Attenuation of Junctional Adhesion Molecule-A Is a Contributing Factor for Breast Cancer Cell Invasion

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Abstract

The metastatic potential of cancer cells is directly attributed to their ability to invade through the extracellular matrix. The mechanisms regulating this cellular invasiveness are poorly understood. Here, we show that junctional adhesion molecule A (JAM-A), a tight junction protein, is a key negative regulator of cell migration and invasion. JAM-A is robustly expressed in normal human mammary epithelium, and its expression is down-regulated in metastatic breast cancer tumors. In breast cancer cell lines, an inverse relationship between JAM-A expression and the ability of these cells to migrate on a collagen matrix was observed, which correlates with the known ability of these cells to metastasize. The T47D and MCF-7 cells, which migrate least, are found to express high levels of JAM-A, whereas the more migratory MDA-MB-468 cells have lower levels of JAM-A on the cell surface. MDA-MB-231 cells, which are highly migratory, express the least amount of JAM-A. Overexpression of JAM-A in MDA-MB-231 cells inhibited both migration and invasion through collagen gels. Furthermore, knockdown of JAM-A using short interfering RNAs enhanced the invasiveness of MDA-MB-231 cells as well as T47D cells. The ability of JAM-A to attenuate cell invasion correlated with the formation of increased numbers of focal adhesions and the formation of functional tight junctions. These results show for the first time that an immunoglobulin superfamily cell adhesion protein expressed at tight junctions could serve as a key negative regulator of breast cancer cell invasion and possibly metastasis. Furthermore, loss of JAM-A could be used as a biomarker for aggressive breast cancer. [Cancer Res 2008;68(7):2194–203]

Introduction

Epithelial cells act as a barrier between organisms and the external milieu. Epithelial cells exhibit two unique phenotypic characteristics: (*a*) closely joined membrane structures, called tight junctions, and (*b*) an apical-basolateral polarization with distinct biochemically and functionally unique plasma membrane domains. These domains are established by the tight junctions, which act as a fence, blocking the free diffusion of lipids and proteins between the apical to the basolateral surface of a cell (1, 2). Tight junctions are established by interactions between three major families of transmembrane proteins claudins, occludin, and junctional adhesion molecules (JAM; refs. 3–5), which interact with peripheral membrane proteins such as PDS-95/Discs large/ZO family (PDZ) domain–containing proteins that play important roles in maintaining junctional integrity (4, 6, 7).

Global analyses of gene expression have shown that cancer cells have many differences in gene expression from their normal precursors, particularly, many genes involved in cell-cell adhesion, including those contributing to the tight junction, are underexpressed or overexpressed in different carcinomas (8, 9). Cell-cell adhesion molecules have been well-documented to regulate cancer cell motility and invasion. For example, it has been proposed that the loss of E-cadherin from the breast epithelium with the subsequent onset of N-cadherin expression may be responsible for breast cancer cell invasion (10, 11). However, these results remain controversial because some breast cancer cell lines that do not express N-cadherin still posses highly invasive characteristics (12, 13).

JAMs are members of the Cortical Thymocyte marker for *Xenopus* family of type I membrane proteins predominately localized to endothelial and epithelial cell tight junctions, platelets, and some leukocytes (14, 15). The classic JAMs are JAM-A, JAM-B, and JAM-C, which all can regulate leukocyte-endothelial cell interaction through their ability to undergo heterophilic binding with integrins $\alpha_L\beta_2$ or $\alpha_v\beta_3$, $\alpha_4\beta_1$ and $\alpha_M\beta_2$, respectively. It is also known that JAM-C is involved in the binding of tumor cells to the blood vessel endothelium during tumor cell metastasis (16). The other members of the family, ESAM, CAR, JAM-4, and JAM-L, are involved in transepithelial migration of neutrophils and regulation of permeability (17–22). The cytoplasmic tail of JAMs contain a PDZ-domain–binding motif that can interact with PDZ domain containing cytoplasmic molecules such as ZO-1, ASIP/PAR-3, or AF-6 (23, 24). Additionally, their junctional localization and their proclivity to participate in homophilic interactions suggest that JAMs may participate in regulating the tight junctions and maintaining paracellular permeability (25, 26). Recently, it has been shown that JAM-A regulates epithelial cell morphology by modulating the activity of the small GTPase Rap1 (9). In addition, ectopic expression of JAM-A in Chinese hamster ovary cells promotes localization of ZO-1 and occludin to points of cell contact, which suggests a role for JAM-A in tight junction assembly (24, 27, 28).

The lack or loss of tight junctions in tumors seems to increase paracellular permeability and cause a decrease in transepithelial resistance (TER) contributing to cancer development (8, 28). However, little is known about the role of JAM-A in cancer progression. Thus, we investigated the role of JAM-A in regulating the invasive behavior of breast cancer cells. We show that JAM-A is expressed in breast cancer tissues and cell lines and that its protein levels inversely correlate to tumor aggressiveness. Furthermore, we show that restoration of JAM-A expression in advanced breast cancer cells results in a change in cell morphology from spindle-like to cobblestone-like clusters with decreased cell migration and invasion. Conversely, knockdown of JAM-A using short interfering

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RNA (siRNA) enhanced breast cancer cell invasiveness. The ability of JAM-A to attenuate cell invasion was found to be due to the formation of stable focal adhesions. Furthermore, JAM-A also contributes to the formation of functional tight junctions, as observed by distinct accumulation of JAM-A and ZO-1 at the tight junctions and increased TER. These results show for the first time that a tight junctional cell adhesion protein can serve as a key negative regulator of breast cancer cell invasion and possibly metastasis.

Materials and Methods

Breast cancer microarrays. A microarray set containing paraffin sections of 20 normal and 50 localized, low-grade breast neoplasia specimens were obtained from Biomedia. Another microarray consisting of paraffin sections of 12 tumor (low grade) and their corresponding nonneoplastic tissue (adjacent tissue 1.5 cm away from tumor) were obtained from Biomax. Additionally, a breast cancer tissue array consisting of paraffin sections derived from 50 independent malignant primary breast tumors with their corresponding lymph node metastases was purchased from Biomax. The sections were processed using manufacturer's protocols and stained with anti–JAM-A antibodies (22). The immunostained sections were viewed using a Zeiss 510 LSM confocal laser-scanning microscope (Carl Zeiss, Inc.), then counterstained with H&E to ensure the tumor grade. Mean fluorescence pixel intensity of each image captured under same detection settings was determined using the Zeiss software.

Cell culture and transfection. T47D, MCF-7, MDA-MB-468, and MDA-MB-231 cells were all obtained from the American Type Culture Company. The cells were maintained in a growth medium containing DMEM or DMEM-F12 (MCF-7) or RPMI-1610 (T47D) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL insulin (Invitrogen). MDA-MB-231 cells were stably transfected with a JAM-A construct or with the empty vector as described previously (27). The extent of JAM-A protein up-regulation in JAM-A stable clones was analyzed by Western blotting, and the surface expression of JAM-A was analyzed by flow cytometry (Becton Dickinson). Cells cultures were maintained in a 37°C incubator containing 5% CO_2 and 95% humidity. Stable clones were routinely maintained in growth medium containing 300 μg/mL G418 (Invitrogen).

Transfection of siRNAs targeting JAM-A. Design and synthesis of siRNA specific to JAM-A and a luciferase control siRNA were previously described (29, 30). MDA-MB-231 or T47D cells were transfected with siRNA as described previously (29, 30). After 72 h of transfection, cells were lysed and protein (10–50 μg) were assayed by Western blotting for JAM-A, and the blots were reprobed with anti–α-tubulin or heat-shock protein (HSC-70) to ensure equal loading. Surface expression of JAM-A was also assessed by flow cytometry.

Immunofluorescence. Immunofluorescence studies were performed as previously described (31). Briefly, untransfected or transfected cells, as indicated, were allowed to attach on glass coverslide chambers. Cells were fixed, permeabilized, and blocked in 3% bovine serum albumin (BSA) in PBS and then incubated with anti–JAM-A (monoclonal antibody F11; BD Pharmigen) or with anti–ZO-1 (Zymed) overnight at 4°C, followed by appropriate incubation with Texas-red conjugated donkey anti-mouse and FITC-conjugated goat anti-rabbit secondary antibodies from Jackson Immuno-Res Laboratories or FITC-conjugated phalloidin (Invitrogen). In a separate set of experiments, cells were stained with an anti-vinculin antibody (generous gift from Dr. K. Burridge, University of North Carolina at Chapel Hill, Chapel Hill, NC) to visualize focal adhesions. Optical sections were taken through the middle of the cells to highlight cell-cell junctions by confocal microscopy using ×40 or ×100 oil immersion objectives (Zeiss).

Cell migration assays. A quantitative haptotactic migration assay was performed essentially as described previously (32). Nontransfected T47D, MCF-7, MDA-MB-468, MDA-MB-231, mock or JAM-A–overexpressing MDA-MB-231 cells were serum starved (DMEM supplemented with 0.5% BSA), and 5×10^3/100 μL of cells were allowed to migrate across the precoated inserts for 5 h at 37°C. Unmigrated cells from the top of the insert chambers were removed, and the migrated cells from the lower side of the inserts were stained using Diff-Quik solution. The migrated cells were viewed using an inverted microscope equipped with ×20 objective, and images were captured by a Cool-Pix digital camera (Nikon). The average number of migrated cells in 10 randomly chosen fields of view per insert in triplicate was taken to quantify the extent of migration.

Immunoblotting. Untransfected and transfected cells were grown in monolayer culture until they attained 80% confluence, then they were lysed using a lysis buffer [1% NP40, 150 mmol/L NaCl, and 50 mmol/L Tris-HCl (pH. 7.5)] in the presence of protease inhibitors for 10 min at 4°C. The protein concentration was estimated using the BCA protein assay kit (Pierce). Protein (10–50 μg/mL) prepared in Laemelli sample buffer was separated by SDS-PAGE and Western blotted as described previously (31). A densitometric analysis was performed with Quantity One software (Bio-Rad).

Flow cytometry assay. T47D, MCF-7, MDA-MB-468, and MDA-MB-231 cells as well as mock and JAM-A stably transfected MDA-MB-231 cells were harvested using Versene (Invitrogen). Cells (1×10^6 cells per mL) were resuspended in a blocking solution (3% BSA in PBS) and incubated on ice for 30 min. Cells were centrifuged and resuspended in an incubation buffer (0.5% BSA in PBS), and 100 μL of each were incubated with or without anti–JAM-A (1:100) or isotype-specific IgG_1 on ice for 1 h. The cells were then washed thrice in an incubation buffer and then incubated with FITC-conjugated secondary antibodies for 45 min in the dark. Stained cells were washed and fixed using 2% paraformaldehyde in PBS for 10 min, washed, resuspended in 0.5% BSA in PBS, and processed for flow cytometric analysis. Data were analyzed using CellQuest software (Becton Dickinson). Images were processed using Adobe Photoshop.

Time-lapse microscopy. MDA-MB-231 mock or JAM-A–overexpressing cells were plated on an eight-chambered cover glass slide and placed on an inverted microscope with Hoffman ×40 objectives (Zeiss Axiovert 200) equipped with 37°C-heated chamber containing 5% CO_2. Live images were captured by the charge coupled device camera every 5 min for a period of 18 h (Axiovision 4.3 software). On average, the migratory paths and velocity of 50 cells per movie were analyzed using Metamorph software (Nikon).

Invasion assay. MDA-MB-231 cells stably tranfected with empty vector (Mock) or JAM-A construct were used in the invasion assay. In a separate set of experiments, MDA-MB-231 and T47D cells transiently transfected with JAM-A siRNA or with a Luciferase siRNA as control were used. A prepolymerized collagen gel or growth factor–reduced (GFR)-Matrigel (BD Bioscience) was placed on top of 8-μm pore transwell inserts whose lower surface was precoated with either 30 μg/mL collagen or 0.02% gelatin. Cells were serum starved overnight, and a cell suspension containing 1×10^3cells/100 μL in serum-free medium was placed in the top of the transwell chamber, whereas conditioned medium was used as a chemo-attractant in the lower compartment. Cells were allowed to invade for 18 h at 37°C, uninvaded cells were removed, then the invaded cells were fixed and stained with Diff-Quik solution. The stained cells were viewed using an inverted microscope equipped with a ×20 objective (Nikon) and 10 different views counted from triplicates.

TER. Mock and JAM-A–overexpressing MDA-MB-231 cells were plated and grown to a monolayer for 4 d on polyester membranes with a 0.4-μm pore size (Costar). The medium was changed every day, and cells were observed under a phase-contrast microscope before taking the readings. TER was measured using an EVOMX epithelial voltohmmeter with an STX2 electrode (World Precision Instruments).

Statistical analysis. Data analysis was performed using Standard statistical tests (mean value, SE, and paired Student's t test). Results were expressed as mean ± SE. P values of ≤0.05 were regarded as statistically significant.

Results

JAM-A is expressed in breast cancer tissue. JAM-A was the first immunoglobulin-like molecule identified at the cell-cell junction of endothelial and epithelial cells (14, 15). However, not

much is known about the expression of JAM-A in breast epithelium and its derived cancers. To determine the level of JAM-A expression in human breast tumor tissue by immunohistochemistry, we used a tissue microarray consisting of normal mammary gland and low-grade breast tumor sections. The slides were counterstained with H&E to detect the cancerous tissue. As expected, expression of JAM-A in normal tissue was confined to the ductal epithelium (Fig. 1*A*). Interestingly, JAM-A staining was detected throughout each of the low-grade tumors present on the array (Fig. 1*B*). When the sections were stained with preimmune serum, no staining was observed (data not shown). Additionally, to determine how the progression of cancer affected the expression of JAM-A, we used tissue microarrays. We examined the expression levels of JAM-A in 12 tumors and their corresponding nonneoplastic tissue (adjacent tissue 1.5 cm away from tumor) as well as 50 malignant and their corresponding metastatic to lymph nodes samples. The expression of JAM-A was found to decrease significantly as the disease progressed (Fig. 1*C* and *D*). These results suggest that JAM-A, which is only expressed in epithelial cells of the mammary duct of normal tissue, is also widely expressed in breast carcinomas.

Expression of JAM-A is inversely related to the ability of the breast cancer cells to migrate. We first sought to determine the expression levels of JAM-A in four human breast cancer cell lines with distinct metastatic potential: T47D, MCF-7, MDA-MB-468, and MDA-MB-231 (33) by Western blot analysis. The most JAM-A protein was detected in T47D cells and the least in MDA-MB-231 cells; whereas MCF-7 and MDA-MA-468 cells expressed intermediate amounts (Fig. 2*A*). We next asked if the differential expression of JAM-A in these cell lines has an effect on its localization at the tight junction. We found that in T47D cells, JAM-A is localized at the tight junctions and appeared in a cobblestone pattern in the regions of cell-cell contact (Fig. 2*B*). The cells expressing the least amount of JAM-A, the MDA-MB-231 cells, were morphologically similar to fibroblasts with extensive actin stress fibers, whereas the typical junctional expression of JAM-A was not visible (Fig. 2*B*). The surface expression of JAM-A in these four breast cancer lines was quantitated by flow cytometric analysis (Fig. 2*C*). Quantitation of these data indicated that the surface expression of JAM-A was greatest in T47D cells with MCF-7 > MDA-MB-468 > MDA-MB-231 cells (Fig. 2*C*). Together, these data suggested that the expression of JAM-A inversely correlates to the known metastatic behavior of these cells (33).

We have previously shown that JAM-A expression induces endothelial cell migration (32). We therefore tested whether the expression level of JAM-A correlates with the migration of breast cancer cells. Given that human breast tissue has an abundance of collagen matrix, we used collagen as a substratum for haptotactic migration assays. Interestingly, the expression of JAM-A inversely correlated to their ability to migrate on collagen (Fig. 2*D*). Quantification of these data indicated that T47D cells, which express the highest level of JAM-A, migrated least, followed by MCF-7 and MDA-MB- 468 cells. MDA-MB-231 cells, which express the least amount of endogenous JAM-A, migrated the most (Fig. 2*D*). These results suggest that JAM-A expression inversely

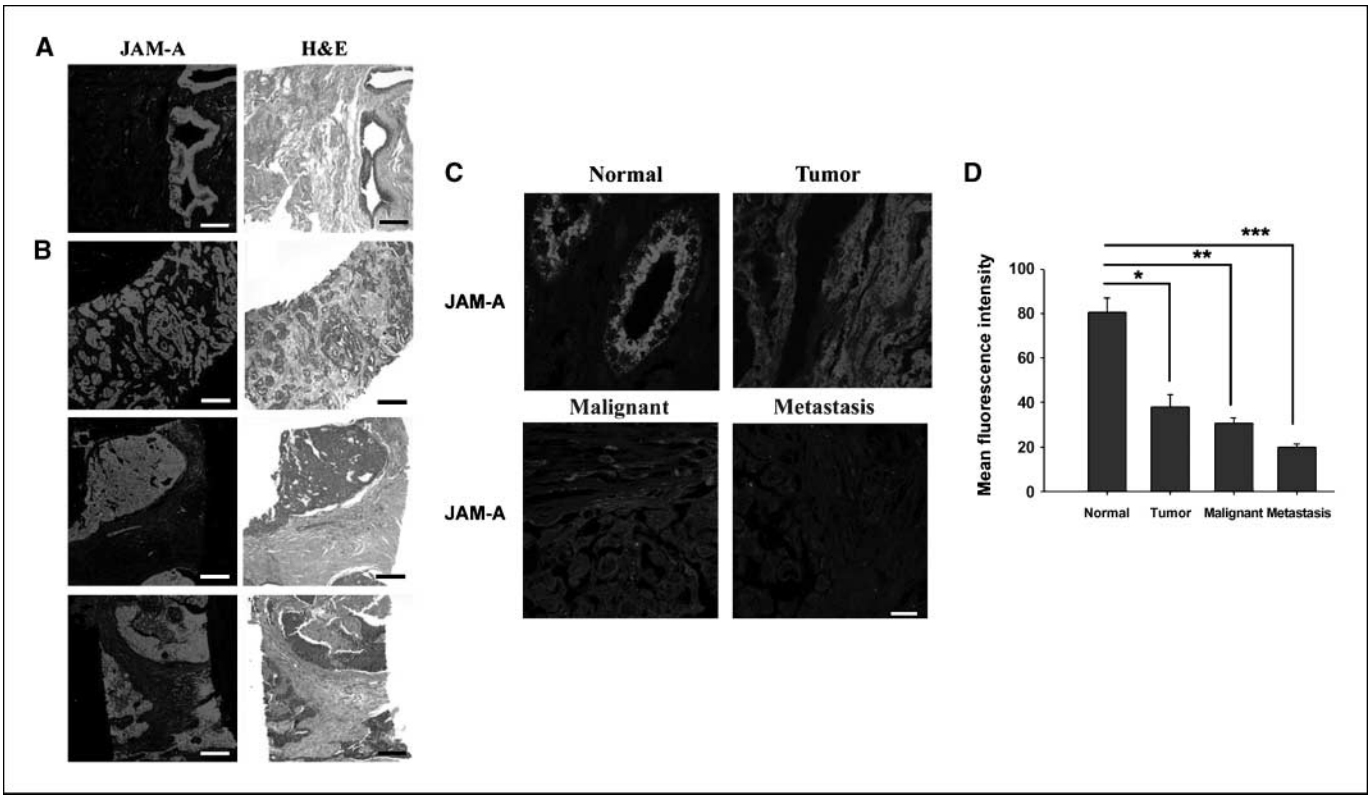

Figure 1. JAM-A is differentially expressed in breast tumor tissues. *A,* immunohistochemical images showing the expression of JAM-A in normal and in low-grade breast tumor tissue sections (*left*). *B,* sections were counterstained with H&E to identify tumor tissue (*right*); *bar,* 154 μm. *C,* representative immunostaining images showing the expression of JAM-A in normal breast epithelium, adjacent primary tumors, malignant tumors, and matched lymph node breast metastases as indicated. *Bars,* 20 μm. *D,* mean fluorescence intensity of normal, tumor, malignant tumor, and metastatic tissue sections. *, $P = 0.7 \times 10^{-4}$; **, $P = 0.9 \times 10^{-7}$; ***, $P = 0.1 \times 10^{-9}$.

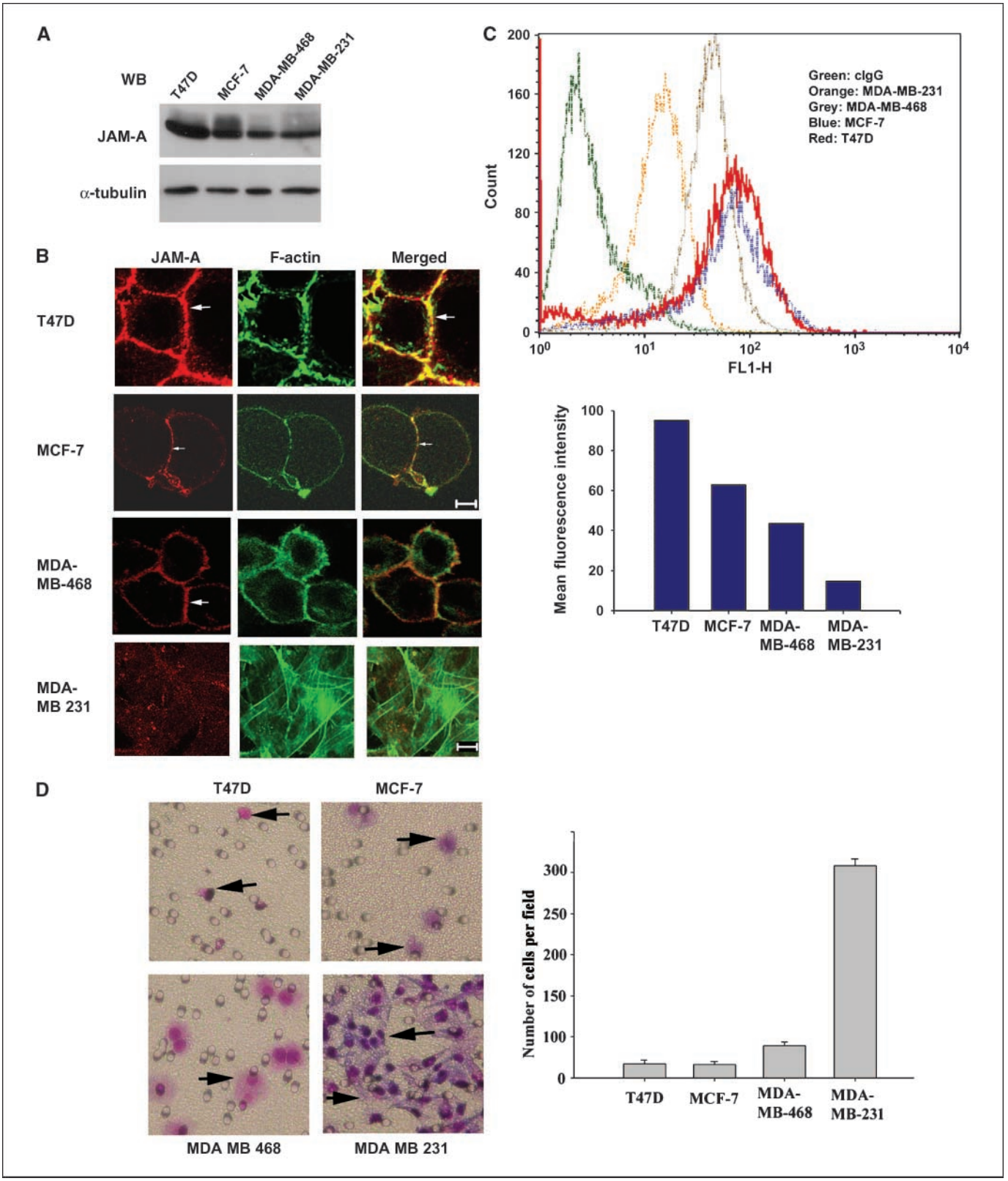

Figure 2. Expression of JAM-A is inversely related to the ability of breast cancer cells to migrate. *A,* Western blot analysis showing the expression of JAM-A protein in various breast cancer cell line as indicated. α-tubulin expression is shown as loading control. *B,* immunofluorescence staining of JAM-A localization at the tight junctions. Note that the MDA-MB-231 cells do not make distinct tight junctions compared with other cells shown by arrows. *Bar,* 5 μm. *C,* surface expression (flow cytometry) of JAM-A in the breast cancer cell lines as indicated and the quantitation of their normalized mean fluorescence intensity (*C*). Data are representative of two independent experiments. *D,* light microscopic images of inserts of haptotactic transwell migration assay of various breast cancer cells stained with Diff-Quik (*left*). *Arrows,* migrated cells. Quantitative analysis of the cell migration assays (*right*). *Columns,* mean of three independent experiments in triplicate; *bars,* SE. *Bar,* 20 μm.

correlates with the ability of the breast cancer cells to migrate on collagen matrix.

Overexpression of JAM-A in MDA-MB-231 cells affected their morphology and their ability to migrate. To test if the level of expression of JAM-A has any effect on MDA-MB-231 cells, we ectopically overexpressed JAM-A in these cells. A Western blot analysis indicated a substantial increase in the level of JAM-A protein in JAM-A–overexpressing MDA-MB-231 cells (Fig. 3*A*). By flow cytometry, surface expression of JAM-A was also increased in these cells (Fig. 3*A*). Interestingly, when JAM-A–overexpressing cells were grown at low density and observed under the phase-contrast microscope, we found that the cells were rounded and tend to cluster together (Fig. 3*B*). In contrast, the parental or mock-transfected cells were spindle shaped and showed distinct fibroblast-like morphology (Fig. 3*B*). These results show that overexpression of JAM-A in these cells affected their fibroblastic morphology and, thus, may impair their migratory behavior.

MDA-MB-231 cells show a high level of spontaneous random motility in the absence of any specific stimulation. To determine the effect of overexpression of JAM-A in these cells, we performed time-lapse microscopy. As expected, the MDA-MB-231 mock cells showed a random migratory phenotype with a high rate of migration, whereas JAM-A–overexpressing cells showed significantly ($P < 0.001$) reduced motility (Fig. 3*C*). When the individual cells were tracked using Metamorph software, the MDA-MB-231 mock cells showed a jagged line representing random motion. In contrast, the MDA-MB-231 cells overexpressing JAM-A showed a smooth line indicating very little motion (Fig. 3*C*). This pattern was similar to the pattern seen with T47D cells that express a high level of endogenous JAM-A and low random motility (data not shown). These results suggested that overexpression of JAM-A renders MDA-MB-231 cells less motile. This effect of JAM-A was further confirmed by a transwell haptotactic motility assay on collagen. We found that mock-transfected MDA-MB-231 cells migrated to a similar extent as that of the parental MDA-MB-231 cells, whereas JAM-A–overexpressing cells failed to migrate or migrated very little (Fig. 3*D*). Quantitation of these data showed that the migration of MDA-MB-231 cells was significantly ($P < 0.001$) inhibited upon overexpression of JAM-A (Fig. 3*D*). These results suggested that JAM-A inhibits motility of highly migratory breast cancer cells.

JAM-A inhibits MDA-MB-231 cell invasion. It has been shown that invasion is a hallmark of cancer cell metastasis (34). We next asked if overexpression of JAM-A will also inhibit cancer cell invasion. To test this, we used a transwell collagen gel invasion assay using conditioned medium as an attractant in the lower chamber. We found that MDA-MB-231 mock cells invaded through the collagen gel efficiently; however, JAM-A–overexpressing cells failed to invade (Fig. 4*A*). Quantitation of these data indicated that JAM-A significantly ($P < 0.001$) blocked MDA-MB-231 cell invasion through the collagen gel (Fig. 4*A*). We next asked if this ability of JAM-A to block cell invasion is specific to collagen. To achieve this, we used GFR-Matrigel in place of collagen gel. We found overexpression of JAM-A also inhibited cell invasion through Matrigel (Fig. 4*A*). These results indicate that overexpresssion of JAM-A in MDA-MB-231 cells affects overall cell invasiveness and is not matrix specific.

To further determine conclusively the role of JAM-A in cancer cell invasion, we reasoned that loss of endogenous JAM-A should enhance invasiveness of MDA-MB-231 cells further. To achieve reduction in the expression of endogenous JAM-A, we used RNAi technology. The MDA-MB-231 cells were transfected with siRNA corresponding to JAM-A transcript. This siRNA was previously shown to specifically knockdown JAM-A in endothelial cells (30). An siRNA corresponding to a luciferase transcript was used as a control. Western blot analysis indicated that we were able to substantially knockdown the expression of endogenous JAM-A protein from MDA-MB-231 cells (Fig. 4*B*). Quantitation of these data suggested that a >80% reduction in JAM-A protein was achieved using this technique (data not shown). To further determine the loss of JAM-A from the cell surface, we performed a fluorescence-activated cell sorting (FACS) analysis. We found that transfection of JAM-A siRNA substantially down-regulated the surface expression of JAM-A in these cells (Fig. 4*B* and *C*). Having successfully down-regulated JAM-A expression in MDA-MB-231 cells, we next determined the effect of this knockdown on the ability of these cells to invade through collagen gel. As seen in Fig. 4*D*, overexpression of JAM-A protein significantly blocked invasion of MDA-MB-231 cells compared with mock as expected. Luciferase siRNA transfection had little effect. Interestingly, down-regulation of endogenous JAM-A significantly enhanced the ability of MDA-MB-231 cells to invade through the collagen gel (Fig. 4*D*). Similar results were obtained when Matrigel was used instead of collagen gel (data not shown). It has been reported that knockdown of JAM-A in colonic epithelial cells results in down-regulation of β_1 integrin through inhibition of Rap1b activity (9). When tested for the effect of JAM-A down-regulation on β_1 integrin expression in MBA-MD-231 cells, we did not see any difference in integrin β_1 expression compared with mock cells (data not shown), suggesting that the observed increase in invasion is not due to β_1 integrin up-regulation. These results suggest that JAM-A is one of the key proteins involved in the regulation of tumor cell invasion and possibly metastasis.

Knockdown of JAM-A in T47D enhances cell invasion. Our results show that T47D cells showed a high expression of JAM-A protein and have low invasive ability. We next investigated the effects of knockdown of JAM-A on the ability of T47D cells to invade a collagen matrix. JAM-A siRNA successfully reduced the endogenous JAM-A protein, whereas no reduction in JAM-A protein was observed when luciferase siRNA was used as a control (Fig. 5*A*). Confocal images revealed that T47D cells transfected with luciferase siRNA showed a well-defined cobblestone morphology of tight junctions with an abundance of JAM-A (Fig. 5*B*), whereas the JAM-A siRNA–transfected cells had no detectable JAM-A staining, and the cells seemed to be spindle shaped (Fig. 5*B*). This observed change in cell morphology due to the loss of JAM-A from tight junctions is consistent with previous studies reported using human hepatocellular carcinoma cells and colonic epithelial cells (9, 35, 36). Next, when we tested the invasive ability of these cells, as predicted, we observed a 3-fold increase in the invasion in JAM-A siRNA–transfected T47D cells compared with the control siRNA–transfected cells (Fig. 5*C*). This result suggested that knockdown of JAM-A led to the augmentation of invasive phenotype through its effect on tight junction integrity.

JAM-A overexpression in MDA-MB-231 cells increases focal adhesions and restores functional tight junctions. As observed earlier, ectopic expression of JAM-A in MDA-MB-231 cells resulted in rounded cell morphology compared with the mock-transfected (fibroblastic) cells (Fig. 3). To further investigate the localization and the distribution of JAM-A in these cells, we performed immunofluorescence microscopy. We found that the mock cells had no detectable cell-cell junctions, and JAM-A staining was diffuse over the membrane. Interestingly, JAM-A–overexpressing

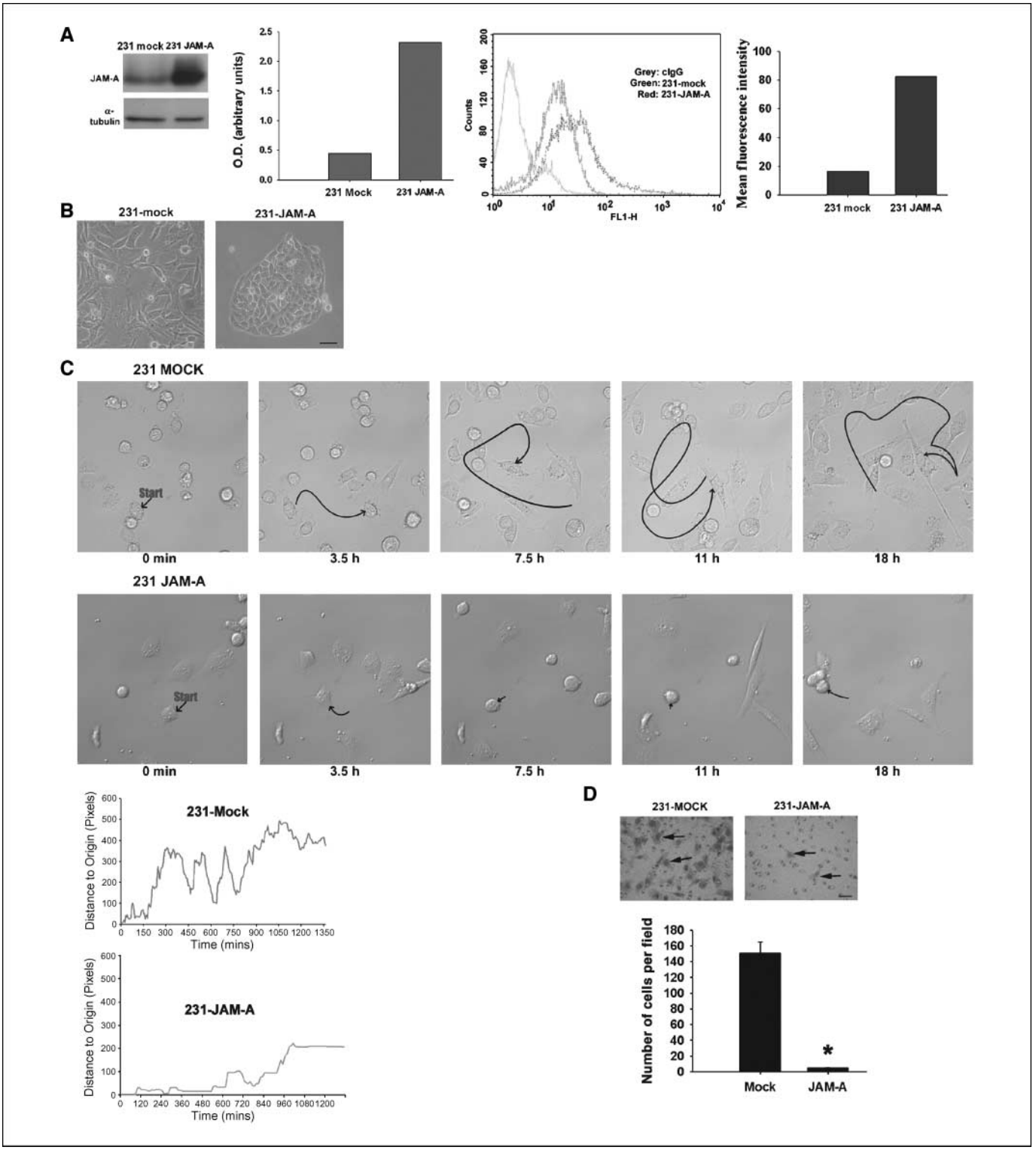

Figure 3. Overexpression of JAM-A in MDA-MB-231 cells causes a morphologic change and inhibits cell migration. *A,* ectopic expression of JAM-A in MDA-MB-231 cells, mock-transfected (empty vector) cells used as control. Western blot was performed using anti–JAM-A antibody. The same blot was reprobed with anti–α-tubulin to ensure equal loading (*bottom*). Quantitation of Western blot by densitometric analysis for the expression of JAM-A (*left*). FACS analysis showing the surface expression of JAM-A in MDA-MB-231–overexpressing cells compared with mock-transfected cells and quantitation by normalized mean fluorescence intensity (*right*). OD, absorbance. *B,* phase-contrast microscopic images of mock and JAM-A–overexpressing MDA-MB-231 cells. Note that the JAM-A–overexpressing cells are grouped together and show a cobblestone-like morphology compared with mock cells. *Bar,* 20 μm. *C,* time-lapse microscopic images showing the migration of mock and MDA-MB-231–JAM-A–overexpressing cells. Images of live cells were captured every 5 min for a period of 18 h. Data shown are representative series of images captured from one of the three independent experiments. Note the movement of the cell from the point of origin is traced. Data obtained from time-lapse experiment were analyzed, and the distance from the origin of a representative cell was calculated using Metamorph software. *D,* light microscopic images of the haptotactic transwell migration assay inserts of MDA-MB-231 mock and JAM-A–overexpressing cells. *Arrows,* Diff-Quik–stained migrated cells (*top*). Quantitative analysis of the migration assay (*bottom*). *, $P < 0.001$. *Columns,* mean of three independent experiments in triplicate; *bar,* SE. *Bar,* 20 μm.

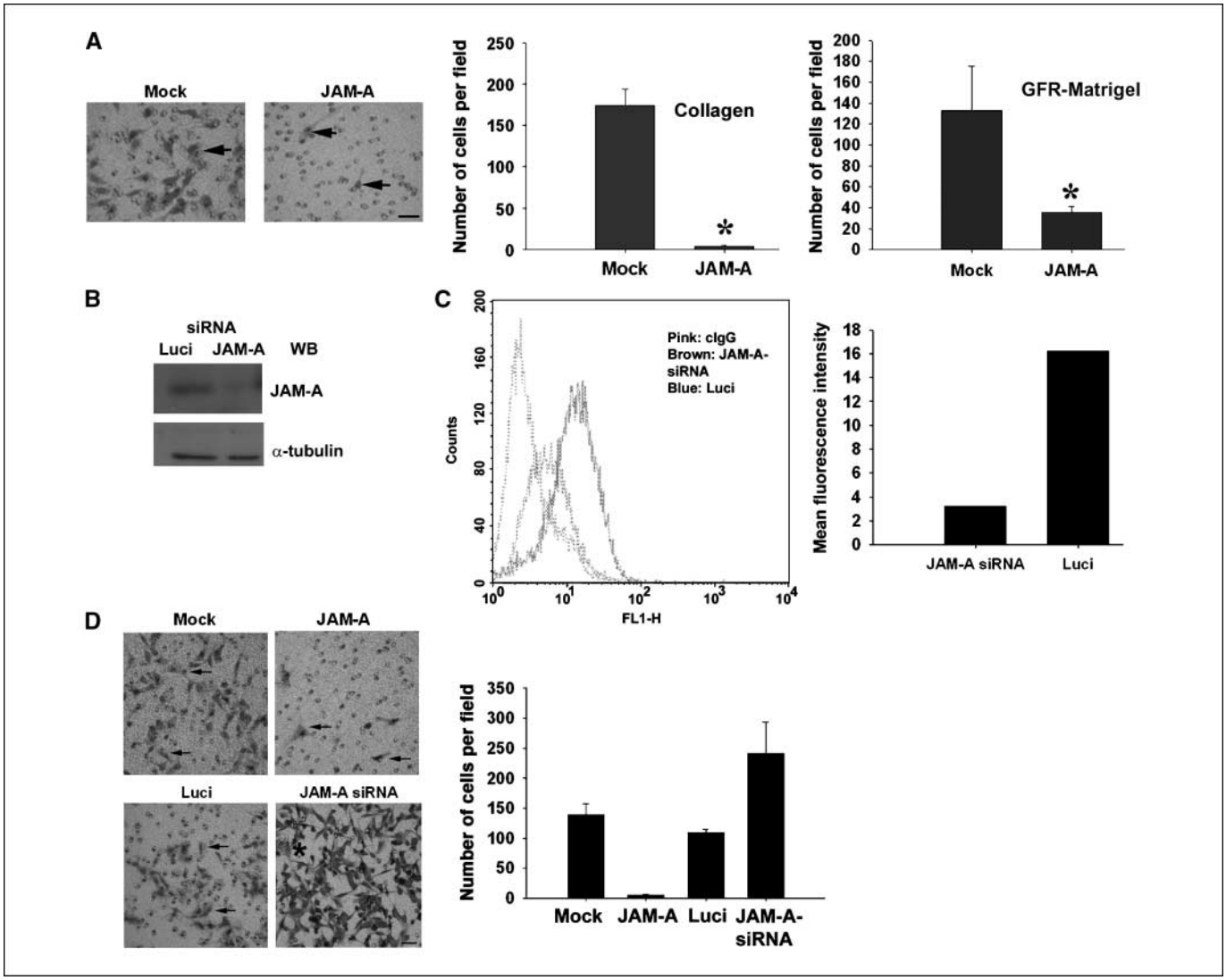

Figure 4. JAM-A inhibits MDA-MB-231 cell invasion. *A,* light microscopic images of transwell invasion assay inserts of MDA-MB-231 mock and JAM-A–overexpressing cells. *Bar,* 20 μm. Quantitative analysis of the invasion assay on collagen and GFR-Matrigel. *, $P < 0.001$. *B,* Western blot analysis showing expression of JAM-A protein in MDA-MB-231 cells transfected with control siRNA (*Luci*) and JAM-A siRNA. α-tubulin is shown as a loading control. *C,* surface expression of JAM-A in control siRNA and JAM-A-siRNA–transfected cells. Normalized mean fluorescence intensity of *C. D,* light microscopic images of transwell invasion assay inserts transfected with empty vector (*mock*), JAM-A expression vector (*JAM-A*), control siRNA, and JAM-A–siRNA (*left*) and quantitative analysis of the invasion assay (*right*). *, $P < 0.001$. *Bar,* 20 μm. *Columns,* mean of three independent experiments; *bars,* SE.

cells showed distinct cell-cell junctions, and JAM-A was localized at these cell-cell contacts (Fig. 6*A*). To test if JAM-A overexpression and accumulation to the cell-cell junctions affected any known tight junction protein, we analyzed the accumulation of ZO-1. As expected, in mock cells, JAM-A staining was diffuse, the tight junctions could not be detected, and ZO-1 staining was indistinct, which is consistent with a previous report (37). Interestingly, we found that JAM-A–overexpressing cells had profound cell-cell junctions, and both ZO-1 and JAM-A were colocalized at the tight junctions (Fig. 6*B*). This staining was comparable with MCF-7 cells used as a positive control where the tight junctions are well-defined (Fig. 6*B*). To test the tight junctional integrity of these junctions, we analyzed their transepithelial resistance. We found that the JAM-A–overexpressing cells had ~2-fold increased TER compared with the mock-transfected cells (Fig. 6*C*). These results suggest that ectopic expression of JAM-A results in the accumulation of JAM-A at the cell-cell junctions, which then recruits ZO-1 and possibly other tight junction proteins to establish functional tight junctions, thus reverting these cells to the parental epithelial phenotype. To investigate the mechanism of how overexpression of JAM-A results in reduced migration and invasion of MDA-MB-231 cells, we examined the organization of the actin cytoskeleton. We found that the mock-transfected cells have a spindle-shaped actin cytoskeleton organized with long stress fibers throughout the length of the cell. Intense filamentous actin (F-actin) staining was also observed at the leading edge, indicating membrane ruffles. In contrast, JAM-A–overexpressing cells exhibit short cortical actin stress fibers with membrane ruffles all around the cell periphery (Fig. 6*D*). Furthermore, we observed that in mock cells, the focal adhesions as visualized by vinculin staining were small and inconspicuous. On the contrary, JAM-A overexpressing cells showed many intense peripheral focal adhesions. These results suggest that JAM-A supports formation of increased number of stable focal adhesions, thus inhibiting cell migration and invasion.

Discussion

Uncontrolled growth of normal cells results in the development of tumors. Some of these tumor cells acquire invasive characteristics. They detach from their neighbors and invade into the surrounding tissue. The mechanism of the ability of the cell to invade is not well-understood. Here, we show that JAM-A specifically regulates the invasive behavior of breast cancer cells. The level of expression of JAM-A dictates the ability of breast cancer cell invasion. In highly metastatic MDA-MB-231 cells, overexpression of JAM-A reduced their migratory and invasive ability, whereas down-regulation of endogenous JAM-A enhanced those abilities. Furthermore, the loss of invasiveness with JAM-A overexpression correlates with cytoskeletal rearrangements leading to the formation of more stable focal adhesions at the periphery and the establishment of functional tight junctions. Thus, we have identified a cell adhesion molecule whose reduced expression seems to be directly responsible for the invasive behavior of breast cancer cells.

JAM-A is a cell-adhesion protein predominantly expressed at the tight junctions of both endothelial and epithelial cells, including those of the mammary epithelium. We have previously shown that JAM-A induces endothelial cell migration (32). Thus, we hypothesized that increasing amounts of JAM-A expression in breast cancer cells would enhance their migratory ability. Surprisingly, we found an inverse relation between the expression level of JAM-A in breast cancer cells and their ability to migrate upon and invade into extracellular matrices. Furthermore, overexpression of JAM-A in a highly invasive breast cancer cell line also changed the morphology of these cells from spindle shaped to round, and cobblestone like with increased TER. This is consistent with a previous report showing that down-regulation of JAM-A expression from epithelial cells using siRNA resulted in a loss of epithelial cell morphology (9), which was attributed to the disruption of epithelial cell barrier function (9). However, unlike in the colonic epithelium previously studied (9), JAM-A knockdown did not lead to a reduction of β_1 integrin expression in MDA-MB-231 cells.

JAM-A has been shown to be important in maintaining tight junction integrity (9, 38–41). Disruption of tight junctions has been implicated to play a role in cancer cell metastasis by inducing epithelial-mesenchymal transition (EMT; ref. 42). Several laboratories, including ours, have shown that cytokines and growth factors redistribute JAM-A from tight junctions (43, 44). Consistent with this finding, it has been shown that hepatocyte growth factor disrupts tight junctions in human breast cancer cells and down-regulates expression of several tight junction proteins (37). It is therefore conceivable that the loss of JAM-A in highly metastatic cells is a consequence of the disruption of tight junctions. This was further supported by the findings that overexpression of JAM-A leads to the formation of functional tight junctions.

Our results presented here are the first report correlating an inverse relationship of JAM-A expression in breast cancer cells to their invasive ability. The reduction in JAM-A expression enhances cell migration, and replenishing the JAM-A levels blocks cell migration. However, the observed effect of overexpression of JAM-A does not seem to be simply due to the formation of tight junctions because individual cells that express increased JAM-A also show reduced migration. This is not surprising, considering the fact that JAM-A in addition to its function of regulating tight junction integrity is also shown to participate in intracellular signaling. Our results presented here further provide a mechanism for the ability of JAM-A to block cell migration by affecting cytoskeletal rearrangement. JAM-A alters the actin stress fibers and supports stable focal adhesions that arrests cell migration. Such effect on cell migration is well-documented in the literature, where transforming growth factor (TGF)β_1 treatment and SMAD4 overexpression also show increased focal adhesions and reduced

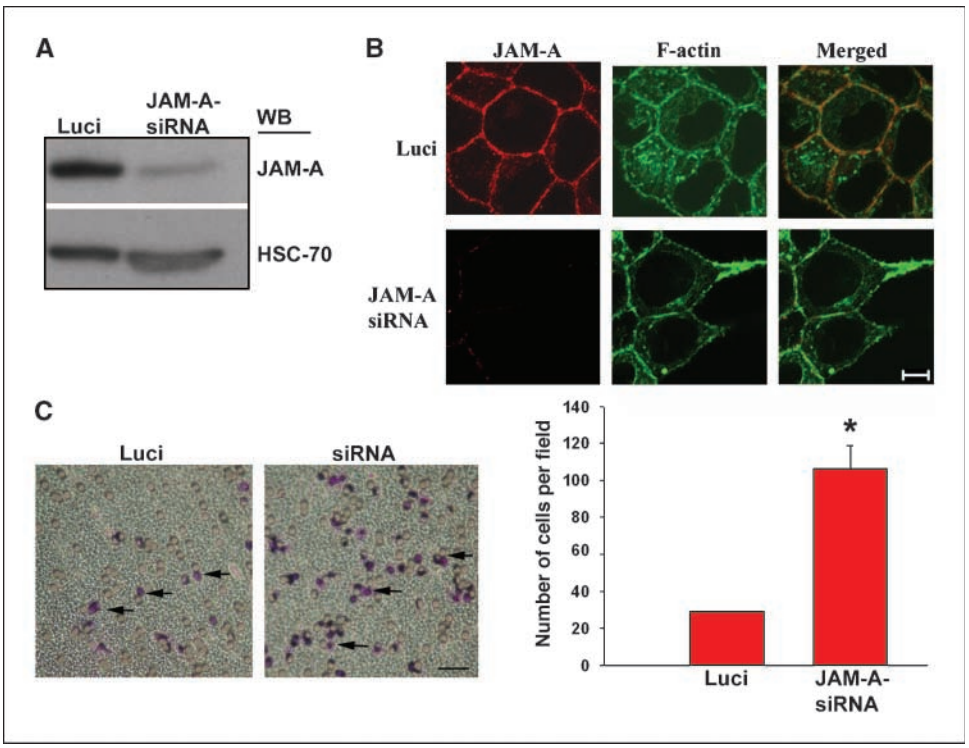

Figure 5. Depletion of JAM-A in T47D cells leads to enhanced invasion. *A,* Western blot of endogenous JAM-A protein in JAM-A siRNA–transfected and the control luciferase siRNA–transfected T47D cells probed using anti–JAM-A. The same blot was reprobed with anti–HSC-70 to ensure equal loading (*bottom*). *B,* immunofluorescence staining using anti–JAM-A of T47D cells transfected with luciferase siRNA (*top*) and JAM-A siRNA (*bottom*). *Bar,* 10 μm. *C,* light microscopic images of inserts of invasion of T47D cells transfected with control siRNA and JAM-A–siRNA (*left*). *Bar,* 20 μm. *Right,* quantitative analysis of the invasion assay. *, $P < 0.003$. *Columns,* mean of three independent experiments; *bar,* SE.

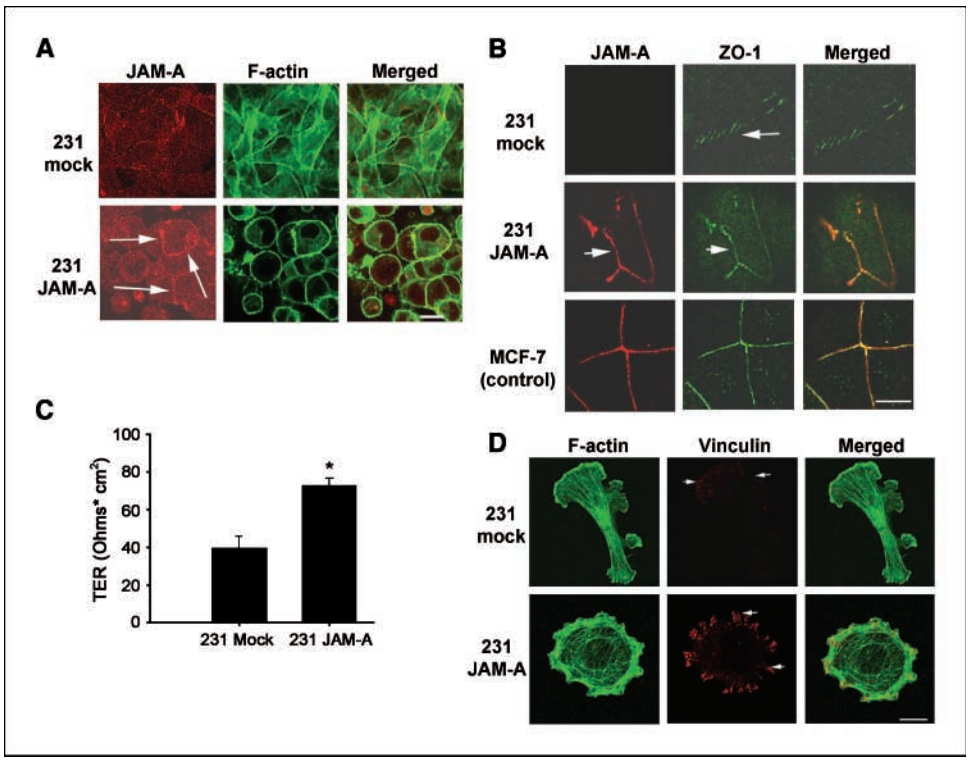

Figure 6. Overexpression of JAM-A in MDA-MB-231 cells restores functional tight junctions. *A,* confocal images of mock (*top*) and JAM-A–overexpressing cells (*bottom*) stained for JAM-A and F-actin. *Arrows,* cell-cell junctions. *B,* confocal images of cells stained with anti–JAM-A (*red*) and anti–ZO-1 (*green*). Mock-transfected MDA-MB-231 (*top*), JAM-A–overexpressing (*middle*), and MCF-7 cells used as a control (*bottom*). Shown is a representative confocal image of three independent experiments. *Bar*, 10 μm. *C,* TER of 4-d-old cultures of mock and JAM-A–overexpressing MDA-MB-231 cells was measured using a voltohmmeter. *, $P < 0.001$. Data are representative of two independent experiments. *D,* confocal images of mock (*top*) and JAM-A–overexpressing cells (*bottom*) stained for vinculin and F-actin. *Arrows,* focal adhesions. *Bar,* 10 μm.

migration and invasion in Car C cells (45). Further work is in progress to see if JAM-A would revert EMT, a hallmark of invasive cancer and is regulated by TGFβ_1 and SMAD signaling (46). JAM-A is capable of interacting homotypically as well as heterotypically on the cell surface (47, 48). It has also been shown that it interacts with several cytoplasmic proteins through its PDZ domain–binding motif and recruits signaling proteins at the tight junctions. We have also shown that JAM-A is a positive regulator of fibroblast growth factor-2 (FGF-2)–induced angiogenesis (30, 49). Furthermore, we have shown that down-regulation of JAM-A in endothelial cells inhibits FGF-2–induced mitogen-activated kinase activity (30). Although, the molecular mechanism responsible for this effect on cell migration is not clear and requires further investigation, the ability of JAM-A to regulate intracellular signaling is undisputable.

In conclusion, we have shown for the first time that JAM-A, an immunoglobulin superfamily member expressed at the tight junction, is responsible in regulating the invasive phenotype of breast cancer cells. We have provided possible mechanism of how loss of JAM-A may result in enhanced cell migration and invasion. Our results also imply that the down-regulation of JAM-A in carcinoma cells may be detrimental to the survival of breast cancer patients. It would be very important to determine the molecular determinants that are responsible for the down-regulation of JAM-A during cancer progression. Thus, loss of JAM-A expression, a molecule that represses breast cancer cell invasion, could be used as a prognostic marker for metastatic breast cancer.

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References

1. Dragsten PR, Blumenthal R, Handler JS. Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? Nature 1981;294:718–22.
2. van Meer G, Gumbiner B, Simons K. The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. Nature 1986;322:639–41.
3. Fanning AS, Anderson JM. Protein modules as organizers of membrane structure. Curr Opin Cell Biol 1999;11:432–9.
4. Faris M, Ensoli B, Kokot N, Nel AE. Inflammatory cytokines induce the expression of basic fibroblast growth factor (bFGF) isoforms required for the growth of Kaposi's sarcoma and endothelial cells through the activation of AP-1 response elements in the bFGF promoter. AIDS 1998;12:19–27.
5. Matter K, Balda MS. Signalling to and from tight junctions. Nat Rev Mol Cell Biol 2003;4:225–36.
6. Fanning AS, Anderson JM. PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. J Clin Invest 1999;103:767–72.
7. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 1998;141:1539–50.
8. Gonzalez-Mariscal L, Lechuga S, Garay E. Role of tight junctions in cell proliferation and cancer. Prog Histochem Cytochem 2007;42:1–57.
9. Mandell KJ, Babbin BA, Nusrat A, Parkos CA. Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on β1 integrins and Rap1 activity. J Biol Chem 2005;280:11665–74.
10. Sommers CL, Gelmann EP, Kemler R, Cowin P, Byers SW. Alterations in β-catenin phosphorylation and plakoglobin expression in human breast cancer cells. Cancer Res 1994;54:3544–52.
11. Sommers CL, Thompson EW, Torri JA, Kemler R, Gelmann EP, Byers, SW. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. Cell Growth Differ 1991;2:365–72.

12. Nieman MT, Kim JB, Johnson KR, Wheelock MJ. Mechanism of extracellular domain-deleted dominant negative cadherins. J Cell Sci 1999;112:1621–32.

13. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol 1999;147:631–44.

14. Martin-Padura I, Lostaglio S, Schneemann M, et al. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 1998;142:117–27.

15. Naik UP, Eckfeld K. Junctional adhesion molecule 1 (JAM-1). J Biol Regul Homeostatic Agents 2003;17:341–7.

16. Santoso S, Orlova VV, Song K, Sachs UJ, Andrei-Selme CL, Chavakis T. The homophilic binding of junctional adhesion molecule-C mediates tumor cell-endothelial cell interactions. J Biol Chem 2005;280:36326–33.

17. Bazzoni G. The JAM family of junctional adhesion molecules. Curr Opin Cell Biol 2003;15:525–30.

18. Ebnet K, Suzuki A, Ohno S, Vestweber D. Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci 2004;117:19–29.

19. Hirabayashi S, Tajima M, Yao I, Nishimura W, Mori H, Hata Y. JAM4, a junctional cell adhesion molecule interacting with a tight junction protein, MAGI-1. Mol Cell Biol 2003;23:4267–82.

20. Moog-Lutz C, Cave-Riant F, Guibal FC, et al. JAML, a novel protein with characteristics of a junctional adhesion molecule, is induced during differentiation of myeloid leukemia cells. Blood 2003;102:3371–8.

21. Zen K, Liu Y, McCall IC, et al. Neutrophil migration across tight junctions is mediated by adhesive interactions between epithelial coxsackie and adenovirus receptor and a junctional adhesion molecule-like protein on neutrophils. Mol Biol Cell 2005;16:2694–703.

22. Naik MU, Mousa SA, Parkos CA, Naik UP. Signaling through JAM-1 and αvβ3 is required for the angiogenic action of bFGF: dissociation of the JAM-1 and αvβ3 complex. Blood 2003;102:2108–14.

23. Ebnet K, Schulz CU, Meyer Zu Brickwedde MK, Pendl GG, Vestweber D. Junctional adhesion molecule (JAM) interacts with the PDZ domain containing proteins AF-6 and ZO-1. J Biol Chem 2000;275:27979–88.

24. Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, Dejana E. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. J Biol Chem 2000;275:20520–6.

25. Kostrewa D, Brockhaus M, D'Arcy A, et al. X-ray structure of junctional adhesion molecule: structural basis for homophilic adhesion via a novel dimerization motif. EMBO J 2001;20:4391–8.

26. Williams LA, Martin-Padura I, Dejana E, Hogg N, Simmons DL. Identification and characterisation of human Junctional Adhesion Molecule (JAM). Mol Immunol 1999;36:1175–88.

27. Naik UP, Naik MU, Eckfeld K, Martin-DeLeon P, Spychala J. Characterization and chromosomal localization of JAM-1, a platelet receptor for a stimulatory monoclonal antibody. J Cell Sci 2001;114:539–47.

28. Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM, Mullin JM. Increased tight junctional permeability is associated with the development of colon cancer. Carcinogenesis 1999;20:1425–31.

29. Cui W, Ning J, Naik UP, Duncan MK. OptiRNAi, an RNAi design tool. Comput Methods Programs Biomed 2004;75:67–73.

30. Naik MU, Vuppalanchi D, Naik UP. Essential role of junctional adhesion molecule-1 in basic fibroblast growth factor-induced endothelial cell migration. Arterioscler Thromb Vasc Biol 2003;23:2165–71.

31. Naik UP, Naik MU. Association of CIB with GPIIb/IIIa during outside-in signaling is required for platelet spreading on fibrinogen. Blood 2003;102:1355–62.

32. Naik MU, Naik UP. Junctional adhesion molecule-A-induced endothelial cell migration on vitronectin is integrin αvβ3 specific. J Cell Sci 2006;119:490–9.

33. Thompson EW, Paik S, Brunner N, et al. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 1992;150:534–44.

34. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

35. Konopka G, Tekiela J, Iverson M, Wells C, Duncan SA. Junctional adhesion molecule-A is critical for the formation of pseudocanaliculi and modulates E-cadherin expression in hepatic cells. J Biol Chem 2007;282:28137–48.

36. Yano T, Hernandez-Blazquez FJ, Omori Y, Yamasaki H. Reduction of malignant phenotype of HEPG2 cell is associated with the expression of connexin 26 but not connexin 32. Carcinogenesis 2001;22:1593–600.

37. Martin TA, Watkins G, Mansel RE, Jiang WG. Hepatocyte growth factor disrupts tight junctions in human breast cancer cells. Cell Biol Int 2004;28:361–71.

38. Balda MS, Matter K. Tight junctions. J Cell Sci 1998;111:541–7.

39. Balda MS, Matter K. Transmembrane proteins of tight junctions. Semin Cell Dev Biol 2000;11:281–9.

40. Bazzoni G. Endothelial tight junctions: permeable barriers of the vessel wall. Thromb Haemost 2006;95:36–42.

41. Dejana E, Corada M, Lampugnani MG. Endothelial cell-to-cell junctions. FASEB J 1995;9:910–8.

42. Gumbiner BM. Regulation of cadherin adhesive activity. J Cell Biol 2000;148:399–404.

43. Ozaki H, Ishii K, Horiuchi H, et al. Cutting edge: combined treatment of TNF-α and IFN-γ causes redistribution of junctional adhesion molecule in human endothelial cells. J Immunol 1999;163:553–7.

44. Shaw SK, Perkins BN, Lim YC, et al. Reduced expression of junctional adhesion molecule and platelet/endothelial cell adhesion molecule-1 (CD31) at human vascular endothelial junctions by cytokines tumor necrosis factor-α plus interferon-γ does not reduce leukocyte transmigration under flow. Am J Pathol 2001;159:2281–91.

45. Santibanez JF, Quintanilla M, Martinez J. TGF-β ([1]) and Smad4 overexpression induce a less invasive phenotype in highly invasive spindle carcinoma cells. FEBS Lett 2002;520:171–6.

46. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 2006;172:973–81.

47. Bazzoni G, Martinez-Estrada OM, Mueller F, et al. Homophilic interaction of junctional adhesion molecule. J Biol Chem 2000;275:30970–6.

48. Ostermann G, Weber KS, Zernecke A, Schroder A, Weber C. JAM-1 is a ligand of the β2 integrin LFA-1 involved in transendothelial migration of leukocytes. Nat Immunol 2002;3:151–8.

49. Cooke VG, Naik MU, Naik UP. Fibroblast growth factor-2 failed to induce angiogenesis in junctional adhesion molecule-A-deficient mice. Arterioscler Thromb Vasc Biol 2006;26:2005–11.

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