in MDA-MB-231 cells with siRNAs targeting CD146; siRNAs targeting GFP were used as control. As shown in Fig. 4B, CD146 expression dramatically decreased to about 50% of that of the control. Consequently, the epithelial marker E-cadherin was up-regulated, the mesenchymal markers vimentin, fibronectin, and Slug were down-regulated. We observed that MDA-MB-231 cells with reduced CD146 expression partly lost their fibroblastic phenotype and grew into tight cell clusters, their migratory and invasive behaviors were also significantly inhibited (Fig. 4C–F). Similar results were also obtained in Hs578T cells transfected with siRNAs targeting CD146 (Fig. S5), suggesting that CD146 contributes to the invasive behaviors of the mesenchymal breast cancer cells.

**CD146 Promotes Tumor Invasion in Vivo.** We then addressed the key question that whether CD146-induced EMTs increase tumor invasion in vivo. We implanted Mock and A5 cells into the mammary fat pads of SCID/Beige mice and terminated the experiment at week 10 postimplantation. As shown in Fig. 5A, 80% of the mice implanted with A5 cells developed tumors, compared with only 30% of the mice implanted with Mock cells. Moreover, the average volume of A5 tumors was much higher than that of Mock tumors, suggesting that CD146 significantly promotes breast tumorigenesis and growth in the orthotopic breast cancer model.

We first confirmed that A5 and Mock tumors continued to maintain their mesenchymal or epithelial phenotypes in vivo. As shown in Fig. S6A, A5 tumors maintained a high level of Flag-tagged CD146 expression even at week 10 postimplantation, whereas Mock tumors showed no CD146 expression. A5 tumors were positive for vimentin, but Mock tumors were positive for E-cadherin. Furthermore, there were significant differences between Mock and A5 tumors in cytology and growth pattern (Fig. 5B). The cells of Mock tumors were normal in appearance and developed into focal tubules, showing a well-differentiated pattern. However, A5 tumors were composed of irregular cells with larger, more prominent nucleoli, indicating a poorly differentiated phenotype that corresponds to a higher histological grade and poorer prognosis in human breast cancer.

Next we examined Mock and A5 tumors and corresponding neighboring tissues to evaluate tumor invasion. As anticipated, Mock tumors were tightly surrounded by fibrotic capsules, indicating their noninvasive phenotype (Fig. 5C, a and b). In contrast, A5 tumors showed apparent local invasion, with small aggregates of tumor cells invading into the adjacent stroma (Fig. 5C, c and d), muscle (Fig. 5C, e), skin, fat tissue, and ribs (Fig. S6B). We observed that A5 cells apparently invaded into blood vessels on the edge of the tumor mass (Fig. 5C, f), indicating possible metastasis. Taken together, our results show that overexpression of CD146 confers an invasive phenotype on noninvasive MCF-7 cells.

To determine whether CD146-induced EMTs would affect tumor angiogenesis, we performed immunofluorescence staining for endothelial cells and Flag for tumor cells. We found that the invasion fronts of A5 tumors exhibited a much higher level of angiogenesis in contrast with Mock tumors, although there was no significant difference in vessel density in the center of these tumors (Fig. S6C–E). We observed that new vessels were accompanied by islands of A5 tumor cells that had invaded into the stroma, indicating that CD146-expressing tumors induce further angiogenesis, facilitating tumor invasion and metastasis.

**CD146 Initiates Tumor Metastasis in Vivo.** We next questioned whether CD146 could initiate tumor metastasis in vivo. Organs of mice carrying Mock and A5 tumors were dissected out 10 wk after implantation and fixed for further analysis. As shown in Fig. 6A, lungs and livers from mice carrying A5 tumors displayed large numbers of visible breast tumor metastases, compared with normal lungs and livers from mice carrying Mock tumors, indicating relatively late dissemination of A5 cells from the primary tumors.

Metastases in the lungs and livers of mice carrying tumors were confirmed by H&E and immunohistochemical staining for Flag and CD146 (Fig. 6B and C). On average, we found six metastases per 9-mm section in lungs, and four micro-metastases in livers from mice carrying A5 tumors, in stark contrast to no micrometastases in the lungs and livers from mice carrying Mock tumors.

In summary, 80% of mice that carrying A5 tumors exhibited numerous lung metastases and 30% of these mice displayed apparent liver metastasis, whereas no lung and liver metastases were found in the mice carrying Mock tumors (Fig. 6D). These observations demonstrate that CD146 strongly promotes breast cancer metastasis in vivo.

**CD146 Is Significantly Associated with TNBC.** A critical question raised from our in vitro and in vivo data was whether CD146 expression clinically correlated with human breast cancer progression. To address this issue, we performed immunohistochemistry to detect CD146 expression in 505 human primary breast cancers. Although only 35% of tumors were positive for CD146 staining, CD146 expression was significantly associated with advanced tumor grade, with a positive status for Ki-67, and with poor prognosis in breast cancer (Tables S1 and S2). Furthermore, there was significant correlation between CD146+ tumors and a shorter progression-free survival or overall survival (Fig. S7). These observations demonstrate that CD146 expression significantly correlates with invasive breast cancer.

Interestingly, we observed abnormally high expression of CD146 in TNBC, the most aggressive breast cancer with EMT-
like features. As shown in Fig. 7A, there was a striking correlation between CD146 expression and the triple-negative phenotype (P < 0.001). Of TNBC samples, 68.9% were CD146+, in contrast to 21.2% in non-TNBC samples. CD146 expression was also significantly correlated with ER-, PR-, and HER2-negative status (P < 0.001), respectively.

To investigate whether the abnormally high expression of CD146 in TNBC accounts for its EMT-like features, we further analyzed correlations between CD146 and E-cadherin expression in 90 TNBC samples. As shown in Fig. 7B, CD146 was frequently expressed at the leading edge of invasion. CD146+ TNBC samples tended to be either E-cadherin-negative or to express E-cadherin in the cytoplasm. In contrast, CD146− TNBC samples expressed E-cadherin in membranes, the normal expression pattern for E-cadherin. CD146 expression was significantly associated with a reduction of E-cadherin in TNBC samples (P = 0.01). These clinical data are consistent with our observations in cell cultures and the orthotopic breast cancer mouse model, demonstrating the pathological relevance of CD146 in the regulation of EMTs.

Discussion

In this study, we are unique in demonstrating that CD146 is a regulator of the EMTs in breast cancer progression. We confirm this finding by providing the following evidence. First, CD146 overexpression in noninvasive epithelial breast cancer cells represses the epithelial phenotype, induces a mesenchymal phenotype, and dramatically increases migratory and invasive behaviors and CSC-like properties. Second, CD146 down-regulation in invasive mesenchymal breast cancer cells reverses their malignant phenotypes. Third, CD146 induces tumorigenesis and a poorly differentiated phenotype, and promotes tumor invasion and metastasis in an orthotopic breast cancer mouse model. Finally, by examining 505 human primary breast cancer tissues, we found that CD146 expression is significantly associated with high tumor stage, poor prognosis, and TNBC, providing pathological support for our in vitro and animal studies. Our studies are consistent with a recent report describing a significant correlation between CD146 expression and invasive breast cancer (16), and have assessed the mechanism underlying this correlation.

Another important objective of this study was to clarify the molecular mechanism underlying CD146-induced EMTs. First, we found that the small GTPase RhoA is activated in CD146-overexpressing cells, and we further demonstrated that activation of RhoA is responsible for CD146-induced EMTs rather than being a consequence of them. Previous reports have shown that RhoA activation results in disruptions of cell-cell adhesion and EMTs in TGF-β1–stimulated mammary epithelial cells (20), colon carcinoma cells (21), and podoplanin-overexpressing MDCK cells (22). Second, we observed that CD146-induced EMTs increase Slug expression, whereas other transcriptional factors SIP1, Snail, and Twist were not correlated with CD146 expression. Slug is a member of the Snail family, and it is both necessary and sufficient to repress E-cadherin transcription and trigger the EMT process in breast cancer (23). We demonstrate that both CD146 silencing and RhoA inhibition in CD146-overexpressing cells significantly decreased Slug expression. A previous report shows that Slug down-regulation is sufficient to restore E-cadherin expression in breast epithelial cells (24). Third, the fact that CD146 is able to activate RhoA without affecting Rac1 and Cdc42 activity suggests a direct link between CD146 and RhoA. Our data show that CD146 physically interacts with ERMs and then recruits RhoGDI1 via the phosphorylated ERMs, and finally induces RhoA activation in melanoma cells (25), providing evidence for this function of CD146 in breast cancer cells. However, unraveling the detailed signaling pathways involved in RhoA and the CD146-induced EMTs will require further research.

Our studies on the role of CD146 in breast cancer progression have promising clinical implications. Foremost, our findings indicate that CD146 could be a unique therapeutic target, with particular relevance to clinically aggressive TNBC. TNBC is the most lethal subtype for its high incidence of metastasis and resistance to current targeted therapies (26). CD146 accounts for the aggressiveness and stenness of TNBC, targeting the EMT-like phenotype becomes a unique strategy for TNBC treatment. Our results have shown that CD146 is expressed at abnormally...
high levels and is associated with a reduction of E-cadherin in TNBC. CD146 silencing in the so-called “TNBC cell line” MDA-MB-231 partly reverses its mesenchymal phenotype, implying that CD146-induced EMTs partially explain the mesenchymal and malignant characteristics of TNBC. In addition, increasing evidences support the critical role played by tumor angiogenesis in breast cancer progression (26). Because CD146 has also been proposed as a marker for tumor angiogenesis (8), targeting CD146 could have a double role, targeting the mesenchymal phenotype and tumor angiogenesis at the same time, thus will be a promising strategy in TNBC treatment.

Although previous reports have shown that CD146 significantly correlates with advanced tumor stage in malignant melanoma (13), prostate cancer (14), epithelial ovarian cancer (15), and mesothelioma (27, 28), little is known about the underlying mechanisms. Here, we report a unique role for CD146; the induction of EMTs to promote breast cancer progression. As the EMTs are regarded as a mechanism that is conserved in developmental processes and the progression of pathological diseases (2), we wonder whether CD146-induced EMTs might play a role in tumor progression in other CD146-positive cancers. A previous report showed a negative correlation between CD146 and E-cadherin expression in prostate cancer cell lines (29), implying that CD146-induced EMTs are not limited to breast cancer and might have a role in various types of cancers. More studies are needed to confirm the implication.

In conclusion, we demonstrate that CD146 plays a critical role in promoting breast cancer progression by up-regulating active RhoA and Slug to promote EMTs. Furthermore, the striking correlation between abnormally high CD146 expression and TNBC at least partially explains its role as an EMT inducer in the most aggressive breast cancer. Thus, CD146 can be used as a potential therapeutic target for breast cancer, especially for TNBC.

Materials and Methods

Reagents, cell lines, and transfections are listed in SI Materials and Methods.

In vitro migration and invasion assays, immunofluorescence, mammaphone assays, and RhoA activity assays were conducted using standard procedures described in SI Materials and Methods. Construction of orthotopic breast cancer animal models and collection of clinical samples followed established procedures described in SI Materials and Methods.

ACKNOWLEDGMENTS. This work was partially supported by grants from 973 Program (2009CB521704, 2011CB933503, 2012CB933403, and 2011CB935002), the National Natural Science Foundation of China (91029732 and 30930308), and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-M15).


All chemicals were obtained from Sigma, and all cell culture media were purchased from Gibco. The following antibodies were used in this study: anti-E-cadherin (Abcam), antivimentin (Sigma), anti-fibronectin (Abcam), anti-pan cytokeratin (Abcam), anti-Slug (Abcam), anti-RhoA (Cell Signaling), anti-Cdc42 (Cell Signaling), anti-Rac1 (BD Biosciences), anti-GAPDH (Abcam), anti-EMR and anti-p-ERM (Cell Signaling). Mouse anti-human CD146 mAbs are asics generated in our laboratory. Alexa fluor 488-phalloidin for F-actin was purchased from Invitrogen. Exonuclease C3 transferase was obtained from Cytoskeleton.

**Cell Lines and Transfections.** MCF-7, HS578T, MDA-MB-231, and Madin-Darby canine kidney (MDCK) cell lines were obtained from the American Type Culture Collection. The p3xFlag Mock vector and the vector containing human CD146 were transduced into MCF-7 and MDCK cells using Fugen HD (Roche). Stable clones were selected and established in the presence of 418 (500 μg/mL; Invitrogen). SiRNAs targeting CD146 and control siRNAs targeting GFP were synthesized by Invitrogen. The sequences of the siRNAs are available as follows: siRNA-CD146: forward, 5′-CCA GCU CGG CUU CAA AdTdT-3′; reverse, 5′-UUU GUU GAC GCC GCG CUG GdTdT-3′; siRNA-GFP: forward, 5′-CUU CAG CCG CUG CUU GCC GdTdT-3′; reverse, 5′-CCG CAA GGU GCC GAC GGU GdTdT-3′. Lipofectamine 2000 (Invitrogen)-mediated siRNA transfections were performed according to the manufacturer’s instructions.

**Supporting Information**

Zeng et al. 10.1073/pnas.1111053108

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**Cell Lines and Transfections.** MCF-7, HS578T, MDA-MB-231, and Madin-Darby canine kidney (MDCK) cell lines were obtained from the American Type Culture Collection. The p3xFlag Mock vector and the vector containing human CD146 were transduced into MCF-7 and MDCK cells using Fugen HD (Roche). Stable clones were selected and established in the presence of 418 (500 μg/mL; Invitrogen). SiRNAs targeting CD146 and control siRNAs targeting GFP were synthesized by Invitrogen. The sequences of the siRNAs are available as follows: siRNA-CD146: forward, 5′-CCA GCU CGG CUU CAA AdTdT-3′; reverse, 5′-UUU GUU GAC GCC GCG CUG GdTdT-3′; siRNA-GFP: forward, 5′-CUU CAG CCG CUG CUU GCC GdTdT-3′; reverse, 5′-CCG CAA GGU GCC GAC GGU GdTdT-3′. Lipofectamine 2000 (Invitrogen)-mediated siRNA transfections were performed according to the manufacturer’s instructions.

**In Vitro Migration and Invasion Assays.** For the transmigration assay, 5 × 106 to 1 × 106 cells were plated on the top of noncoated membranes (96-well inserts, 8 μm; Corning). For the invasion assay, 1 × 105 to 1.5 × 105 cells were seeded in the top chamber on Matrigel-coated (BD Biosciences) membranes. For both assays, cells were seeded in medium without FBS in the top chamber, and medium containing 10% FBS in the lower chamber to stimulate migration or invasion. Cells were incubated for 12 to 36 h before they were stained with Crystal violet and counted.

**Immunofluorescence.** Cells were plated on round cover slips, and cultured in six-well plates until the confluence reached about 80%. Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton/PBS for 3 min at room temperature, and blocked by 5% normal goat serum for 30 min. The cells were then incubated with primary antibodies for 1 h, followed by incubation with the Alexa Fluor 488 (Fab2) fragment of goat anti-mouse or rabbit IgG (H+L) for 30 min at 37 °C. Finally, the cells on the round cover slips were photographed with a confocal laser scanning microscope (FV-1000, Olympus).

**Mammosphere Assay.** Single cells were plated at a density of 5,000 cells per well in ultralow attachment plates (35 mm; Corning). Cells were grown in serum-free DMEM/F12, supplemented with B27 (1:50, Invitrogen), 20 ng/mL EGF and 20 ng/mL bFGF (BD Biosciences), and 4 μg/mL heparin (Sigma). The mammospheres were cultured for 7–10 d. Then the mammospheres with diameter >50 μm were counted.

**RhoA, Rac1, and Cdc42 Activity Assay.** The level of active RhoA in cell lysates was measured using a GST fusion protein with the RhoA-binding domain of Rhotekin (RBD). For Rac1 and Cdc42 activities, a GST fusion protein of the binding domain of PAK (GST-PBD) was used. Briefly, GST-RBD or GST-PBD was expressed in BL21 and purified using glutathione Sepharose beads (GE Healthcare). Cell lysates were incubated with GST-RBD or GST-PBD precoupled to glutathione Sepharose beads at 4 °C for 45 min to precipitate GTP-bound RhoA, or Rac1, and Cdc42. The product was separated on a 12% SDS-PAGE gel, and immunoblotting was used to detect the protein level of total and GTP-bound RhoA, Rac1, or Cdc42.

**Orthotopic Breast Tumor Model in SCID/Beige Mice.** All animal experiments were performed in compliance with the Institution of Biophysics guidelines. Four-week-old SCID/Beige female mice (Charles River Laboratories) were anesthetized by 5% Chloral hydrate. The mammary fat pad was exposed and 5 × 105 tumor cells in 50 μL PBS plus with 50 μL matrigel (10 mg/mL) were injected. Slow-release estrogen pellets (Innovative Research of America) were implanted into the dorsum of the each mouse. Every week, tumor volume was measured with calipers and calculated based on the formula (length × width × width × π/3). Mice were killed when they became moribund. Tumors, lungs, and livers were fixed in 4% paraformaldehyde and embedded in paraffin. H&E staining and immunohistochemical detection were performed on tissue slices.

**Human Breast Cancer Tissue Collection.** Fifty and five samples of human breast cancer specimens were obtained from patients who underwent breast cancer surgery from 2003 to 2008 at the Cancer Hospital of Tianjin Medical University (Tianjin, China). Use of human tissues in this study was with informed consent and was approved by the Ethics Committee of the Cancer Hospital of Tianjin Medical University and the Institute of Biophysics, Chinese Academy of Science. Immunohistochemistry was performed using anti-CD146 and anti-E-cadherin antibodies. Statistical analysis was performed using SPSS software (SPSS).

**RNA Isolation and RT-PCR.** TRizol (Invitrogen) reagent was used to isolate RNA from cultured cells. cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) using random primers. PCR reactions were performed using 1 μL cDNA and a pair of primers specific for each gene. For semiquantitative PCR, PCR products were visualized on a 1% agarose gel with EB staining. The sequences of the semiquantitative RT-PCR primers are as follows: CD146: forward, 5′-GCT GCC CAG TGG GAA CCA CA-3′; reverse, 5′-ATG GTG TCC AAG TTC CAG GC-3′. E-cadherin: forward, 5′-ATT CTG ATT CTG CTG TCG-3′; reverse, 5′-AGT AGT CAT AGT CCT GGT CT-3′. Vimentin: forward, 5′-GAA CGC CAG ATG GGT AGT-3′; reverse, 5′-CCA GAG GGA GTG AAT CCA GAT TA-3′. Fibronectin: forward, 5′-GAA GGT CTC TCT CAG ACA ACC A-3′; reverse, 5′-GCC CAC GGT AAC AAC CTC TT-3′. Cytokeratin-19: forward, 5′-GCT CAC CTG TTT GTC TCG CT-3′; reverse, 5′-AGT GAA GTG GAA GAT TGC TTC-3′. GAPDH: forward, 5′-AGG TCG TAC ACG GAT GGT TT-3′; reverse, 5′-ATC AGT GGG TGC ATG GTG-3′. Slug: forward, 5′-CAT ACC TCA GAT GTA GAT-3′; reverse, 5′-CGA TCG TAC TCG TCT-3′. SIP1: forward, 5′-GTT TAC AGG CAA TGG ACC ATG TCA-3′; reverse, 5′-TTG TAC AGG CAA TGG ACC ATG TCA-3′.

**Statistical Analysis.** All values were shown as means ± SEM. Statistical differences were assessed using the Student’s t test. The differences were considered statistically significant when P < 0.05.
**Fig. S1.** (A) RT-PCR analysis of CD146 and epithelial-mesenchymal transition (EMT) markers in Mock, B10, and A5 cells. (B) Quantifications of mRNA levels of CD146 and EMT markers in Mock, B10, and A5 cells. Data were collected from three trials. *P < 0.05; **P < 0.01; ***P < 0.001, compared with Mock cells.

**Fig. S2.** CD146 silencing reverses the EMT phenotype of A5 cells. (A) Expression of CD146 and EMT markers after CD146 silencing analyzed by immunoblotting. (B) Morphology of A5 cells transfected with siRNA-GFP or siRNA-CD146. Magnification, 100×. (C and D) Migration and invasion assays of A5 cells after siRNA transfection. Data were collected from three wells; *P < 0.05, compared with the control.
Fig. S3. Overexpression of CD146 induces EMTs in MDCK cells. (A) Morphology of vector control clone MDCK-Mock and CD146 clones MDCK-B7 cells. Magnification, 200×. (B) FACS analysis of CD146 expression in MDCK-Mock and MDCK-B7 cells. (C) Expression of CD146 and the EMT markers analyzed by immunoblotting in MDCK-Mock and MDCK-B7 cells. (D) Immunofluorescence of CD146 and the EMT markers in MDCK-Mock and MDCK-B7 cells. (Scale bars, 20 μm.) (E and F) Migration and invasion assays of MDCK-Mock and MDCK-B7 cells. Data were collected from three wells; ***P < 0.001. Representative images of migrated or invaded cells are shown below each graph.

Fig. S4. (A) The active form of Rac1 (GTP-Rac1) and Cdc42 (GTP-Cdc42) are unchanged in Mock, B10, and A5 cells, as analyzed by Rac1 and Cdc42 activity assays. (B) SIP1 is unchanged, Snail is decreased in A5 cells and Twist is slightly increased compared with Mock cells, the expression of which are all not correlated with CD146 expression in these three cells, as analyzed by semiquantitative RT-PCR.
Fig. S5. CD146 silencing in Hs578T cells reverses the mesenchymal and invasive phenotype. (A) Immunoblotting of CD146, fibronectin, vimentin, and Slug after CD146 silencing in Hs578T cells. (B) Morphology of Hs578T cells after CD146 silencing. Magnification, 100×. (C and D) Migration and invasion assay of Hs578T cells after siRNA transfection. Data were collected from three wells; *P < 0.05, compared with the control.
Fig. S6. (A) MCF-7-A5 tumors continue to express high levels of CD146 and maintain their mesenchymal phenotype, but Mock tumors continue to maintain their epithelial phenotype in vivo. Mock and A5 tumor sections were stained with anti-CD146, anti-Flag, anti-E-cadherin, and anti-vimentin antibodies. (Scale bars, 100 μm.) (B) A5 tumors invade into the dermis (a and b), fat (c and d), and ribs (e and f). Asterisks, tumor mass. Arrows in “e” indicate ribs. (Scale bars, 100 μm.) (C–E) Prominent intratumoral vessels are associated with the invasion front of A5 tumors, as demonstrated by immunofluorescence staining for CD31 (green) and Flag (red) in primary tumors formed by Mock and A5 cells at the edge (C) and the center (D). Quantification of vessels at the edge and the center of the tumors (E). Dotted line indicates the tumor edge. (Scale bars, 20 μm.) *P < 0.05, compared with the vessel density in the edge of Mock tumors.
Fig. S7. Kaplan–Meier curves of progression-free survival (PFS) (A) and overall survival (OS) (B) for CD146 expression in all patients with breast cancer shows that CD146 expression was associated with reduced PFS ($P = 0.001$) and OS ($P = 0.001$). $P$ values were calculated by the log-rank test.

Table S1. Association between CD146 expression and clinicopathological parameters in invasive ductal carcinomas of breast

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<tr>
<th>Characteristics</th>
<th>Total no. (%)</th>
<th>Negative, n (%)</th>
<th>Positive, n (%)</th>
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<td><strong>Age at diagnosis</strong></td>
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<tr>
<td>≤50 y</td>
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<td>161 (64.1)</td>
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<td>&gt;50 y</td>
<td>254 (50.5)</td>
<td>167 (65.7)</td>
<td>87 (34.3)</td>
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<td><strong>Tumor size</strong></td>
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<td>87 (64.9)</td>
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<td>371 (73.5)</td>
<td>241 (65.0)</td>
<td>130 (35.0)</td>
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Table S2. Association between CD146 expression and patient prognosis in breast cancers

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