Integrin αvβ3-associated DAAM1 is essential for collagen-induced invadopodia extension and cell haptotaxis in breast cancer cells *

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Abstract

The formin protein dishevelled-associated activator of morphogenesis 1 (DAAM1) polymerizes straight actin filaments and mediates migration of cancer cells. However, how DAAM1 governs cell haptotaxis in response to collagen remains unexplored in breast cancer cells. We hypothesized that DAAM1 mediates invadopodia extension and cell haptotaxis in response to type IV collagen in association with integrin receptors. Using Boyden chamber membranes coated with type IV collagen, we show here that type IV collagen activates both DAAM1 and Ras homolog family member A (RHOA) and promotes haptotaxis of MDA-MB-231 and MDA-MB-453 breast cancer cells, a process abolished by treatment with the integrin αvβ3 inhibitor cyclo(-RGDfK). ShRNA-mediated knockdown of DAAM1 or a dominant-negative DAAM1 mutation (N-DAAM1) significantly decreased collagen-induced RHOA activity and the assembly of stress fibers, invadopodia extension, and cell haptotaxis. Immunoprecipitation and pulldown assays revealed that integrin αvβ3 is associated with, but only indirectly binds to, the C-terminal DAD domain of DAAM1 in mammalian cells. Blockade of RHOA activation with a specific inhibitor (CCG-1423) or via a dominant-negative RHOA mutation (RHOA-N19) suppressed collagen-induced invadopodia extension and haptotaxis of the MDA-MB-231 and MDA-MB-453 cells. Immunoblotting and immunofluorescence assays indicated high DAAM1 and RHOA expression in invadopodia, which was abolished by cyclo(-RGDfK) treatment or DAAM1 knockdown. These findings have uncovered an integrin αvβ3/DAAM1/RHOA signaling pathway for type IV collagen-induced invadopodia extension and haptotaxis in breast cancer cells. Targeting this pathway may be a means for reducing invasiveness and metastasis of breast cancer.
Introduction

Although metastases of epithelial cancers are responsible for 90% of human cancer-related deaths, the mechanisms regulating the development and metastasis of carcinomas are not fully understood. Haptotaxis, a motility directed by a gradient of cellular adhesion sites or substrate-bound chemoattractants, frequently conducts at the early stage of epithelial tumor metastasis (1). During haptotaxis, cancer cells are guided by gradients of surface-bound extracellular matrix (ECM) proteins (2).

ECM is composed of highly diverse and dynamic components that regulate cell activities. Fibrillar collagens, fibronectin, hyaluronan and matricellular protein are matrix components commonly found in involution and cancer (3). ECM serves as an adhesive substrate for cell migration and, by binding morphogens and growth factors, creates concentration gradients for haptotactic migration or pattern formation (4,5). ECM protein rich in laminin and type IV collagen makes up the specialized basement membrane that provides a barrier for cell invasion and substrates for adhesion of migrating tumor cells (6). The initial phases of tumor cell invasion and migration require transducing extracellular signaling from the surrounding ECM to trigger the intracellular signaling controlling motility.

Integrins are a family of 24 αβ heterodimeric transmembrane cell-surface receptors that modulate cell behavior, transducing spatiotemporal messages from the extracellular environment (7). The roles of collagen-binding integrins in physiological and pathological settings emphasize on wound healing, fibrosis and tumor-stroma interactions (8-10). Dysregulation of integrin αvβ3 expression and/or signaling correlates with development of cancer, e.g. promoting epithelial-mesenchymal transition (EMT), mediating metastasize to bone, modulating cell adhesion and invasion (11-13). Although recent literature reports that integrin αvβ3 activates intracellular kinases to regulate the motile behavior of tumor cells (13,14), little is known about which novel molecule interacts with integrin αvβ3 and then modulates cellular actin cytoskeleton.

Invadopodia are actin-rich protrusions of the plasma membrane that are associated with degradation of the ECM in cancer invasiveness and metastasis (15). Actin polymerization is crucial for the formation and function of invadopodia. Formin proteins, elements of the cellular actin cytoskeleton, can polymerize actin filaments at the barbed end (16). Based on their sequences and domain architectures in mammals, a total of fifteen formin proteins are grouped into eight different sub-families (17). The protein dishevelled-associated activator of morphogenesis 1 (DAAM1) is an autoinhibited formin protein. DAAM1 is identified as interaction factor of dishevelled (Dvl) and mediates the non-canonical Wnt/PCP (planar cell polarity) signaling pathway (18,19). Our previous study finds that active DAAM1 is the downstream target of Wnt5a/Dvl2 and its activation is required for Wnt5a-induced cell migration (19). Here, we report that DAAM1 is associated
with integrin β3 and then promotes invadopodia extension and cell haptotaxis in response to type IV collagen. These results reveal the molecular signaling mechanism in the haptotaxis of breast cancer cells, and identify DAAM1 as a target for anticancer therapy.

Results

Type IV collagen induces haptotaxis of breast cancer cells

To determine whether collagen induced directional cell haptotaxis, we examined the migration of MDA-MB-231 for 8 h in 8.0-μm porous Boyden chamber membranes coated with 0, 1, 5 10, 20 μg/mL type IV collagen on the lower sides of membranes, respectively. Ten and 20 μg/mL type IV collagen largely elevated the haptotaxis of MDA-MB-231 cells (Supplementary Figure S1). Next, we tested the migration of MDA-MB-231 and MDA-MB-453 breast cancer cells and MCF10A mammary epithelial cells through 8.0-μm porous membranes coated with vehicle, or 10 μg/mL type IV collagen on upper sides, both sides, or lower sides. An approximately fifteen-fold increase was observed in the number of migrant breast cancer cells induced by type IV collagen coated on the lower sides than that of vehicle groups (Figure 1A, 1B, 1C). Moreover, three-fold increase was observed in the number of migrant breast cancer cells induced by type IV collagen coated on the lower sides than that of both-side coated groups (Figure 1A, 1B, 1C). A comparison of migrant MCF10A cells in the Boyden chamber membranes coated with collagen on both sides with those in the Boyden chamber membranes coated with collagen on the lower sides revealed that collagen-induced haptotaxis only exists in breast cancer cells (Figure 1A, 1D). In addition, collagen coated on the upper sides exhibited little effect on cell migration of both breast cancer cells and mammary cells (Figure 1A, 1B, 1C, 1D). To exclude the effect of Wnt5a on cell migration under the condition of collagen coated Boyden chamber membranes, we examined the secretion of Wnt5a in MDA-MB-231 and MDA-MB-453 cells seed on coverslips coated with type IV collagen (10 μg/mL) or vehicle. MDA-MB-231 and MDA-MB-453 cells exhibited the low endogenous secretion of Wnt5a, which was not statistically changed under collagen treated condition (Supplementary Figure S2). Accordingly, 10 μg/mL type IV collagen treated for 8 h was used to identify the mechanism for the haptotaxis in breast cancer cells.

DAAM1 is involved in collagen-induced cell haptotaxis

For oriented cell migration, dishevelled and DAAM1 are recruited in the reorganization of actin cytoskeleton (19,20). We examined whether DAAM1 also mediated collagen-induced haptotaxis of breast cancer cells. Pulldown assays and immunoblotting showed a significant elevation of active DAAM1 levels after 4 h induction by type IV collagen (Figure 1E, 1F, 1G). Moreover, the active DAAM1 of cells in Boyden chamber membranes coated with collagen on the lower sides was further increased than that in Boyden chamber membranes coated with collagen on the upper and both sides.
(Figure 1E, 1F, 1G). To analyze the role of endogenous DAAM1 activation in collagen-induced haptotaxis, we knocked down DAAM1 expression using shRNA (#1 and #2) and selected the stable DAAM1 knockdown cells (Figure 1H). ShRNA targeting DAAM1 reduced the protein levels of DAAM1 by more than 80% (Figure 1H). The haptotaxis of stable DAAM1 knockdown cells was significantly retarded after the induction of type IV collagen coated on the lower sides of Boyden chamber membranes (Figure 1I, 1J). Moreover, full-length DAAM1 cDNA with three mutation sites which could be unpaired with DAAM1 shRNA #2 rescued the haptotaxis of DAAM1-knockdown breast cancer cells (Supplementary Figure S3). We also transiently transfected a panel of DAAM1 truncations into MDA-MB-231 and MDA-MB-453 cells and tested their roles in cell haptotaxis. In order to measure the percentage of the transfected cells, we co-transfected DAAM1 truncations with EGFP construct. The expression efficiency of EGFP puncta was 60~70% as observed under a fluorescence microscope. Interestingly, the expression of full-length endogenous DAAM1 is downregulated by the expression of C-DAAM1 (C-terminal of DAAM1), but not by N-DAAM1 (dominant negative DAAM1) (Figure 1K, 1N, and 1P). N-DAAM1 retarded collagen-induced cell haptotaxis (Figure 1L, 1M), while C-DAAM1 failed to accelerate cell haptotaxis (Figure 1N, 1O). Taken together, these experiments demonstrated that active DAAM1 is involved in collagen-induced haptotaxis of breast cancer cells.

**DAAM1 acts as a downstream target of integrin and is associated with integrin αvβ3**

To determine whether integrin receptor mediated collagen-induced directional haptotaxis, we examined the migration of MDA-MB-231 or MDA-MB-453 cells through 8.0-μm porous membranes coated with type IV collagen on the lower side after treating with integrin inhibitors. Large decreases were observed in the number of migrant cells incubated with cilengitide trifluoroacetate (an integrin inhibitor for αvβ3 receptor and αvβ5 receptor) or cyclo(-RGDfK) (a specific inhibitor for integrin αvβ3) (Figure 2A), suggesting that αvβ3 integrin mediated collagen-induced cell haptotaxis. To examine whether integrin regulated DAAM1 in breast cancer cells, we measured the activity of DAAM1 after treating MDA-MB-231 or MDA-MB-453 cells with cyclo(-RGDfK). We found the significant decrease of DAAM1 activity in the presence of the αvβ3 integrin inhibitor (Figure 2B, 2C). These data strongly implicated αvβ3 integrin in collagen-induced DAAM1 activation and haptotaxis of MDA-MB-231 and MDA-MB-453 cells.

Next, we tested whether the physical interaction of DAAM1 with integrin was collagen-dependent in human cancer cells. Interestingly, DAAM1 was strongly associated with integrin β3 in MDA-MB-231 or MDA-MB-453 cells, which was enhanced upon collagen type IV stimulation (Figure 2D, 2E). To test which domain of DAAM1 indeed interacted with integrin β3, we
expressed HA-tagged DAAM1 constructs in HEK-293T cells followed by immunoprecipitation with anti-HA antibody. Blotting with a HA-antibody confirmed the high expression of full-length DAAM1 and truncations (Figure 2F). The immunoprecipitated HA-DAAM1 and C-DAAM1 pulled down integrin β3 (Figure 2F). However, the immunoprecipitated HA-ΔDAD-DAAM1 failed to pull down integrin β3 (Figure 2F). Using an in vitro binding assay, we demonstrated that purified bacterially expressed recombinant DAAM1 did not directly bind to the C-terminal domain of integrin β3 (Figure 2G). Thus, we verified that the C-terminal DAD domain of DAAM1 was associated with, but indirectly bound to, integrin β3 in mammalian cells.

**RHOA acts as a downstream target of DAAM1 and mediates collagen-induced cell haptotaxis**

Given that Rho GTPases play a central role for all types of cell migration (21), we suggest that Rho GTPases may promote haptotaxis of breast cancer cells in response to the collagen gradient. Rho GTPase activation assays showed the significantly increased activity of RHOA, ras-related C3 botulinum toxin substrate 1 (RAC1), and cell division control protein 42 homolog (CDC42) after 4 h of type IV collagen treatment (Figure 3A, 3B, 3C). To elucidate which specific Rho GTPases participated in the DAAM1-mediated cell haptotaxis, we performed Rho GTPase activation assays after DAAM1 knockdown. The activation of RHOA, not RAC1 or CDC42, was downregulated in stable DAAM1 knockdown MDA-MB-231 cells (Figure 3D). Next, we found that RHOA activation was largely inhibited by cyclo(O-RGDfK) treatment in MDA-MB-231 cells (Figure 3E). These results indicated that RHOA functioned as a downstream target of integrin αvβ3/DAAM1 in breast cancer cells.

We used CCG-1423 (a RHOA specific inhibitor) to study the role of RHOA activation in cell haptotaxis. Pre-incubation with 1 µmol/L CCG-1423 for 1 h completely inhibited the haptotaxis of MDA-MB-231 and MDA-MB-453 cells induced by collagen coated on the lower sides of Boyden chamber membranes (Figure 3F, 3H). Also, we transfected MDA-MB-231 and MDA-MB-453 cells with RHOA-N19 (dominant negative mutant) and selected the stable RHOA-N19 overexpressed cells. Overexpression of N19-RHOA completely abolished the haptotaxis of MDA-MB-231 and MDA-MB-453 cells induced by collagen coated on the lower sides of Boyden chamber membranes (Figure 3G, 3H). To examine whether RHOA regulated DAAM1 in MDA-MB-231 cells, we measured the activity of DAAM1 in RHOA-N19 overexpressed cells. A comparison of DAAM1 activity in RHOA-N19 overexpressed cells with that in control cells had no statistical differences in the absence of collagen coated on the lower sides of Boyden chamber membranes (Figure 3I, 3J). N-DAAM1 largely retarded the haptotaxis of MDA-MB-231 cells, which could be rescued by constitutively active RHOA (RHOA-V14) overexpression (Figure 3K). These results indicated that RHOA acted as a downstream target of
DAAM1. Thus, we concluded that DAAM1/RHOA activation is involved in collagen-induced haptotaxis of breast cancer cells.

DAAM1 is highly expressed in invadopodia and mediates their extension

To determine whether collagen induced invadopodia extension, MDA-MB-231 and MDA-MB-453 cells were seeded onto 3.0-μm porous membranes coated with vehicle, or 10 μg/mL type IV collagen on the upper sides, both sides, or lower sides. collagen coated on the upper sides of Boyden chamber membranes or vehicle were unable to induce invadopodia extension (Figure 4A). An approximately three fold increase was observed in the invadopodia extension induced by collagen coated on the lower sides than that by collagen coated on both sides (Figure 4A, 4B). Next, we examined whether DAAM1 mediated collagen-induced invadopodia extension. The stable DAAM1 knockdown cells showed a significant decrease of invadopodia extension after the induction of type IV collagen coated on the lower sides (Figure 4A, 4E). We also treated MDA-MB-231 and MDA-MB-453 cells with cyclo(-RGDFIK) and found that this integrin αvβ3 inhibitor retarded collagen-induced invadopodia extension (Figure 4D, 4E).

We next tested whether DAAM1 was significantly highly expressed in invadopodia of breast cancer cells in response to collagen. Cell bodies (the top sides of membranes) and invadopodia (the lower sides of membranes) of MDA-MB-231 or MDA-MB-453 cells were mechanically separated. The quality of the fractionation was assessed by immunoblotting for Cortactin and Histone H3 (Figure 4F). Indeed, most DAAM1 proteins concentrated in invadopodia of MDA-MB-231 and MDA-MB-453 cells (Figure 4F, 4G, 4H). Therefore, we preliminarily demonstrated that active DAAM1 participates in collagen-induced invadopodia extension and highly expressed in invadopodia of breast cancer cells.

DAAM1-dependent activation of RHOA mediates collagen-induced invadopodia extension

To study the role of RHOA activation in invadopodia extension, we treated breast cancer cells with a RHOA specific inhibitor (CCG-1423). Pre-incubation with 1 μmol/L CCG-1423 for 1 h completely inhibited collagen-induced invadopodia extension in MDA-MB-231 and MDA-MB-453 cells (Figure 5A, 5B). Furthermore, we found that N19-RHOA overexpression completely abolished collagen-induced invadopodia extension (Figure 5A, 5B).

Immunofluorescence showed that RHOA was highly expressed in invadopodia of MDA-MB-231 cells induced by type IV collagen (Figure 5C, 5D). Knockdown of DAAM1 or cyclo(-RGDFIK) treatment significantly decreased the number of RHOA-positive invadopodia after collagen induction (Figure 5C, 5D). However, the stable DAAM1 knockdown cells also showed a significant decrease of invadopodia extension (Figure 4C). We next used Z-axis scan to observe the RHOA location in pores of 3.0-μm porous membranes (Figure 5E, 5F).
Knockdown of DAAM1 or cyclo(-RGDfK) treatment significantly blocked the high RHOA expression in invadopodia (Figure 5E, 5F). Thus, we concluded that DAAM1-dependent activation of RHOA mediates collagen-induced invadopodia extension of breast cancer cells.

**DAAM1 and RHOA signaling regulate the assembly of stress fibers**

We also examined whether DAAM1 and RHOA signaling regulates the assembly of actin filaments in breast cancer cells. We performed fluorescent phalloidin and myosin II staining to investigate the distribution of filamentous actin (F-actin) in MDA-MB-231 cells or stable DAAM1 knockdown cells treated with type IV collagen (Figure 6A, Supplementary Figure S4). We found that the knockdown of DAAM1 disrupted the formation of stress fibers and decreased the length of stress fibers (Figure 6A, 6B, 6C). Simultaneously, we also found that CCG-1423 and cyclo(-RGDfK) disrupted the formation of stress fibers and decreased the length of stress fibers (Figure 6A, 6B, 6C). Thus, the findings indicated that integrin αvβ3, DAAM1 and RHOA signaling regulates the assembly of stress fibers in breast cancer cells induced by collagen.

**Discussion**

The underlying mechanism governing cancer cell haptotaxis remains largely undefined. We conclude that DAAM1 associating with integrin αvβ3 is indispensable for type IV collagen-induced haptotaxis of breast cancer cells. To exclude the effect of Wnt5a, we examined the secretion of Wnt5a and found that MDA-MB-231 and MDA-MB-453 cells exhibited the low endogenous secretion of Wnt5a, which did not statistically change under collagen treated condition. In the previous study, the high concentration of Wnt5a (at least 300 ng/mL) is able to induce cell migration of MDA-MB-231 cells (19). Here, the low endogenous secretion of Wnt5a (less than 8 ng/mL) is unable to improve cell migration in Boyden chamber assays. Type IV collagen coated on the lower sides of Boyden chamber membranes largely stimulates cell haptotaxis. On the contrary, a small number of cells migrating to the lower component of Boyden chamber membranes coated with collagen on the upper sides reveals that most of cells lose the migratory ability and adhere on the upper sides.

DAAM1, a member of formin family, exists in an auto-inhibited state with its N-terminal GBD and C-terminal DAD domains intramolecularly interacted (18,22). Our results show that collagen/integrin αvβ3 remarkably activates DAAM1 in MDA-MB-231 and MDA-MB-453 cells. Our prior work demonstrates that N-DAAM1 retards Wnt5a-induced cell migration which is a typical chemotaxis evoked by a chemical stimulus (19). Here, we find that interference of DAAM1 function via N-DAAM1 expression, or knockdown of DAAM1 expression via shRNA transfection, inhibits RHOA activation and type IV collagen-induced haptotaxis of breast cancer cells. However, the expression of C-DAAM1 does not accelerate collagen-induced haptotaxis. One possible explanation is that the expression of full-length
endogenous DAAM1 is downregulated by C-DAAM1 expression.

Integrins are transmembrane receptors activating signal transduction pathways that mediate cellular signals such as regulation of the cell migration, organization of the intracellular cytoskeleton (23). Actin remodeling and cell movement is triggered via rapid signaling to integrin αβ3, Src, phosphatidylinositol 3-OH kinase (PI3K), and FAK in breast cancer T-47D cells (14). Integrin β3 also could employ the PI3K-Akt and the MAPK pathway for enabling cell survival and proliferation in MDA-MB-231 cells (24). Integrin β3 participates in invadopodia formed by lung carcinoma cells, and it is associated with poor prognosis and increased metastasis in lung cancer (25). The MENA-mediated haptotactic response in breast cancer cells depends on MENA’s binding to α5β1 integrin receptor, adhesion protein signaling, and fibronectin fibrillogenesis (26,27). In this study, we find that integrin αβ3 associating with, but indirectly binding to, the C-terminal DAD domain of DAAM1 promotes collagen-induced haptotaxis of breast cancer cells.

Actin-rich protrusions anchoring the plasma membrane, invadopodia can decompose ECM during cancer invasiveness and metastasis (15). In this study, we test the ability of DAAM1 in stimulating invadopodia extension in Boyden chambers (a three-dimensional cell culture system). Its two chambers offer different chemical environments in which cell migration can be stimulated. To imitate cell haptotactic migration, just add soluble chemoattractants into the bottom compartment or coat the lower membrane with ECM proteins (28). Our Boyden chamber assays show that integrin αβ3 inhibitor or DAAM1 knockdown blocks collagen-induced invadopodia extension.

DAAM1 and Rho GTPases cooperatively regulate the signaling pathways being responsible for the dynamics of actin filaments (18,19,29). Two type of actin assembly factors, DAAM1 and mDia1 exhibit distinct efficiencies when directly regulated by Rho GTPases (30). Aspenstrom et al. reported that their study are not consistent with a model in which DAAM1 activates RHOA, instead the protein is likely to be a downstream effector of RHOA (29). Here, we were unable to detect any collagen-induced activation of DAAM1 with the nucleotide-free mutant RHOA-N19. N-DAAM1 largely retarded the haptotaxis of breast cancer cells, which could be rescued by constitutively active RHOA (RHOA-V14) overexpression. This study concludes that RHOA acts as a downstream target of integrin αβ3/DAAM1 and mediates collagen-induced stress fiber formation, and haptotaxis of breast cancer cells. On the contrary, WAVE complex brings high motility to the membranes of junctions in epithelial cells, but DAAM1-mediated actin regulation restrains this motility (31). Guillabert-Gourgues et al. report that Kif26b and DAAM1 cooperatively regulate the initiation of epithelial cells’ sprouting and their directional migration via microtubule reorganization (20). Bile acid stimulates haptotaxis in colorectal cancer cells via RHOA/Rho-kinase pathway and signaling cascades including protein kinase C, mitogen-activated protein kinase, and
cyclooxygenase-2 (32). For the first time, our study determines the importance of integrin αvβ3/DAAM1/RHOA signaling pathway in maintaining stress fiber formation and cell haptotaxis for breast cancer metastasis.

Although this study offers a mechanistic understanding on the role of DAAM1 in cancer cell migration, how DAAM1 precisely regulates haptotaxis and motility of breast cancer cells is to be answered. Together, our data prove the ability of integrin αvβ3/DAAM1/RHOA-dependent mechanism that leads to increased collagen-induced invadopodia extension and haptotaxis of breast cancer cells. These findings elucidate a molecular pathway linking integrin αvβ3/DAAM1 signaling with invadopodia extension and cell haptotaxis, which may shed new light into the invasive and metastatic mechanisms of breast cancer.

**Experimental Procedures**

**Cell culture and transfections**

MDA-MB-231, MDA-MB-453, HEK-293T, and MCF10A cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). MDA-MB-231, MDA-MB-453, and HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, high glucose) (Cat. SH30022.01, Hyclone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (FBS) (Cat. SH30068.03, Hyclone) in a humidified incubator at 37°C with 5% CO2. MCF10A cells were grown in DMEM/F12 media (Cat. 12500096, Gibco, Grand Island, NY) supplemented with 5% (v/v) horse serum (Cat. SH30074.03, Hyclone), 20 ng/mL human EGF, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, penicillin, streptomycin and 100 ng/mL cholera toxin. All cell lines were verified to be mycoplasma negative monthly.

The constructs of N-DAAM1 (N-terminal of DAAM1), C-DAAM1 (C-terminal of DAAM1), ΔDAD-DAAM1 (lacking the DAD (Diaphanous-like autoregulatory domain) domain), RHOA-V14 (constitutively active RHOA) and RHOA-N19 (dominant-negative RHOA) were previously generated by our lab (19,33).

For the construct of GST-tagged integrin β3 cytoplasmic tail, the fragment of integrin β3 tail (716-762 amino acids) was amplified by PCR and inserted into pGEX-6p-1 vector (34). The cells were seeded in 6-well plates (Costar, Corning, NY) and cultured to 80–90% confluence, and then transfected with plasmids (4 μg/well) using Lipofectamine 2000 reagent (10 μL/well)(Cat. 11668-019, Invitrogen, Carlsbad, CA) in serum-free OPTI-MEM. The cells were switched to fresh medium containing 10% FBS 6 h after the transfection and cultured for 48 h.

The targeting sequences of the shRNAs are: DAAM1 #1 (5’-GCCACTTTGTATCCTATCGG-3’), and DAAM1 #2 (5’-GCAGGATTTCTTGTGAACAG-3’). The shRNAs were designed and cloned into Tet-pLKO-puro vector (kindly gifted from Dr. Yu Jiang, University of Pittsburgh). The resulting plasmids were transfected into MDA-MB-231 and MDA-MB-453 cells using Lipofectamine 2000 reagent (Cat. 11668-019, Invitrogen, Carlsbad, CA) and selected against puromycin. Clones
stably expressing the doxycycline-inducible shRNAs were isolated. The inducible cells were cultured in the presence of puromycin (1 μg/mL) and the expression of shRNA was induced by including 100 ng/mL of doxycycline in the culture medium. All cells were maintained in a 37°C incubator with 5% CO₂ and cultured as the parental cells.

**Boyden chamber assays**

Cell migration was assessed in a modified Boyden chamber system (Costar, Corning, NY), in which the two chambers were separated by a polycarbonate membrane (8.0 μm pore diameter) (Cat. 3422, Costar). For invadopodia extension, Boyden chamber membranes with 3.0 μm pores (Cat. 3415, Costar) were employed. Lower or two sides of Boyden chamber membranes were coated with human type IV collagen (10 μg/mL) (Cat. Sigma-Aldrich) for 2 h at 37°C. MDA-MB-231, MDA-MB-453, or MCF10A cells were grown to subconfluence in tissue culture plates, detached, centrifuged and rendered into single cell suspensions in serum-free culture medium supplemented with 5 μg/mL BSA. The suspensions containing 1×10⁵ cells were added to wells with a membrane placed at the bottom. The cells were allowed to migrate or extend invadopodia for the indicated periods of time at 37°C. Thereafter, the medium was discarded, stationary cells were removed with a cotton-tipped applicator, and the membranes were cut off the chamber and stained with 0.5% crystal violet or indicated fluorescently-labeled antibodies. The response was evaluated in a microscope (Mshot MF53, Micro-shot Technology Co., Guangzhou, China) by counting the number of cells or invadopodia that had passed through the membrane.

**Migrating cells, invadopodia and cell body isolation**

Cells were seeded on the upper surface of the Boyden chamber membranes (8.0 μm pores) and allowed to migrate into the bottom chamber. Non-migrating cells stood on the upper sides of membranes and were manually removed with a cotton swab. Migrating cells were adhered on the lower sides of membranes and scraped into lysis buffer as described below.

To isolate invadopodia and cell bodies of cells induced to migrate with collagen, we followed the protocol described previously with some modifications (28,35,36). Cells were seeded on the upper surface of the Boyden chamber membranes (3.0 μm pores) and allowed invadopodia to extend into the bottom chamber. After indicated time, the cells were briefly rinsed with PBS and fixed with 0.3% methanol-free formaldehyde in PBS for 10 min at room temperature. Glycine was added to 250 mmol/L for 5 min at room temperature and the cells were washed twice with PBS. To isolate invadopodia, cell bodies on the upper sides of membrane surface were manually removed with a cotton swab and laboratory paper and invadopodia on the lower sides of the membranes were scraped into crosslink reversal buffer (100 mmol/L Tris pH 6.8, 5 mmol/L EDTA, 10 mmol/L dithiothreitol and 1%SDS). Cell bodies were similarly isolated except that invadopodia on the
lower sides of the membranes were manually removed and cell bodies were scraped into crosslink reversal buffer. Extracts were incubated at 70°C for 45 min to reverse the formaldehyde-induced crosslinks.

**Immunoblotting**

Cells grown in Boyden chambers were washed twice with PBS, and then lysed with ice-cold RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L EDTA, 1 mmol/L PMSF, and 1% cocktail of protease inhibitors) (pH 7.4). The lysates were then clarified by centrifugation at 12,000 g for 20 min at 4°C. The equal amount of protein extracts were separated by 8% or 10% SDS-PAGE. The following antibodies were used: anti-integrin β3 (1:1000 dilution, Cat. 18309-1-AP, ProteinTech Group, Wuhan, China), anti-GAPDH (1:5000 dilution, Cat. 60004-1-Ig, ProteinTech), anti-β-actin (1:5000 dilution, Cat. 60008-1-Ig), ProteinTech, anti-Histone H3 (1:5000 dilution, Cat. 17168-1-AP, ProteinTech), and anti-HA tag (1:2000 dilution, Cat. 51064-2-AP, ProteinTech), anti-DAAM1 (1:1000 dilution, Cat. sc-100942, Santa Cruz Biotechnology), and anti-Cortactin (1:2000 dilution, Cat. ab81208, Abcam) antibodies. Protein bands were detected after incubation with horseradish peroxidase conjugated antibodies and visualized with Pierce™ ECL Western Blotting Substrate (Cat. 32209, Thermo Scientific, Rockford, IL).

**Pulldown assays**

For detection of active DAAM1, GST-RHOA beads were incubated with 0.1 mmol/L GTPγS (Sigma-Aldrich, St. Louis, MO) at 30°C for 15 min with constant agitation. Cells grown in Boyden chambers were washed twice with PBS, and then lysed with lysed with lysis buffer containing 1% Triton X-100, 50 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, and 1×protease inhibitor mixture. The equal amount of total cellular protein were incubated with GTPγS-incubated GST-RHOA beads captured on MagneGST glutathione particles (Promega, Madison, WI) at 4°C with constant rotation for 90 min. The beads were washed three times with washing buffer (4.2 mmol/L Na2HPO4, 2 mmol/L KH2PO4, 280 mmol/L NaCl, and 10 mmol/L KCl, pH7.2). At the end of this period, beads were captured by magnet in a magnetic stand.

For an in vitro binding assay, GST-tagged integrin β3 tail (716-762 amino acids) was purified by using Glutathione Sepharose 4B (Cat. 17-0756-01, GE Healthcare, Uppsala, Sweden). HA-tagged full length DAAM1 was purified by using Pierce anti-HA agarose (Cat. 26181, Thermo Scientific). The beads were washed three times with washing buffer and captured by centrifugation at 12,000 g for 5 min.

After washing with ice-cold buffer for three times, beads were resuspended in Laemmli buffer, boiled, and subjected to immunoblotting analysis. SDS-PAGE and immunoblotting were performed using standard methods.

**Immunoprecipitation (IP)**

To test interaction between
DAAM1 and integrin, cells were lysed with lysis buffer containing 50 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L PMSF, and 1×protease inhibitor mixture. Cell debris and unbroken cells were removed by centrifugation at 10,000 g for 10 min. Clarified lysates were incubated with antibodies overnight followed by addition of protein A/G conjugated agarose beads (Pierce, Rockford, IL). After incubation for additional 1.5 h with agitation, beads were washed four times with lysis buffer, once with 20 mmol/L Tris-HCl (pH 7.4), and boiled for 5 min in 60 l of 2×SDS sample buffer. Samples were subjected to SDS-PAGE and blotted with anti-integrin β3 (1:1000 dilution) or anti-DAAM1 antibodies (1:1000 dilution).

HEK-293T cells were transfected with indicated constructs of HA-tagged DAAM1. After 24 h, cell lysates were harvested, and incubated with anti-HA antibody and protein A/G conjugated agarose beads at 4 °C with constant rotation for overnight. The immunoprecipitates were analyzed by immunoblotting with anti-HA (1:2000 dilution) or anti-integrin β3 antibodies (1:1000 dilution). Whole cell lysates were used as controls.

**Rho GTPase activation assays (G-LISA small GTPase activation assays)**

In Rho GTPases (RHOA, CDC42, and RAC1) activation assays (Cat. BK121 for RHOA, Cat. BK127 for CDC42, Cat. BK126 for RAC1, Cytoskeleton Inc., Denver, CO), MDA-MB-231, MDA-MB-453, or stable DAAM1 knockdown MDA-MB-231 cells were seeded onto Boyden chambers with the lower sides of membranes coated with human type IV collagen. Cells were allowed to migrate for 4 h. Then, equal volumes of total cellular protein extracted from Boyden chambers were subjected to Rho GTPase activation assays. The experiments were then performed according to the manufacturer’s protocol.

G-LISA small GTPase activation assays offer a fast and sensitive method for performing small G-protein activation assays. Briefly, equal protein concentration in all samples is a prerequisite for accurate comparison between samples in GTPase activation assays. Cell extracts were equalized with ice-cold Lysis Buffer containing protease inhibitor cocktail to give identical protein concentrations. The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. Add 10 µL of each lysate or Lysis Buffer into the well of a 96 well plate. Add 290 µL of Precision Red Advanced Protein Assay Reagent to each well. Incubate for 1 min at room temperature. Blank spectrophotometer with 290 µL of Precision Red plus 10 µL of Lysis Buffer at 600 nm. Read absorbance of lysate samples.

**Immunofluorescence and actin cytoskeleton staining**

Invadopodia and cell bodies adhering Boyden chamber membranes were fixed in 4% paraformaldehyde in PBS for 20 min, permeabilized in 0.2% Triton X-100 and blocked in PBS containing 1% BSA for 60 min at room
temperature. Next, invadopodia and cell bodies were incubated with anti-RHOA (1:100 dilution, Cat. 10749-1-AP, ProteinTech Group) antibodies for 1 h at room temperature, and then treated with FITC-labelled secondary antibodies (Sigma-Aldrich) for 1 h at room temperature. For accounting the number of invadopodia extended, invadopodia on the lower sides of Boyden chamber membranes (3.0 μm pores) were stained with anti-Cortactin (1:100 dilution, Cat. ab81208, Abcam) for 1 h at room temperature, and then treated with FITC-labelled secondary antibodies (Sigma-Aldrich) for 1 h at room temperature. For actin cytoskeleton staining assay, cells were grown on glass slides and stained with TRITC-labeled phalloidin (5 μg/mL, Sigma-Aldrich) or anti-myosin II (1:200 dilution, Cat. 10609-1-AP, ProteinTech Group) for 40 min at room temperature, and then treated with FITC-labelled secondary antibodies (Sigma-Aldrich) for 1 h at room temperature. After washing with PBS, the membranes were mounted on glass slides with DAPI Fluoromount G (Southern Biotech, Birmingham, AL). The images were acquired with a fluorescence microscope (Zeiss LSM710, Oberkochen, Germany). To assess invadopodia extension, images were acquired in Z-axis for fluorescence scan. The fluorescence intensity and assembled Z-axis images were analyzed and merged by ZEN software (Zeiss).

Statistical analysis
All statistical analyses were done using SPSS 23.0 software (Chicago, IL). Most of the data were analyzed by one-way ANOVA or Student’s t-test. Scatter plot charts show scatter plots and means±SD of six independent experiments if not noted. For all analyses, a two-sided p value of less than 0.05 was deemed statistically significant.

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Conflict of Interest

The authors declare no conflicts of interest with the contents of this article.

Author Contributions

T. Y., A. Z., F. S., F. C., and J. M. performed experiments. T. Y. and F. S. conducted the statistical analysis. Y. L. and Y. Z. conceived and planned the experiments and interpreted data. Y. Z. wrote the manuscript.
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Figure Legends

Figure 1. DAAM1 activation is required for collagen-induced cell haptotaxis.
(A, B, C, and D) Type IV collagen triggered haptotaxis of MDA-MB-231 and MDA-MB-453 breast cancer cells. MDA-MB-231, MDA-MB-453, and MCF10A cells were examined for cell haptotaxis for 8 h in 8.0-µm porous Boyden chamber membranes coated with vehicle (Uncoated) or type IV collagen on upper sides (Upper), both sides (Both), or lower sides (Lower). Migratory cells on the lower-side of the membrane were counted per field of microscope. Bar = 50 µm. Objective lens, magnification=×20, numerical aperture=0.75. (E, F, and G) DAAM1 activation was significantly elevated by type IV collagen coated on the lower sides of Boyden chamber membranes. MDA-MB-231 or MDA-MB-453 cells were seeded on Boyden chamber membranes coated with vehicle (Uncoated), or type IV collagen on upper sides (Upper), both sides (Both), or lower sides (Lower). MDA-MB-231 or MDA-MB-453 cells were allowed to migrate toward collagen for 4 h. Celluar lysates were assayed for the active DAAM1 by pulldown assay using a GST-RHOA as a bait. (H) Efficiency of gene knockdown was analyzed by immunoblotting for DAAM1. MDA-MB-231 and MDA-MB-453 cells were transfected with scrambled or DAAM1 shRNAs (#1 and #2). Total protein extracts from MDA-MB-231 and MDA-MB-453 cells transfected with scrambled or DAAM1 shRNAs were analyzed by immunoblotting for DAAM1. (I, J) Knockdown of DAAM1 significantly inhibited cell haptotaxis induced by collagen coated on the lower sides of Boyden chamber membranes. MDA-MB-231 and MDA-MB-453 cells transfected with DAAM1-shRNA or scrambled shRNAs were allowed to migrate for 8 h and the migration rate was determined by Boyden chamber assays. (K, P) The expression of empty vector and HA-tagged N-DAAM1 (N-terminal of DAAM1) was verified using total protein from cells and immunoblotted using anti-HA antibody. The expression level of DAAM1 was tested using immunoblotting and proven the efficiency of N-DAAM1 expression. (L, M) The expression of N-DAAM1 significantly inhibited cell haptotaxis induced by collagen coated on the lower sides of Boyden chamber membranes. MDA-MB-231 and MDA-MB-453 cells transfected with N-DAAM1 were allowed to migrate for 8 h and the number of migratory cells was counted per field of microscope. (N, P) The expression of empty vector and HA-tagged C-DAAM1 (C-terminal of DAAM1) was verified using total protein from cells and immunoblotted using anti-HA antibody. The expression level of DAAM1 was tested using immunoblotting and proved the efficiency of C-DAAM1 expression. (O) MDA-MB-231 cells transfected with C-DAAM1 were seeded on Boyden chamber membranes and allowed to migrate for 8 h and the number of migratory cells was counted per field of microscope.

Figure 2. DAAM1 is associated with integrin β3 and acts as a downstream target of integrin receptor.
(A) Integrin inhibitors blocked type IV collagen-induced haptotaxis of breast cancer
cells. MDA-MB-231 and/or MDA-MB-453 cells, treated with cilengitide trifluoroacetate (an integrin inhibitor for αvβ3 receptor and αvβ5 receptor, 10 nmol/L) or cyclo(-RGDfK) (an αvβ3 integrin inhibitor, 20 nmol/L), were examined for cell haptotaxis for 8 h in 8.0-μm porous Boyden chamber membranes coated with type IV collagen in lower sides. CT, cilengitide trifluoroacetate. Cy, cyclo(-RGDfK). (B, C) Collagen-induced DAAM1 activation was blocked by αvβ3 integrin inhibitor treatment. MDA-MB-231 or MDA-MB-453 cells were seeded on Boyden chamber membranes coated with collagen type IV in both sides (Both) or lower sides (Lower), and treated with cyclo(-RGDfK) (20 nmol/L). MDA-MB-231 or MDA-MB-453 cells were allowed to migrate toward collagen for 4 h. Cellular lysates were assayed for the active DAAM1 by pulldown assay using a GST-RHOA as a bait. Cy, cyclo(-RGDfK).

(D, E) The lysates of MDA-MB-231 or MDA-MB-453 cells were subject to immunoprecipitation with antibody to DAAM1, followed by immunoblotting with antibody to integrin β3 and DAAM1. (F) HA-tagged DAAM1 constructs were expressed in HEK-293T cells. Cell lysates were harvested and cleared, and integrin immunoprecipitated with anti-HA antibodies. Immune precipitates were analyzed by immunoblotting for anti-HA or anti-integrin β3. Whole cell lysates were used as a control. (G) Purified GST or GST fused C-terminal (amino acids 716-762) of integrin β3 was incubated with purified HA-tagged full-length DAAM1. The amounts of HA-DAAM1 co-purified with GST or GST-integrin (Pull down) were analyzed by immunoblotting.

Figure 3. RHOA is a downstream target of DAAM1 and its activation is required for collagen-induced cell haptotaxis.

(A, B, and C) RHOA, RAC1 and CDC42 activations were elevated by type IV collagen treatment. MDA-MB-231 cells were seeded on Boyden chamber membranes coated with vehicle (Uncoated), or type IV collagen on upper sides (Upper), both sides (Both), or lower sides (Lower). Cells were allowed to migrate toward collagen for 4 h. Cellular lysates were assayed for RHOA, RAC1 and CDC42 activation assays. (D) Collagen-induced RHOA activation was inhibited by DAAM1 silence. Stable DAAM1 knockdown MDA-MB-231 cells (DAAM1-shRNA #1 and #2) and control cells were seeded on Boyden chamber membranes coated with collagen type IV on the lower sides. Cells were allowed to migrate toward collagen for 4 h. Cellular lysates were assayed for RHOA, RAC1 and CDC42 activation assays. (E) Collagen-induced RHOA activation was inhibited by cyclo(-RGDfK) treatment. MDA-MB-231 cells were seeded on Boyden chamber membranes coated with collagen type IV on the lower sides. Cells treated with cyclo(-RGDfK) (20 nmol/L) were allowed to migrate toward collagen for 4 h. Cellular lysates were assayed for RHOA activation assays. Cy, cyclo(-RGDfK). (F, G) CCG-1423 (RHOA specific inhibitor) or RHOA-N19 (dominant-negative RHOA) inhibited collagen-induced haptotaxis of MDA-MB-231 cells. MDA-MB-231 cells treated with CCG-1423 (1 μmol/L) or RHOA-N19 overexpressing stable cells were seeded in Boyden chamber membranes coated with type IV collagen on both sides (Both) or lower sides (Lower). Cells were allowed to migrate toward collagen for 8 h. (H) CCG-1423 or RHOA-N19
inhibited collagen-induced haptotaxis of MDA-MB-453 cells. MDA-MB-453 cells treated with CCG-1423 (1 µmol/L) or transfected with RHOA-N19 were seeded in Boyden chamber membranes coated with type IV collagen on both sides (Both) or lower sides (Lower). Cells were allowed to migrate toward collagen for 8 h. (I, J) DAAM1 activation was not altered by RHOA-N19 overexpression. RHOA-N19 overexpressing stable cells were seeded on Boyden chamber membranes coated with type IV collagen on both sides (Both) or on lower sides (Lower). Cells were allowed to migrate toward collagen for 4 h. Cellular lysates were assayed for the active DAAM1 by pulldown assay using a GST-RHOA as a bait. (K) RHOA-V14 rescued the haptotaxis of N-DAAM1-expressed MDA-MB-231 cells. MDA-MB-231 cells transfected with N-DAAM1 and/or RHOA-V14 were seeded in Boyden chamber membranes coated with type IV collagen on lower sides. Cells were allowed to migrate toward collagen for 8 h.

Figure 4. DAAM1 activation is essential for collagen-induced invadopodia extension.
(A, B) Type IV collagen triggered invadopodia extension of MDA-MB-231 and MDA-MB-453 cells. MDA-MB-231 or MDA-MB-453 cells were examined for invadopodia extension for 6 h in 3.0-µm porous Boyden chamber membranes coated with vehicle (Uncoated), or type IV collagen on upper sides (Upper), both sides (Both), or lower sides (Lower). Invadopodia on the lower sides of the membrane were stained with Cortactin antibodies and counted per field of microscope. Bar=10 µm. Objective lens, magnification=×40, numerical aperture=0.95. (C) DAAM1 silence significantly inhibited collagen-induced invadopodia extension. Stable DAAM1 knockdown MDA-MB-231 cells (DAAM1-shRNA #1 and #2) or control cells were allowed to extend invadopodia for 6 h. The number of extended invadopodia was determined by 3.0-µm porous Boyden chamber assays and counted per field of microscope. (D) Cyclo(-RGDFK) suppressed collagen-induced invadopodia extension. MDA-MB-231 cells were treated with 20 nmol/L cyclo(-RGDFK) or vehicle and allowed to extend invadopodia for 6 h. The number of extended invadopodia was determined by 3.0-µm porous Boyden chamber assays and counted per field of microscope. (E) DAAM1 silence or cyclo(-RGDFK) significantly inhibited collagen-induced invadopodia extension. MDA-MB-453 cells transfected with DAAM1-shRNA or treated with cyclo(-RGDFK) were allowed to extend invadopodia for 6 h. The number of extended invadopodia was determined by 3.0-µm porous Boyden chamber assays and counted per field of microscope. Bo, collagen coated in the both sides. Lo, collagen coated in the lower sides. (F, G, and H) Abundant active DAAM1 were located in invadopodia. MDA-MB-231 or MDA-MB-453 cells were seeded on 3.0-µm porous Boyden chamber membranes coated with collagen type IV on the lower sides. The invadopodia of MDA-MB-231 or MDA-MB-453 cells were allowed to extend toward collagen for 4 h. Cell bodies and invadopodia were separated as the description in Experimental Procedures. Cellular lysates were assayed for the active DAAM1 by pulldown assay using a GST-RHOA as a bait.
Figure 5. DAAM1-dependent activation of RHOA mediates collagen-induced invadopodia extension.
(A) Blockade of RHOA inhibited collagen-induced invadopodia extension of MDA-MB-231 cells. MDA-MB-231 cells treated with CCG-1423 (RHOA specific inhibitor, 1 µmol/L) or RHOA-N19 overexpressing stable MDA-MB-231 cells were seeded in 3.0-µm porous Boyden chamber membranes coated with type IV collagen in both sides (Bo) or lower sides (Lo). Cells were allowed to extend invadopodia toward collagen for 6 h. The number of extended invadopodia was counted per field of microscope. (B) Blockade of RHOA inhibited collagen-induced invadopodia extension of MDA-MB-453 cells. MDA-MB-453 cells treated with CCG-1423 (1 µmol/L) or transfected with RHOA-N19 were seeded on 3.0-µm porous Boyden chamber membranes coated with type IV collagen in both sides (Both) or lower sides (Lower). Cells were allowed to extend invadopodia toward collagen for 6 h. The number of extended invadopodia was counted per field of microscope. (C, D, E, and F) The high expression of RHOA in invadopodia was disrupted by cyclo(-RGDfK) treatment or DAAM1 knockdown. MDA-MB-231 cells treated with cyclo(-RGDfK) (20 nmol/L) or stable DAAM1 knockdown MDA-MB-231 cells were seeded on 3.0-µm porous Boyden chamber membranes coated with type IV collagen in both sides (Both) or lower sides (Lower). Cells were allowed to extend invadopodia toward collagen for 6 h. Cy, cyclo(-RGDfK). Invadopodia on the lower sides of the membrane were stained with RHOA antibodies and counted per field of microscope. (C, D) The expression and location of RHOA in invadopodia were showed on the lower sides of 3.0-µm porous Boyden chamber membranes. The number of RHOA-positive invadopodia was counted per field of microscope. Bar=10 µm. Objective lens, magnification=×40, numerical aperture=0.95. (E, F) Z-axis scan of 3.0-µm pores in Boyden chamber membranes. The fluorescence of RHOA (green) in invadopodia (below the yellow lines) were adjusted by ZEN software and normalized to the average value of control groups. Objective lens, magnification=×63, numerical aperture=1.4. n=5. Z-distance=16 µm. Bar=1 µm. Pixel dimensions=99×66 pixel.

Figure 6. DAAM1 and RHOA participate in the rearrangement of stress fibers.
(A) DAAM1-shRNA, CCG-1423 treatment, or cyclo(-RGDfK) treatment disrupted the formation of actin stress fibers in MDA-MB-231 cells. Stable DAAM1 knockdown MDA-MB-231 cells were grown on collagen-coated coverslips. MDA-MB-231 cells grown on collagen-coated coverslips were pre-treated with 1 µmol/L CCG-1423 or 20 nmol/L cyclo(-RGDfK) for 1 h. Subsequently, cells were fixed and F-actin organization was analyzed by phalloidin staining. Bar=10 µm. Objective lens, magnification=×40, numerical aperture=0.95. (B, C) DAAM1-shRNA, CCG-1423, or cyclo(-RGDfK) disrupted the formation of stress fibers in MDA-MB-231 cells. The number of stress fibers in a cell (B) and the length of the longest microfilament (C) were determined in MDA-MB-231 cells (n=20).
Figure 1
Figure 1 continued

K

MDA-MB-231  MDA-MB-453

DAAM1

HA

beta-actin

L

Numbers of migratory cells per field of microscope

MDA-MB-231

MDA-MB-453

Ve  N  Ve  N  Ve  N  Ve  N

Uncoated  Upper  Both  Lower

p=0.63

p=0.856

p=0.001

p=0.302

p=0.303

p=0.001

M

Numbers of migratory cells per field of microscope

MDA-MB-231

MDA-MB-453

Ve  N  Ve  N  Ve  N  Ve  N

Uncoated  Upper  Both  Lower

p=0.859

p=0.882

p=0.303

p=0.302

N

MDA-MB-231

DAAM1

HA

beta-actin

O

Numbers of migratory cells per field of microscope

MDA-MB-231

Ve  C  Ve  C  Ve  C  Ve  C

Uncoated  Upper  Both  Lower

p=0.951

p=0.903

p=0.003

p=0.648

P

Full-length DAAM1 / beta-actin

MDA-MB-231

MDA-MB-453

MDA-MB-231

Ve  N  Ve  N  Ve  N  Ve  N

p=0.560

p=0.435

p=0.0014

p=0.903
Figure 3
Figure 4
Figure 5

A

MDA-MB-231

Numbers of invadopodia per field of microscope

Collagen

Bo Lo Bo Bo Bo Lo Lo

CCG-1423 RHOA-N19

p<0.001 p<0.001

B

MDA-MB-453

Numbers of invadopodia per field of microscope

Collagen

Both Lower Both Lower Both Lower Both Lower

Ctrl CCG-1423 RHOA-N19

p<0.001

C

Both Collagen on lower side

anti-RHOA

Ctrl Ctrl Cy #1 #2 DAAM1-shRNA

D

Numbers of RHOA-positive invadopodia per field of microscope

Collagen

Both Lower Lower Lower Lower

Ctrl Ctrl Cy #1 #2 DAAM1-shRNA

p<0.001 p<0.001 p<0.001

E

RHOA DAPI Merge

Collagen on both sides

Ctrl

F

Relative fluorescence of RHOA in invadopodia (below the yellow lines)

Collagen

Both Lower Lower Lower Lower

#1 #2 DAAM1-shRNA

p<0.001 p<0.001 p<0.001
Figure 6
Integrin αvβ3-associated DAAM1 is essential for collagen-induced invadopodia extension and cell haptotaxis in breast cancer cells
Ting Yan, Ailiang Zhang, Fangfang Shi, Fei Chang, Jie Mei, Yongjian Liu and Yichao Zhu

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