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Breast cancer cell migration is regulated through junctional adhesion molecule-A-mediated activation of Rap1 GTPase

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ABSTRACT

Introduction: The adhesion protein junctional adhesion molecule-A (JAM-A) regulates epithelial cell morphology and migration, and its over-expression has recently been linked with increased risk of metastasis in breast cancer patients. As cell migration is an early requirement for tumour metastasis, we sought to identify the JAM-A signalling events regulating migration in breast cancer cells.

Methods: MCF7 breast cancer cells (which express high endogenous levels of JAM-A) and primary cultures from breast cancer patients were used for this study. JAM-A was knocked down in MCF7 cells using siRNA to determine the consequences for cell adhesion, cell migration and the protein expression of various integrin subunits. As we had previously demonstrated a link between the expression of JAM-A and β1-integrin, we examined activation of the β1-integrin regulator Rap1 GTPase in response to JAM-A knockdown or functional antagonism. To test whether JAM-A, Rap1 and β1-integrin lie in a linear pathway, we tested functional inhibitors of all three proteins separately or together in migration assays. Finally we performed immunoprecipitations in MCF7 cells and primary breast cells to determine the binding partners connecting JAM-A to Rap1 activation.

Results: JAM-A knockdown in MCF7 breast cancer cells reduced adhesion to, and migration through, the β1-integrin substrate fibronectin. This was accompanied by reduced protein expression of β1-integrin and its binding partners αV- and α5-integrin. Rap1 activity was reduced in response to JAM-A knockdown or inhibition, and pharmacological inhibition of Rap1 reduced MCF7 cell migration. No additive anti-migratory effect was observed in response to

simultaneous inhibition of JAM-A, Rap1 and β1-integrin, suggesting that they lie in a linear migratory pathway. Finally, in an attempt to elucidate the binding partners putatively linking JAM-A to Rap1 activation, we have demonstrated the formation of a complex between JAM-A, AF-6 and the Rap1 activator PDZ-GEF2 in MCF7 cells and in primary cultures from breast cancer patients.

Conclusions: Our findings provide compelling evidence of a novel role for JAM-A in driving breast cancer cell migration via activation of Rap1 GTPase and β1-integrin. We speculate that JAM-A over-expression in some breast cancer patients may represent a novel therapeutic target to reduce the likelihood of metastasis.

INTRODUCTION

Breast cancer accounts for approximately 30% of all female cancers diagnosed in the European Union and is the leading cause of female cancer deaths, with over 85,000 women (many in their reproductive and economically productive years) succumbing to the disease in 2006 [1]. Although there have been substantial improvements in breast cancer treatment, targeted adjuvant therapies are restricted to treating those patients whose tumour cells express high levels of the few targetable breast cancer molecular markers; namely the estrogen and HER2 receptors. It is therefore clear that further improvements are needed in the molecular understanding, diagnosis, and treatment of breast cancer.

Most breast cancers originate in the epithelial cells lining breast ducts. Epithelial cell polarity in normal ducts is maintained via intercellular multi-protein adhesion complexes, which facilitate

adhesion and allow communication between neighbouring cells. Loss of epithelial polarity and consequent disruptions in tissue architecture, a hallmark of de-differentiation, is an early feature of breast cancer and other malignancies [2]. Emerging evidence points toward an important role for proteins of the intercellular tight junction (TJ) complex in mediating tumourigenesis. To date, several TJ proteins have been shown to be dysregulated in breast carcinoma, with claudins-3 and -4 highly up-regulated [3], and claudin-7 down-regulated in *in situ* and invasive ductal carcinomas [4]. Furthermore, loss of the tight junction-associated protein ZO-1 in breast cancer correlates both with poor prognosis [5] and increased expression of proteinases important for tumour invasion [6]. Interestingly, adhesion/polarity proteins have recently been shown to be targeted by oncogenes (such as ERBB2 [7] and MYC [8]) resulting in the disruption of tissue organisation often observed during cancer development. Together, these studies provide strong evidence that adhesion proteins may act as key regulators of breast cancer initiation and progression.

The JAM family of TJ proteins has important functions in numerous cellular adhesive processes including intercellular junction assembly and cell polarity [9], cell morphology [10], platelet activation [11], and leukocyte migration [12]. Pathophysiologically, JAM-A has been linked to various inflammatory disorders [13-15] and more recently some cancers [16, 17]. However discordance exists regarding the specific role of JAM-A in breast cancer [18, 19]. Our previous investigations into the role of JAM-A in breast cancer had analysed levels of JAM-A expression in two cohorts of patients with invasive breast cancer. We demonstrated a novel and significant association between JAM-A over-expression in breast tissue and poor prognosis for breast cancer patients [19]. Notably, patients whose tumours had high JAM-A expression levels were

significantly more likely to develop metastasis. Given that migratory capacity is crucial for tumour cell dissemination, and that JAM-A has established functions in leukocyte migration [12]; we sought to determine the contribution of JAM-A to breast cancer cell migration *in vitro*. Our study has demonstrated that antagonism or knockdown of JAM-A in MCF7 human breast cancer epithelial cells significantly decreases cell migration, likely due to a concomitant reduction in expression of the migratory protein β1-integrin [20]. β1-integrin has been strongly linked with murine breast proliferation [21] and the formation of metastases in mouse models of breast cancer [22]. Accordingly, results from ours [19] and other [23] tissue microarray (TMA) studies have shown an association between high β1-integrin expression levels and poor prognosis in breast cancer patients.

In this study, we further investigated the JAM-A signalling events which regulate β1-integrin-dependent migratory events in breast cancer cells. We demonstrated that reductions in cell migration following JAM-A functional antagonism or siRNA-mediated gene knockdown are exerted via downstream effects on the activity of RapGTPase1, a known activator of β1-integrins [24] and a regulator of breast tumourigenesis [25]. Furthermore we present data suggesting that JAM-A may activate Rap1 indirectly through associations with downstream signalling proteins AF-6 and the guanine exchange factor, PDZ-GEF2. Finally, we demonstrate that the JAM-A signalling complexes identified *in vitro* in MCF7 cells also exist in breast cancer patient tissues, using *ex vivo* immunoprecipitation strategies in primary cultures generated from breast cancer patients.

Taking together the regulatory influence of JAM-A on breast cancer cell migration *in vitro* and its link with metastasis in patient tissue samples [19], our studies suggest a role for JAM-A as a potentially-important therapeutic target for the development of future breast cancer therapies.

MATERIALS & METHODS

Cell culture

The MCF7 breast cancer cell line was obtained from the European Collection of Cell Cultures (ECACC) (Sigma-Aldrich, Poole, UK) and maintained in MEM (Sigma-Aldrich, Poole, UK) supplemented with 10% foetal bovine serum (FBS) (Lonza, Basel, Switzerland), 2mM L-glutamine, 50U/ml penicillin, 50µg/ml streptomycin (Invitrogen, Ca, USA) and 1 % non-essential amino acids (NEAA) (Sigma-Aldrich, Poole, UK). Cells were maintained at 37°C in humidified air with 5% CO_2.

Primary culture generation

Human breast tissue samples from lumpectomy or mastectomy patients, gathered with informed consent in protocols approved by the Beaumont Hospital Medical Ethics (Research) committee, were used to generate mammary epithelial primary cell cultures with minor modifications from published methods [26]. In brief, tissue biopsies from within the tumour (T) and from histopathologically-normal non-tumour margins (N) were incubated in penicillin/streptomycin/neomycin (Invitrogen, CA, USA), minced in DMEM/F12 (Sigma-Aldrich, Poole, UK) containing penicillin/streptomycin/neomycin, 10% FBS, 10µg/ml insulin, 5µg/ml fungizone, 100U/ml hyaluronidase 1-S, 200U/ml collagenase (Sigma-Aldrich, Poole,

UK), and agitated for 2 hr at 37°C. Cells were pelleted and washed before being cultured in mammary epithelial growth medium (MEGM; Lonza, Wokingham, UK) at 37ºC with 5% CO_2. Trypsin/EDTA and soybean trypsin inhibitor were used to subculture confluent flasks. Cells were harvested after 1 passage to generate enough material for immunoprecipitation assays, and 1-2 passages for protein isolation. Only patient-matched primary cultures were used (see Table 1).

siRNA-mediated gene-expression knockdown

JAM-A siRNAs were transfected into MCF7 breast cancer cells using the N-TER nanoparticle siRNA transfection system (Sigma-Aldrich, Poole, UK). In brief, cells (1.5 X 10^6 cells/10cm dish) were seeded 16 hr prior to transfection. Cells were washed in PBS, transfected with nanoparticles containing 75nM final concentration of JAM-A siRNAs, negative control siRNA, or mock (NTER reagent only) in MEM media. Assays were performed after transfection for 48hr at 37ºC with 5% CO_2. Two alternative JAM-A siRNAs were used for all experiments. Representative results from a single siRNA are shown for clarity.

Antibodies/inhibitors

All antibodies used for western blot and immunofluorescence analyses were obtained from commercial sources: rabbit polyclonal anti-JAM-A (Zymed Laboratories Inc., Invitrogen, CA, USA), MAb13 rat anti-β1 integrin (BD Biosciences, NJ, USA), rabbit anti-β2 rabbit, anti- β3, rabbit anti- β4, rabbit anti- β5, rabbit anti-αV, rabbit anti-α4, rabbit anti-α5 integrins (Cell Signalling Technologies, MA, USA), rabbit anti-actin (Abcam, Cambridge, UK), rabbit anti-Rap1 (Millipore, MA, USA), rabbit anti-AF-6 (Zymed Laboratories Inc., Invitrogen, CA, USA),

mouse anti-PDZGEF2 (Santa Cruz, CA, USA), and mouse J.10.4 inhibitory anti-JAM-A (Santa Cruz, CA, USA). HRP-conjugated secondary antibodies: anti-mouse and anti-rat (Sigma-Aldrich, Poole, UK), and anti-rabbit (Cell Signalling Technologies, MA, USA). IgG isotype control antibodies: mouse, rabbit and rat (Sigma-Aldrich, Poole, UK). Rap1 pharmacological inhibitor: GGTI-298 (Calbiochem/Merck, Darmstadt, Germany).

Adhesion assay

Cell adhesion strips coated with fibronectin (which binds αVβ1 and α5β1 integrins) or BSA-control substrate (Millipore MA, USA) were rehydrated in 96-well plates with PBS for 30 min at room temperature. Cells (1 X 10^5/well) were added and incubated for 2 hr at 37°C and 5% CO_2, then washed in PBS, stained with 0.2% crystal violet (Sigma-Aldrich, Poole, UK) in 10% ethanol for 5 min at room temperature and rinsed in PBS. Cell-bound stain was solubilised by gently shaking with solubilisation buffer (1:1, 0.1M NaH_2PO_4:50% ethanol) for 5 min. Absorbance was measured at 560nm on a microplate reader. Results from three independent experiments with three replicates per experiment were pooled.

Migration assays

Transwell migration assays were conducted on MCF7 cells following transient JAM-A gene expression knockdown. 8μM pore Transwell chambers were coated with 10μg/ml fibronectin (Sigma-Aldrich, Poole, UK) overnight at 4°C, washed in PBS, and rehydrated with serum-free media for 30 min at 37°C. Media was removed and cells (1 X 10^5 /chamber) were added to upper chambers with 15% FBS in lower chambers as a chemoattractant. Chambers were incubated for 3hr at 37°C and 5% CO_2. Migrated cells on the underside of the filter were fixed in 10% ethanol

for 20min prior to staining with DAPI (Sigma-Aldrich, Poole, UK) for 10min at room temperature. Membranes were mounted on glass slides and cells enumerated using Cell B software (Olympus) to analyze multiple fluorescent micrographs. Results from three independent experiments with three replicates per experiment were pooled.

Scratch wounding migration assays were conducted on wild-type MCF7 cells after treatment with inhibitors. Confluent cell monolayers in 24-well plates were pre-incubated for 2hr with either 5μg/ml mouse anti-JAM-A J.10.4 antibody, 5μg/ml MAb13 anti-β1 integrin antibody, 10μM Rap1 inhibitor GGTI-298, appropriate isotype-control IgG or dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Poole, UK) vehicle control. A scratch-wound was made using a pipette tip. Media was replaced and wounds were photographed at time 0, 2, and 6hr. Scion Image software (Scion Corporation Ltd., USA) was used to measure closure of the wound over time by averaging 6 individual measurements of wound size for each wound at each timepoint. Results from three independent experiments with three replicates per experiment were pooled.

Rap1 activity assay

Adherent cells were washed in tris-buffered saline then scraped and dounced in 1ml Rap1 activation lysis buffer containing 50mM Tris-HCl, 0.5M NaCl, 1% NP40, 2.5mM $MgCl_2$ and 15% glycerol (Millipore, MA, USA) and 1% protease and phosphatase inhibitors (Sigma-Aldrich, Poole, UK). Lysates were incubated with 30μg Ral GDS-RBD agarose slurry (Millipore, MA, USA) for 45min at 4°C. Beads were pelleted and washed in lysis buffer. Bound proteins were recovered by boiling at 95°C for 5min in 40μl 2X sample buffer. Each immunoblot depicted is representative of three independent experiments, with densitometry conducted on

triplicate experiments. Densitometric data was used to determine the average active : total Rap1 protein expression over triplicate experiments.

Western blotting

Cultured cells were washed in 10ml PBS, scraped and dounced in Relax lysis buffer containing 100mM KCl, 3mM NaCl, 3.5mM $MgCl_2$, 10mM HEPES pH 7.4 and 1% Triton-X100, as well as protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Poole, UK). Protein samples were separated by SDS-PAGE under reducing conditions using tris-glycine running buffer. After electrophoresis, proteins were transferred to nitrocellulose membranes, (Optitran-Sigma-Aldrich, Poole, UK). Membranes were blocked in 5% milk for 1hr. Protein expression was detected using primary antibodies incubated overnight at 4°C. Membranes were washed and incubated for 1hr with horseradish peroxidase (HRP)-conjugated secondary antibodies. Antigen-antibody complexes were detected using Western Lightning Enhanced Chemiluminescence reagent (Perkin-Elmer, MA, USA). Each immunoblot depicted is representative of three independent experiments, with densitometry conducted on triplicate experiments.

Immunoprecipitation

Protein extraction was conducted as above. Equal protein concentrations from control siRNA, JAM-A siRNA and mock-transfected cells were subjected to JAM-A immunoprecipitation (IP). Pre-clear was conducted via rotation of protein lysates for 1hr at 4°C with protein G-sepharose beads (GE Healthcare, CT, USA). IP antibodies (J.104 anti-JAM-A antibody or isotype control mouse IgG; 4μg/ml) were rotated for 1hr at 4°C. Bound antibody was retrieved by rotation with proteinG-sepharose beads for 3hr at 4°C. Beads were washed in Relax lysis buffer and bound

proteins were recovered by boiling at 95°C for 5min in 40µl 2X sample buffer. Western blotting was performed as above for JAM-A, AF-6, PDZ-GEF2, Rap1 and β1-integrin. Each IP and associated immunoblot (IB) was conducted three times for cell lines and once each per primary culture.

Statistical Analysis

Averaged data from triplicate adhesion, transwell migration, and western blot experiments were used to generate bar graphs depicting average values + standard deviation (SD), with paired student's t-tests used to measure significance. For scratch-wound migration assays ± inhibitors, linear regression analysis was used to calculate any differences between treatments and controls.

RESULTS

JAM-A knockdown decreases integrin-mediated cancer cell adhesion and migration

Our previous study reported that high JAM-A expression levels in breast tissues from patients with invasive breast cancer correlated with reduced metastasis-free survival [19]. We and others have demonstrated that knockdown of JAM-A gene-expression in epithelial cells results in decreased collective cell migration, likely due to reductions in β1-integrin protein [19, 27, 28]; (Supplementary Figure S1A in Additional file 1). Indeed, integrins perform key roles at several key steps required for cell migration: adhesion assembly, disassembly and turnover [29, 30]. Taken together, our results to date suggest a role for JAM-A in promoting breast cancer cell migration through a β1-integrin dependent pathway. To investigate this hypothesis, we utilised the MCF7 breast cancer cell line which expresses high endogenous levels of JAM-A and β1-

integrin. JAM-A protein levels were knocked down in these cells using a nanoparticle delivery system to transiently transfect cells with short interfering (si)-RNA targeting JAM-A. Transiently-tranfected cells were analyzed after 48hrs for JAM-A knockdown at protein level. JAM-A siRNA transfected cells displayed a 90% reduction in JAM-A protein expression (Figure 1A). Similar effects were observed with a second JAM-A siRNA construct (Supplementary Figure S1B in Additional file 1).

To investigate the link between JAM-A, β1-integrin and cell migration, the ability of transfected cells to adhere to and migrate across a fibronectin substrate was analyzed (Figure 1B, 1C). Fibronectin binds multiple β1-integrin proteins through an RGD domain [31] and thus was used to facilitate analysis of the effects of JAM-A knockdown. Firstly, transfected cells were allowed to adhere to fibronectin-coated transwell supports prior to cell staining and absorbance measurement. JAM-A knockdown cells showed an approximate 40% reduction in adhesion to fibronectin (Figure 1B; $p = 0.02$) but not to control transwells coated with BSA (data not shown). Next, transfected cells were allowed to migrate across fibronectin-coated supports with 8μm pores prior to fluorescent staining and enumeration. Here, in addition to the reduction in collective cell migration demonstrated in previous reports [19, 27, 28], JAM-A knockdown cells showed an approximate 50% reduction in individual cell motility in these matrix-specific Transwell migration assays (Figure 1C; $p = 0.01$); further underlining the important role of JAM-A in the regulation of breast cancer cell migration. However JAM-A antagonism did not reduce invasion of MCF7 cells across Matrigel-coated filters in classic invasion assays (Supplementary Figure S2C in Additional file 2), owing to the fact that MCF7 cells are virtually non-invasive in this model (Supplementary Figure S2A in Additional file 2).

JAM-A regulates β1-integrins and Rap1GTPase in breast cancer cells

We and others have previously reported that JAM-A knockdown induces a concomitant reduction in β1-integrin in epithelial cells [10, 19, 27]. To probe whether this was a specific effect of JAM-A knockdown on β1-integrin in breast cancer cells, the expression of several integrin subunit proteins was analysed following JAM-A knockdown in MCF7 breast cancer cells. Total protein isolates were prepared from MCF7 cells transfected with either JAM-A siRNA or control siRNA, and the protein expression levels of a panel of alpha- and beta- subunit integrins assessed by western blot analysis (Figure 2A and 2B). As previously stated, JAM-A siRNA transfected achieved a >90% reduction in JAM-A protein expression compared to control siRNA transfection. No change in the expression of β3-, β4-, or β5-integrins was observed upon JAM-A knockdown as evidenced by densitometric analysis of triplicate immunoblots (Figure 2B). Expression of α4-integrin was not detected in MCF7 cells. Similar to previous studies [10, 19, 27], a 50% reduction in the expression of β1-integrin was observed upon transient JAM-A knockdown. Furthermore, reductions of approximately 30% and 60% were observed for protein expression levels of αV and α5-integrin subunits respectively, both of which have been widely reported to complex with β1-integrin to form functioning fibronectin receptors [32]. These results further suggest that JAM-A exerts specific effects on β1-integrin heterodimers.

We next sought to investigate the signalling events connecting JAM-A engagement at the cell-cell interface to β1-integrin function at the cell matrix interface. Given the spatial separation of these proteins, we hypothesised that their physical association was unlikely. To test this, JAM-A immunoprecipitations (IP) were conducted on protein isolates from MCF7 cells transfected with

JAM-A or control siRNA (Figure 2C). A reduction of β1-integrin total protein expression was confirmed in input samples of equal protein concentration. However, no bands were observed in JAM-A immunoprecipitated lanes immunoblotted for β1-integrin, confirming an absence of physical association between JAM-A and β1-integrin. We therefore concluded that other signalling proteins must be responsible for linking β1-integrin function to JAM-A.

One candidate protein, the integrin activator Rap1GTPase [33], has been shown to regulate tissue polarity, lumen formation, and invasive potential in human breast epithelial cells [25]. We therefore investigated if JAM-A knockdown using siRNA or functional inhibition using JAM-A inhibitory antibody altered Rap1 expression and activity (Figure 2D). Western blot analyses of MCF7 breast cancer cells showed a marginal decrease in total protein expression of Rap1 following JAM-A knockdown. However, a near abolition of active (GTP-bound) Rap1 was observed in JAM-A knockdown cells. In an effort to further confirm the regulation of Rap1 activation status by JAM-A, MCF7 cells were treated with an inhibitory antibody to JAM-A (J10.4). Western blot analysis demonstrated negligible changes in total Rap1 expression but dramatic reductions in active Rap1 following JAM-A inhibition. Indeed reductions of 50% ($p < 0.01$) and 80% ($p < 0.02$) were observed for expression ratios of active Rap1 to total Rap1 following JAM-A knockdown and inhibition respectively (Figure 2E), strongly indicating that JAM-A regulates downstream activation of Rap1. However it is notable that the *ratios* of active/total Rap1 were not equivalent between knockdown and inhibition conditions, because JAM-A knockdown (but not inhibition) induced a reduction in the *total* levels of Rap1 protein.

Inhibition of putative JAM-A signalling proteins reduces breast cancer cell migration

Our results thus far suggested the existence of a JAM-A signalling pathway in MCF7 breast cancer cells; initiated by JAM-A signalling via Rap1 and β1-integrin and culminating in cancer cell migration. We next reasoned that if this pathway was linear, inhibition of any step should elicit inhibitory effects on cell migration similar to those induced upon inhibition of JAM-A alone. To investigate this hypothesis, we first treated MCF7 cells with the Rap1 pharmacological inhibitor GGTI-298, and verified that active Rap1 is reduced following treatment (Figure 3A). GGTI-298 also significantly reduced MCF7 cancer cell migration in scratch wound assays (Figure 3B; $p = 0.015$). .Next we demonstrated that treatment of MCF7 cells with an inhibitory antibody targeting β1-integrin elicited a similar reduction in MCF7 cell migration, from approximately 32% wound closure to 18% wound closure at 6 hours (Figure 3C; $p = 0.001$). This was mirrored upon JAM-A inhibition, where cell migration was observed to decrease from approximately 32% to 15% wound closure after 6 hours (Figure 3D; $p < 0.0001$). Combined treatment of MCF7 cells with inhibitors of Rap1, β1-integrin and JAM-A resulted in a decrease in cancer cell migration from approximately 35% to 18% wound closure after 6 hours (Figure 3E; $p < 0.0001$). When the migratory differences between treatments and controls were quantitatively compared, no additive effects were observed in response to inhibitor combinations versus single inhibitors alone (Figure 3F). This indicated that JAM-A, Rap1, and β1-integrin are likely to function together in a linear signalling pathway in breast cancer cells.

JAM-A co-associates with AF-6 and PDZ-GEF2

Using immunoprecipitation strategies, we next sought to determine the JAM-A associations and signalling events which may affect downstream Rap1 activation. Total protein was extracted from MCF7 cells transfected with control siRNA or JAM-A siRNA; and JAM-A protein binding

partners were co-immunoprecipitated using an anti-JAM-A antibody (Figure 4). We first tested for the presence of Rap1 in JAM-A immunoprecipitates, but did not detect any co-association. We therefore reasoned that intermediate signalling proteins must link JAM-A to the downstream activation of Rap1. Knockdown of the Rap1-associating protein [34] AF-6 (afadin) had been previously shown to result in reductions in active Rap1 and colorectal cancer cell migration [27]. We therefore investigated if JAM-A co-associated with AF-6 in MC7F breast cancer cells. JAM-A immunoprecipitates from MCF7 protein lysates were immunoblotted with AF-6, and co-association between a pool of JAM-A and AF-6 was detected (Figure 4). Similar effects were observed using a second, independent JAM-A siRNA construct (Supplementary Figure S1B in Additional file 1). In addition to AF-6, previous studies had demonstrated a role for the Rap1 activator PDZ-GEF2 in both lung cancer cell adhesion [35] and colorectal carcinoma cell migration [27]. Similarly, JAM-A immunoprecipitates immunoblotted for PDZ-GEF2 confirmed co-association between a pool of JAM-A and PDZ-GEF2 in MCF7 breast cancer cells (Figure 4).

JAM-A signalling complexes are altered in human breast cancer patient tissues

Our accumulating results suggested a breast cancer signalling pathway where JAM-A associates with AF-6 and PDZ-GEF2 to activate Rap1 and regulate β1-integrin-mediated cell migration. To determine the *ex vivo* relevance of this putative JAM-A signalling pathway, we sought to verify our results in primary cultures isolated from multiple human breast cancer patient tissues (Table 1). Primary cultures were generated from three patient-matched tumour (1T, 2T, 3T) and non-tumour (1N, 2N, 3N) tissues, and total protein extracted for investigation of JAM-A co-associations. JAM-A immunoprecipitation was conducted on all samples followed by immunoblotting for JAM-A, AF-6, PDZ-GEF2, total Rap1, and β1-integrin (Figure 5A).

Analysis of total protein levels demonstrated a small increase in JAM-A expression in patient tumour cultures relative to non-tumour cultures (Figure 5A input and Supplementary Figure S3 in Additional file 3) Negligible differences in protein expression between tumour and non-tumour cultures were observed for AF-6, Rap1 and β1-integrin (Figure 5A input and Supplementary Figure S3 in Additional file 3). Notably, however, after densitometric pooling tumour cultures displayed a reduced total protein expression of PDZ-GEF2 compared to non-tumour cultures (Supplementary Figure S3 in Additional file 3). Analysis of JAM-A immunoprecipitates demonstrated co-association between JAM-A and a pool of AF-6 and PDZ-GEF2 but not Rap1 or β1-integrin (Figure 5A); mirroring data acquired from MCF7 breast cancer cells (Figure 4). Pooled densitometric analysis of tumour:non-tumour ratios following immunoprecipitation experiments revealed a trend toward increased association of both AF-6 and PDZ-GEF2 with JAM-A, suggesting enhanced formation of a JAM-A/AF-6/PDZ-GEF2 signalling complex in tumour cells (Supplementary Figure S3 in Additional file 3).

Together, our findings provide compelling evidence of a novel role for the cell-cell adhesion protein, JAM-A, in influencing breast cancer cell migration at the cell-matrix interface via regulation of Rap1 and β1-integrin.

DISCUSSION

Although recent improvements in breast cancer treatments have resulted in average ten year survival rates of ~72% [36], improved therapeutic strategies are required to target metastasis, the leading cause of death in breast cancer patients. Loss of cell polarity is an early indicator of carcinoma progression [2]. To date several proteins in cell polarity complexes [7, 37] and cell

junctional complexes[9-12] [38, 39], have been implicated in breast cancer [40]. Our previous studies demonstrated a novel and significant association between mammary over-expression of the TJ protein JAM-A and poor prognosis of breast cancer patients [19]. This was initially surprising in light of another study reporting that *loss* of JAM-A promoted invasive behaviour in breast cancer cell lines [18, 19]. However JAM-A likely plays a complex temporal role in cancer. Low JAM-A expression could potentially reduce adhesion and facilitate detachment of cells from early-stage tumours, while signalling events arising from high JAM-A expression may later on promote the migratory events associated with tumour invasion and metastasis. Accordingly, we demonstrated a link between JAM-A and β1-integrin protein expression, and a regulatory influence of JAM-A on breast cancer cell migration *in vitro* [19]. The aim of the current study was to further elucidate the mechanisms whereby JAM-A influences cancer cell migration, in an attempt to explain the increased metastatic events observed in breast cancer patients whose tumours express high levels of JAM-A.

As presented in this manuscript, our evidence is consistent with a pathway whereby JAM-A regulates β1-integrin-mediated breast cancer cell migration *in vitro* via alterations in key signalling proteins downstream of JAM-A. We have also verified that key JAM-related signalling complexes exist *ex vivo* in primary cultures isolated from human breast cancer patient tissues, supporting the clinical relevance of our studies.

We first demonstrated that knockdown of JAM-A protein expression in MCF7 breast cancer cells significantly reduced breast cancer cell adhesion and migration. These results are not surprising, given the well-established functions of JAM-A in promoting epithelial cell spreading

[10] and leukocyte migration [12]. However it is important to note that JAM-A knockdown or antagonism did not *abolish* cell migration, indicating that this is not the sole regulatory pathway controlling a process as fundamental as cell migration.

As the integrin family of proteins are crucial regulators of both cell adhesion and cell migration [41], we probed a putative cross-regulation between JAM-A and integrins in breast cancer by measuring the expression levels of several alpha and beta subunit integrins in JAM-A knockdown MCF7 cells. Transient JAM-A knockdown specifically reduced the expression of β1-integrin and its alpha subunit binding partners αV and α5. Integrin knockdown was predictably not absolute, since integrins form the terminal step of migratory signalling cascades from multiple upstream regulators [42, 43]. Nonetheless, our results suggested that JAM-A specifically affected β1-integrin heterodimers. Furthermore, previous studies had widely validated both αVβ1 and α5β1 integrin as functioning fibronectin receptors; thereby influencing our choices of substrate for migration and adhesion experiments [32]. Other evidence has shown that both αvβ1 and α5β1 integrins are RGD-binding integrins which recognise specific ligands containing an RGD tripeptide active site [32]. RGD-binding integrins bind to several ligands (both extracellular matrix and soluble), suggesting mechanisms to explain the influence of integrins on diverse cellular processes. Our results thus built upon studies in intestinal epithelial cells [10, 27] to show that JAM-A may regulate β1-integrin-mediated migratory processes in breast cancer cells. This is consistent with our previous work suggesting a correlation between protein over-expression of JAM-A and β1-integrin in breast cancer both *in vivo* and *in vitro* [19]. Interestingly, the related family member JAM-C has also been shown to exert regulatory

influence over β1-integrin activation and cell adhesion/migration via a motif in the JAM-C cytoplasmic tail [44].

β1-integrin has long been implicated in breast morphogenesis [45] and malignancy [46] via its regulatory influence on processes such as growth, apoptosis, migration and invasion [20, 47]. Indeed, inhibition of β1-integrin in 3-dimensional breast cell cultures *in vitro* has been shown to phenotypically revert malignant cell aggregates to structures resembling normal breast acini *in vivo* [48]. Expression of β1-integrin in human breast cancer has been associated with poor patient survival [23] and resistance to both radiotherapy [49] and the adjuvant chemotherapy, Trastuzumab (Herceptin) [50]. Given the pro-tumourigenic cellular processes governed by β1-integrin signalling, and the effect of JAM-A expression on β1-integrin protein expression, it was logical to hypothesize that JAM-A may directly regulate downstream β1-integrin-mediated processes.

To test this, we sought to determine if JAM-A signalling culminates in β1-integrin activation in breast cancer cells. Our immunoprecipitation results showed no direct physical co-association between these two proteins. However, similar to studies in colonic epithelial cells [27], we found that JAM-A knockdown or inhibition in breast cancer cells significantly reduced the activity of Rap1GTPase, a known activator of β1-integrins [24] and a regulator of cellular adhesion [51]. Some residual Rap1 activity was observed even in JAM-A knockdown or -antagonised cells, consistent with the fact that there are multiple known upstream regulators of Rap1 function [51].

Rap1 GTPase protein activity is controlled by Rap guanine nucleotide exchange factors (GEFs) and Rap GTPase activating proteins (GAPs), which regulate guanine nucleotide exchange and the thus activity status of the protein [51]. Interestingly, increased Rap1 activity has been implicated in several cancers including thyroid [52] and prostate [53]. Down-regulation of Rap1GAP, a negative regulator of Rap1 activity, has been demonstrated in both pancreatic [54] and thyroid cancer [52]. Furthermore, activation of Rap1 in prostate cancer cells increases cell migration and invasion *in vitro*; and introduction of activated Rap1 in a xenograft prostate cancer mouse model has been reported to enhance metastasis [53]. To date, only one study (utilising mouse xenograft models and 3D cell culture models) has been published detailing the role of Rap1 in breast cancer [25]. This study demonstrated that Rap1 is a regulator of breast architecture, with normal levels of activation maintaining polarity during morphogenesis, and increased activation inducing tumour formation and breast cancer progression *in vivo*. Although several studies have investigated Rap activity via RapGAP loss or RapGEF gain, to our knowledge none have focussed on the role of possible upstream effector proteins such as JAM-A in breast cancer. It has however been shown independently that both JAM-A [55] and Rap1 [56] proteins are required for FGF-induced angiogenesis *in vitro*. Our results provide further evidence that Rap1 may represent an important downstream effector of JAM-A in the development and progression of breast cancer. That JAM-A, Rap1 and β1-integrin exist in a linear pathway was supported by evidence from migration assays in which combined pharmacological inhibition of all three proteins failed to exert additive effects relative to single inhibition of any one protein.

To further probe the association of JAM-A and Rap1 in breast cancer, immunoprecipitation experiments were conducted to identify direct binding partners that might physically link both proteins. As in previous studies [27], we were unable to detect direct co-association between JAM-A and Rap1. However, JAM-A co-associations were detected with both the adhesion protein AF-6 and the RapGEF, PDZ-GEF2. AF-6 largely functions to connect membrane-associated proteins to the actin cytoskeleton [34], and is known to recruit and bind JAM-A at intercellular junctions via a PDZ domain [57]. AF-6 has also been suggested to be a Rap effector [58], and AF6 knockdown in colonic epithelial cells reportedly decreases Rap1 activity and cell migration in vitro [27]. However, knockdown of AF-6 in T cells has been shown to enhance Rap1-induced integrin-mediated cell adhesion [34]. These contrasting roles for AF-6 are perhaps not surprising when one considers that maintenance of cell adhesion is crucial for epithelial cell polarity; while maintenance of a non-adherent state is favoured for resting T-cell function. However, possible mechanisms accounting for this duality are still as yet unknown. Little is known regarding the role of AF-6 in breast cancer, with only a single study suggesting AF-6 as a potential tumour suppressor due to an observed correlation between AF-6 loss and poor patient prognosis [59].

As mentioned above, GEF proteins such as PDZ-GEF2 are crucial activators of Rap1 GTPase [51]. Indeed, studies in colonic epithelial cells have demonstrated that knockdown of PDZ-GEF2 simultaneously reduces β1 integrin protein expression and cell migration [27]. Although there is no data regarding PDZ-GEF2 expression or dysregulation in breast cancer; it has been identified as an upstream activator of Rap1 required for the maturation of adherens junctions in lung carcinoma cells [35]. Given that dynamic adhesion changes are involved in migration and

invasion, it is therefore plausible that PDZ-GEF2 may also regulate these processes which are critical for cancer progression.

In this study, we have presented results indicating a role for JAM-A in the regulation of β1-integrin-mediated migratory processes in breast cancer cells. Our data suggest a linear signalling pathway whereby JAM-A engagement leads to activation of Rap1 via PDZ-GEF2 and AF-6, and culminates in β1-integrin-induced cell migration. These results are similar to those from studies using colonic epithelial cells [27], indicating that this JAM-A signalling pathway may be conserved in several cell types. However site-specific expression of JAM-A in mouse endothelial cells has been reported to prevent spontaneous motility *in vivo* [60], illustrating that the *in vivo* role of JAM-A in regulating migration is complex and spatially-dependent. To exclude the possibility of an artefactual JAM-Rap-β1-integrin pathway in MCF7 cells, however, we have also verified our results in primary breast cell cultures isolated from human breast cancer patient tissues. Our data revealed an increase in JAM-A protein expression in patient tumour cultures compared to non-tumour cultures, corroborating our previous study observing JAM-A over-expression in invasive breast cancer tissue microarrays [19]. Patient primary cultures also showed physical co-associations between JAM-A AF-6 and PDZ-GEF2, but not with Rap1 or β1-integrin. This mirrors *in vitro* cell line data from us and others [27], and further supports the possibility that JAM-A drives a pro-migratory pathway.

Intriguingly, patient tumour primary cultures displayed a trend towards increased co-association of JAM-A with both AF-6 and PDZ-GEF2 compared to that in non-tumour cultures. This suggests the potential for increased signalling via an AF-6/PDZ-GEF2 pathway downstream of

JAM-A overexpression in tumour cells. We speculate that this could lead to hyper-activation of Rap1, and consequent hyper-activation of β1-integrin (Figure 6). However, given the practical limitations of primary cultures (including a finite lifespan, slower growth and thus lower cell numbers than in immortalized cultures), it was not feasible to conduct functional assays investigating integrin-mediated cell migration or invasion secondary to JAM-A protein manipulation. Future work (including animal models) will shed further light upon the mechanistic pathways involved in JAM-A signalling *in vivo*. Nonetheless, our current study has presented a model of JAM-A signalling in breast cancer cells which may help to explain the increase in metastatic events observed in breast cancer patients over-expressing JAM-A [19].

CONCLUSIONS

Our findings provide compelling evidence of a novel role for the cell-cell adhesion protein JAM-A in influencing breast cancer cell migration. We have shown that JAM-A signals via Rap1 and β1-integrin proteins; both of which are crucial for cell adhesion, migration and invasion. Furthermore, our *in vitro* data from cell lines and patient primary cultures, when taken in context with our previous study linking JAM-A and breast cancer, suggest that JAM-A signalling may facilitate metastatic spread. In fact a recent manuscript has identified a micro-RNA (miR-145) whose over-expression reduces invasive and motile behaviour in breast cancer cells by targeting JAM-A for downregulation [61]. We propose that JAM-A mediated "hijacking" of adhesive and migratory functions may represent a new therapeutic target for the development of anti-migratory cancer therapies.

ABBREVIATIONS

BSA, Bovine Serum Albumin; DAPI, 4,6-diamidino-2-phenylindole; DCIS, Ductal Carcinoma In Situ; DMEM, Dulbecco's Modified Eagles Medium; DMSO, Dimethylsulphoxide; ECACC, European Centre of Animal Cell Cultures; ECL, Enhanced Chemiluminescence; FBS, Foetal Bovine Serum; HER2, Human Epidermal growth factor Receptor 2; HRP, Horseradish Peroxidase; IDC, Invasive Ductal Carcinoma; JAM-A, Junctional Adhesion Molecule–A; MEGM, Mammary Epithelial Basal Medium; MEM, Minimum Essential Medium; PBS, Phosphate Buffered Saline; RBD Rap Binding Domain; SDS-PAGE, Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis; TBS, Tris Buffered Saline; TMA, Tissue Microarray; ZO-1, Zona Occludens-1.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

EMcS participated in the design of the study, performed most of the experimental work, interpreted the data and drafted the manuscript. LH performed the primary culture isolations. KB and ADKH participated in analysis and interpretation of the data. AH conceived the study, participated in its design, interpreted the data and revised the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1: JAM-A knockdown reduces breast cancer cell adhesion and migration. (A) Transfection of MCF7 breast cancer cells with siRNA targeting JAM-A reduces JAM-A protein expression. **(B)** Adhesion of MCF7 cells transfected with JAM-A siRNA to a fibronectin substrate was reduced relative to that induced under control conditions. **(C)** Individual cell migration of MCF7 cells transfected with JAM-A siRNA or control siRNA MCF7 cells across fibronectin-coated 8μm pore filters was reduced relative to control conditions. Images show DAPI-stained migrated cells on transwell filters. Error bars refer to SD of pooled triplicate experiments.

Figure 2: JAM-A regulates β1-integrin protein expression and Rap1 GTPase activity in breast cancer cells. (A) Western blot analysis of a panel of alpha and beta subunit integrins was conducted on MCF7 cell lines transfected with control siRNA or JAM-A-siRNA. Assessment of actin expression was performed to control for protein loading. **(B)** Densitometry analysis of triplicate western blot experiments showing relative protein expression of alpha and beta subunit integrins with error bars referring to SD of triplicate experiments. **(C)** β1-integrin western blot analysis of equal total protein lysates (input) and JAM-A immunoprecipitates (IP:JAM-A) from

MCF7 cells transfected with control siRNA or JAM-A-siRNA. **(D)** Western blot analysis of JAM-A, total Rap1, and active Rap1 protein expression in MCF7 cells transfected with control or JAM-A siRNA and in MCF7 cells treated with isotype control antibody or JAM-A inhibitory antibody. **(E)** Densitometric analysis of triplicate JAM experiments showing the ratio of active to total Rap after JAM-A knockdown or antagonism, with error bars referring to SD of triplicate experiments.

Figure 3: Rap1 or β1-integrin protein antagonism reduces breast cancer cell migration. (A) Western blot analysis of active Rap1 protein expression in MCF7 cells following treatment with the Rap1 pharmacological inhibitor GGTI-298 (10μM). Migration of MCF7 cells in scratch-wound assays after pre-treatment for 2hr with Rap1 inhibitor **(B)**, MAb 13 anti-β1-integrin inhibitory antibody **(C)**, J104 anti-JAM-A inhibitory antibody **(D)**, or a combination of all three antagonists **(E)**. Error bars refer to standard deviation (SD) and represent triplicate values in a representative experiment. **(F)** Graphic representation of migration differences between control treatments and inhibitor/antibody treatments alone and in combination. Error bars refer to SD in pooled triplicate experiments.

Figure 4: JAM-A co-associates with AF-6 and PDZ-GEF2 in breast cancer cells. Immunoblot (IB) analysis for JAM-A, Rap1, AF-6, and PDZ-GEF2 in equivalent concentrations of total protein lysates (input) and JAM-A immunoprecipitates (IP:JAM-A) from mock-transfected MCF7 cells and MCF7 cells transfected with control siRNA or JAM-A-siRNA.

Figure 5: JAM-A signalling is increased in breast cancer primary tumour cells. Immunoblot analysis for JAM-A, AF-6, PDZ-GEF2, Rap1, and β1-integrin protein expression conducted on input lysates of equivalent protein concentrations **(A, left)** and JAM-A immunoprecipitates **(A, right)** isolated from matched tumour (T) and non-tumour (N) primary breast cultures. **(B)** Comparison of tumour to non-tumour ratios from pooled samples of input protein and JAM-A immunoprecipitates for JAM-A, AF-6 and PDZ-GEF2 individually.

Figure 6: Model of JAM-A signalling in human breast cancer cells. Our working model hypothesizes that in normal breast cells, a baseline level of JAM-A signalling via AF-6 and PDZ-GEF2 leads to a low level of β1-integrin mediated cell migration required for crucial normal pathological process such as wound healing **(A)**. However, in breast cancer cells, over-expression of JAM-A leads to increased associated with PDZ-GEF2 protein which in turn hyperactivates the GTPase Rap1. We suggest this culminates in increased β1-integrin mediated cancer cell migration leading to increased risk of invasion and metastasis **(B)**.

Table 1: Pathological parameters of human breast tissue primary cultures

Tissue	Age	Diagnosis	Grade	ER	PR	HER2
1T, 1N	53	IDC	2	+	+	-
2T, 2N	32	DCIS	NA	+	+	NA
3T, 3N	42	IDC	3	-	-	-

IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor, HER2, human epidermal growth factor receptor-2; NA, not available.

Note: the ER, PR and HER2 status of each patient tissue was derived directly from the histopathology report on each case.

Additional files

Additional file 1: Supplementary Figure S1. Reduced cell migration downstream of JAM-A knockdown is reproducible with different siRNA constructs. (A) Fold change in % wound closure over time of control MCF7 cells compared with cells transfected with two separate JAM-A siRNAs or a mock control siRNA. **(B)** Representative immunoblots illustrating expression levels of JAM-A, AF-6, and Rap1 (left panel) or co-precipitation of the same proteins with JAM-A (right panel) in MCF7 cells transfected with two separate JAM-A siRNAs or a mock control siRNA.

Additional file 2: Supplementary Figure S2. JAM-A inhibition reduces migration but not invasion of MCF7 cancer cells. (A) Comparison of the relative invasion rates of MDA-MB-231, Hs579T cells and MCF7 cells across Matrigel-coated Transwell filters; confirming the non-invasive nature of MCF7 cells. Accordingly, although JAM-A antagonism with the inhibitory antibody J10.4 exerted a significant anti-migratory effect on MCF7 cells in scratch wound assays by 4h **(B)**, no significant_antagonism of MCF7 invasion across Matrigel-coated filters was observed even after 24h exposure to J10.4 **(C)**.

Additional file 3: Supplementary Figure S3. Pooled analysis of JAM-A signalling proteins in tumour versus normal breast tissue primary cultures. (A) Ratio of pooled tumour to non-tumour densitometric values from JAM-A, AF-6, PDZ-GEF2, Rap1 and β1-integrin protein immunoblots with equal total input protein concentrations. **(B)** Ratio of pooled tumour to non-

tumour densitometric values from concentrations JAM-A, AF-6, PDZ-GEF2, Rap1 and β1-integrin protein immunoblots of JAM-A immmunoprecipitates.

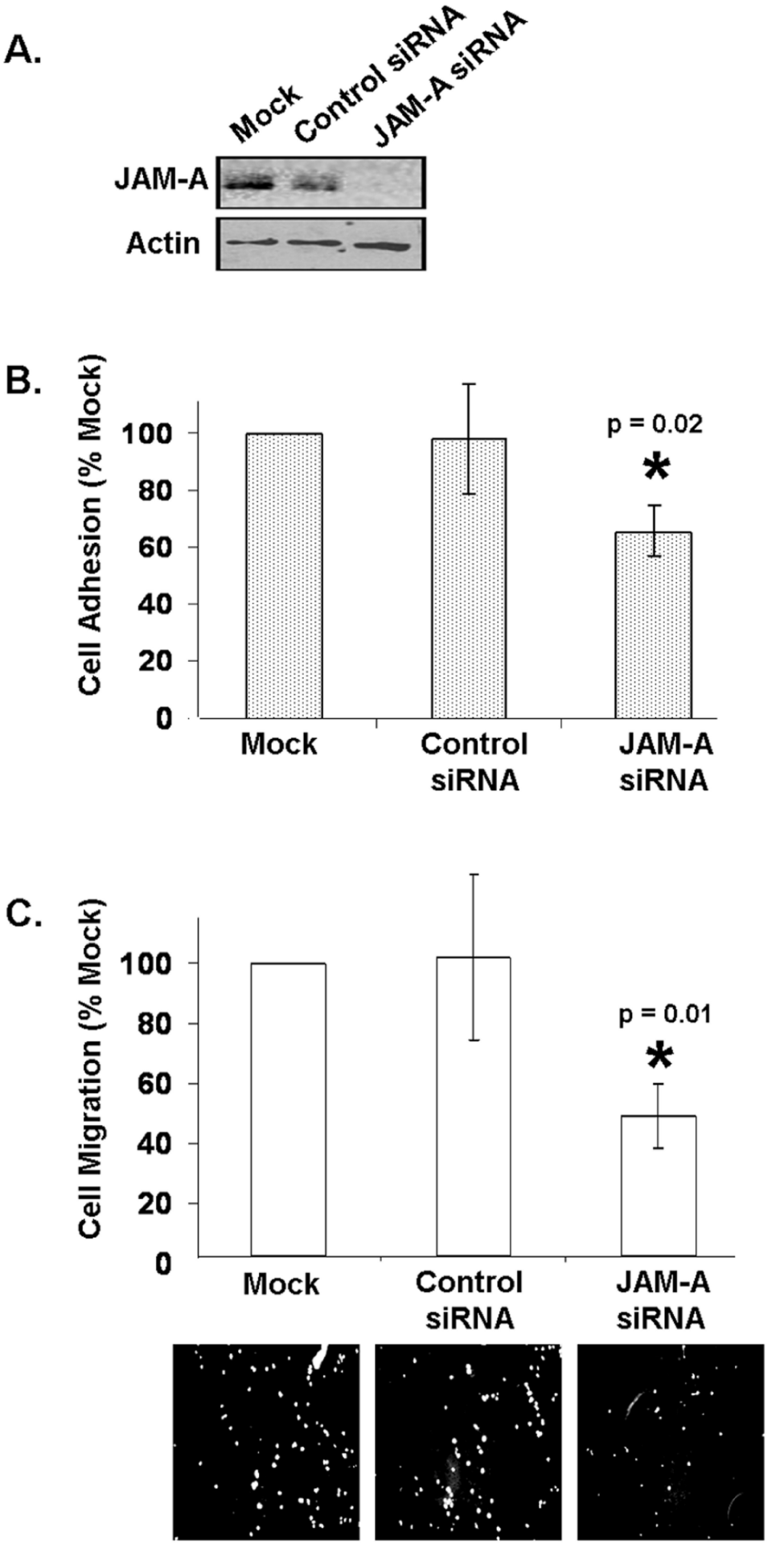
A.
Mock
Control siRNA
JAM-A siRNA
JAM-A
Actin
B.
Cell Adhesion (% Mock)
100
80
60
40
20
0
p = 0.02
*
Mock
Control siRNA
JAM-A siRNA
C.
Cell Migration (% Mock)
100
80
60
40
20
0
p = 0.01
*
Mock
Control siRNA
JAM-A siRNA

Figure 1

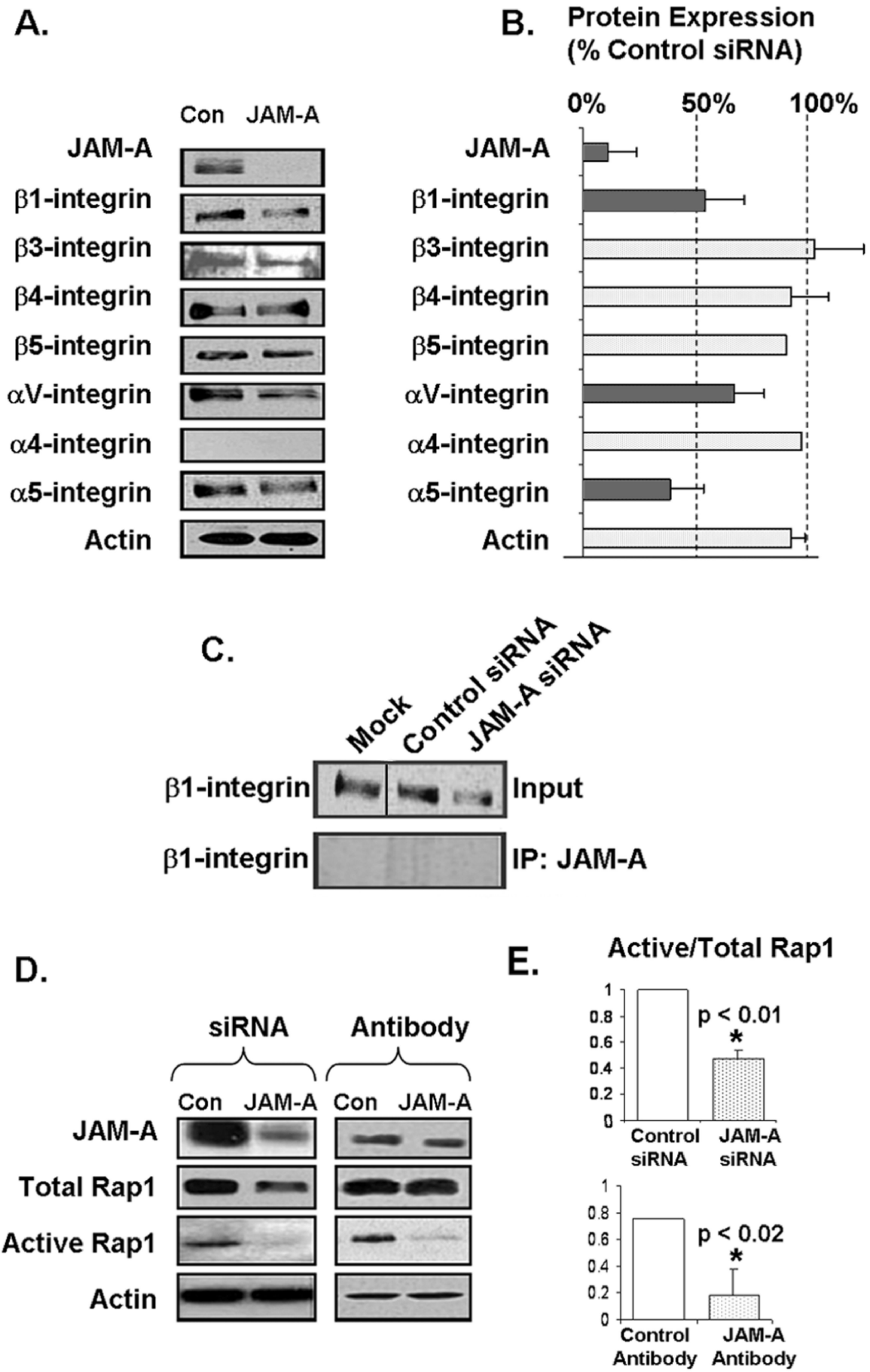
A.
Con
JAM-A
JAM-A
β1-integrin
β3-integrin
β4-integrin
β5-integrin
αV-integrin
α4-integrin
α5-integrin
Actin
B.
Protein Expression
(% Control siRNA)
0%
50%
100%
JAM-A
β1-integrin
β3-integrin
β4-integrin
β5-integrin
αV-integrin
α4-integrin
α5-integrin
Actin
C.
Mock
Control siRNA
JAM-A siRNA
β1-integrin
Input
β1-integrin
IP: JAM-A
D.
siRNA
Antibody
Con
JAM-A
Con
JAM-A
JAM-A
Total Rap1
Active Rap1
Actin
E.
Active/Total Rap1
1
0.8
0.6
0.4
0.2
0
p < 0.01
*
Control siRNA
JAM-A siRNA
1
0.8
0.6
0.4
0.2
0
p < 0.02
*
Control Antibody
JAM-A Antibody

Figure 2

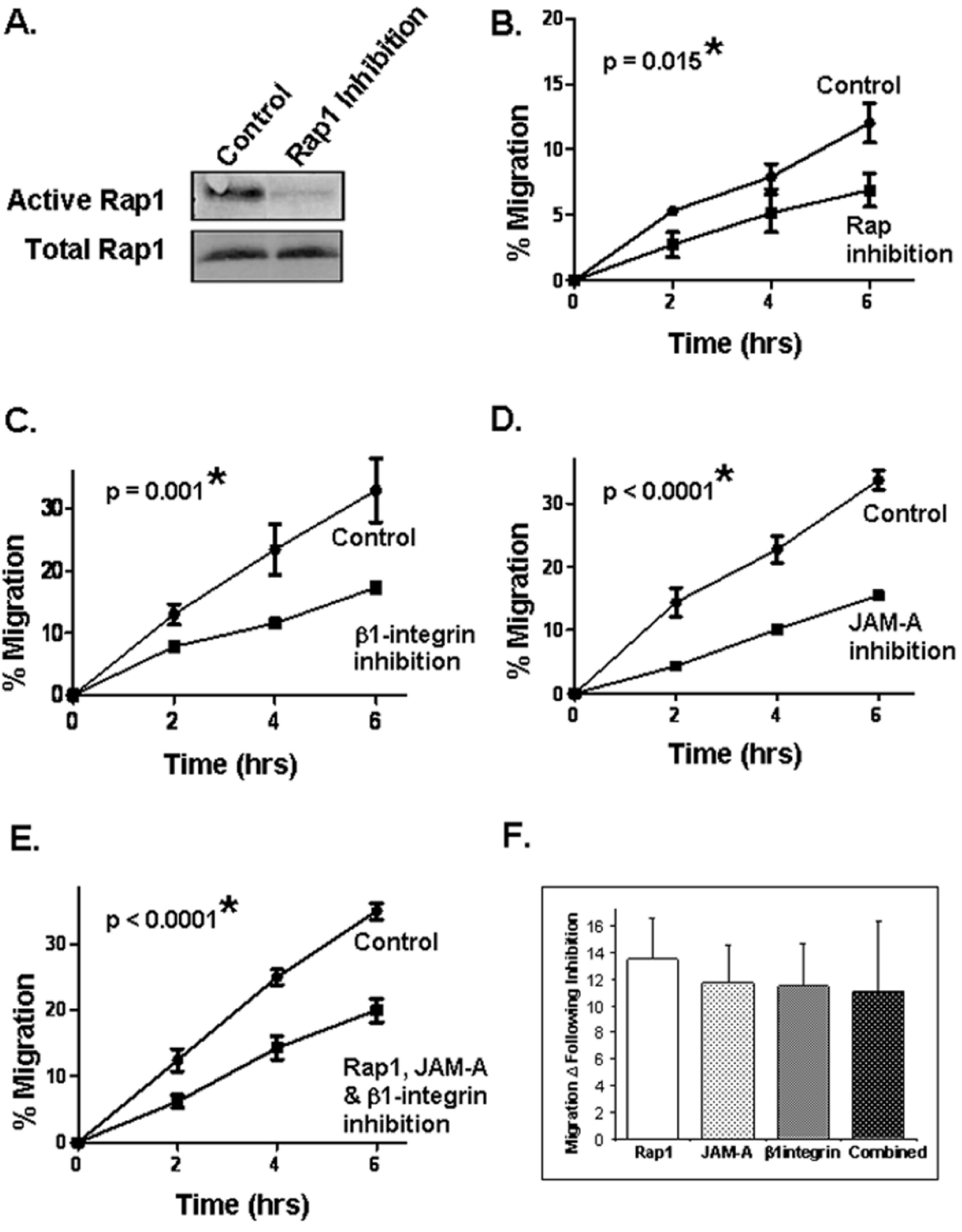
A.
Control
Rap1 Inhibition
Active Rap1
Total Rap1
B.
p = 0.015*
Control
Rap inhibition
% Migration
Time (hrs)
C.
p = 0.001*
Control
β1-integrin inhibition
% Migration
Time (hrs)
D.
p < 0.0001*
Control
JAM-A inhibition
% Migration
Time (hrs)
E.
p < 0.0001*
Control
Rap1, JAM-A & β1-integrin inhibition
% Migration
Time (hrs)
F.
Migration Δ Following Inhibition
Rap1
JAM-A
β1integrin
Combined

Figure 3

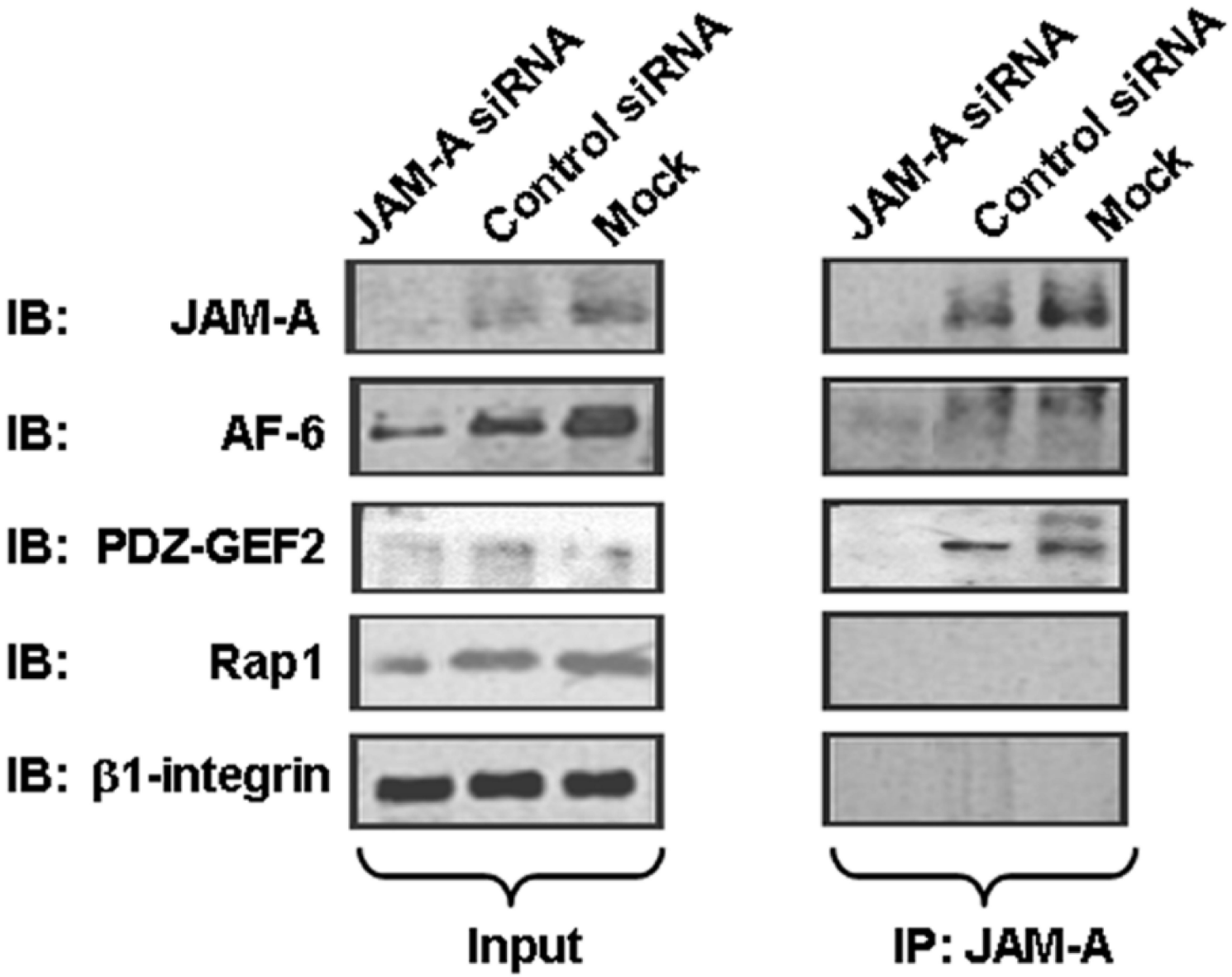
JAM-A siRNA
Control siRNA
Mock
JAM-A siRNA
Control siRNA
Mock
IB: JAM-A
IB: AF-6
IB: PDZ-GEF2
IB: Rap1
IB: β1-integrin
Input
IP: JAM-A

Figure 4

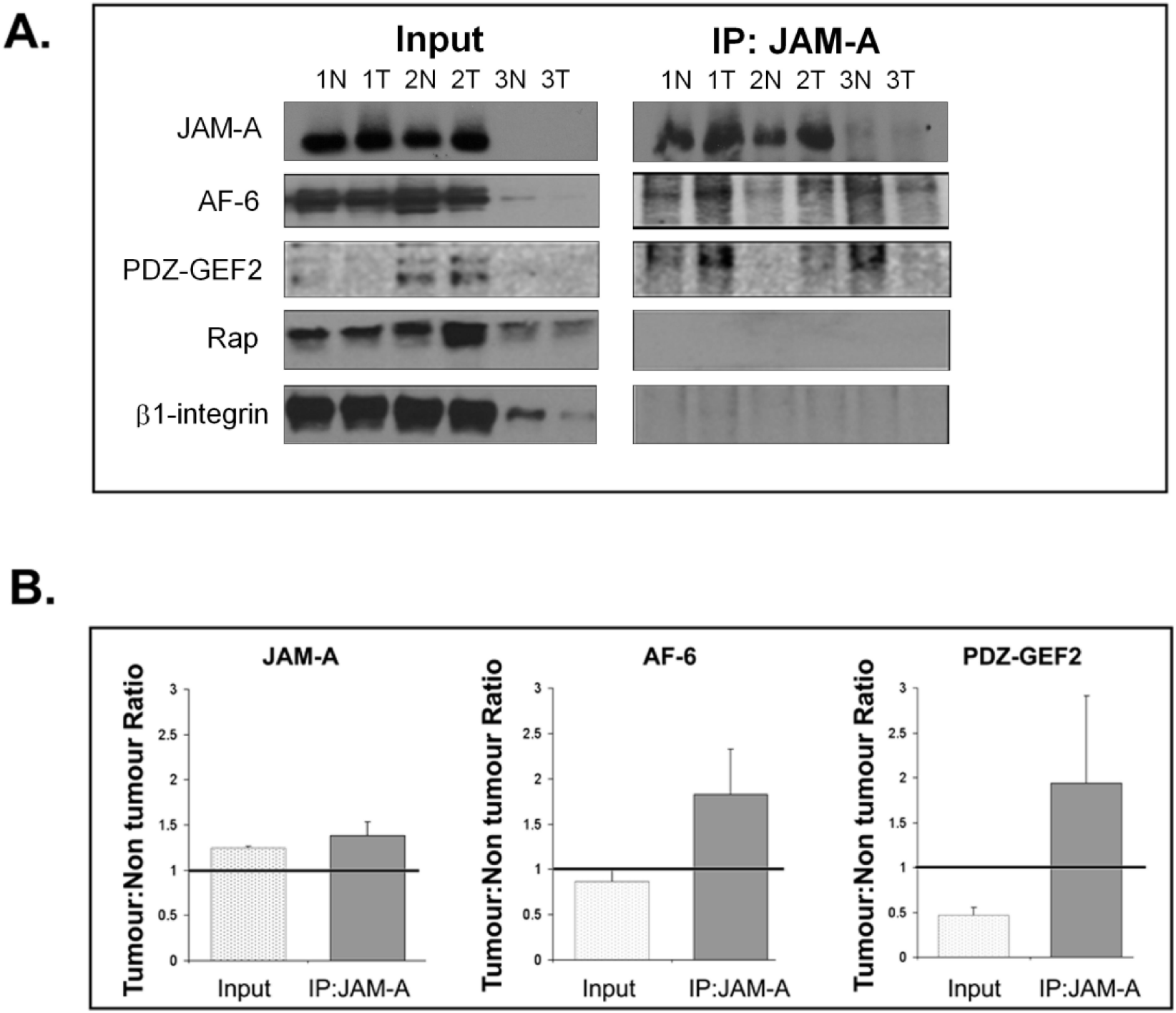
A.
Input
IP: JAM-A
1N 1T 2N 2T 3N 3T
1N 1T 2N 2T 3N 3T
JAM-A
AF-6
PDZ-GEF2
Rap
β1-integrin
B.
JAM-A
AF-6
PDZ-GEF2
Tumour:Non tumour Ratio
Tumour:Non tumour Ratio
Tumour:Non tumour Ratio
0
0.5
1
1.5
2
2.5
3
Input
IP:JAM-A
Input
IP:JAM-A
Input
IP:JAM-A

Figure 5

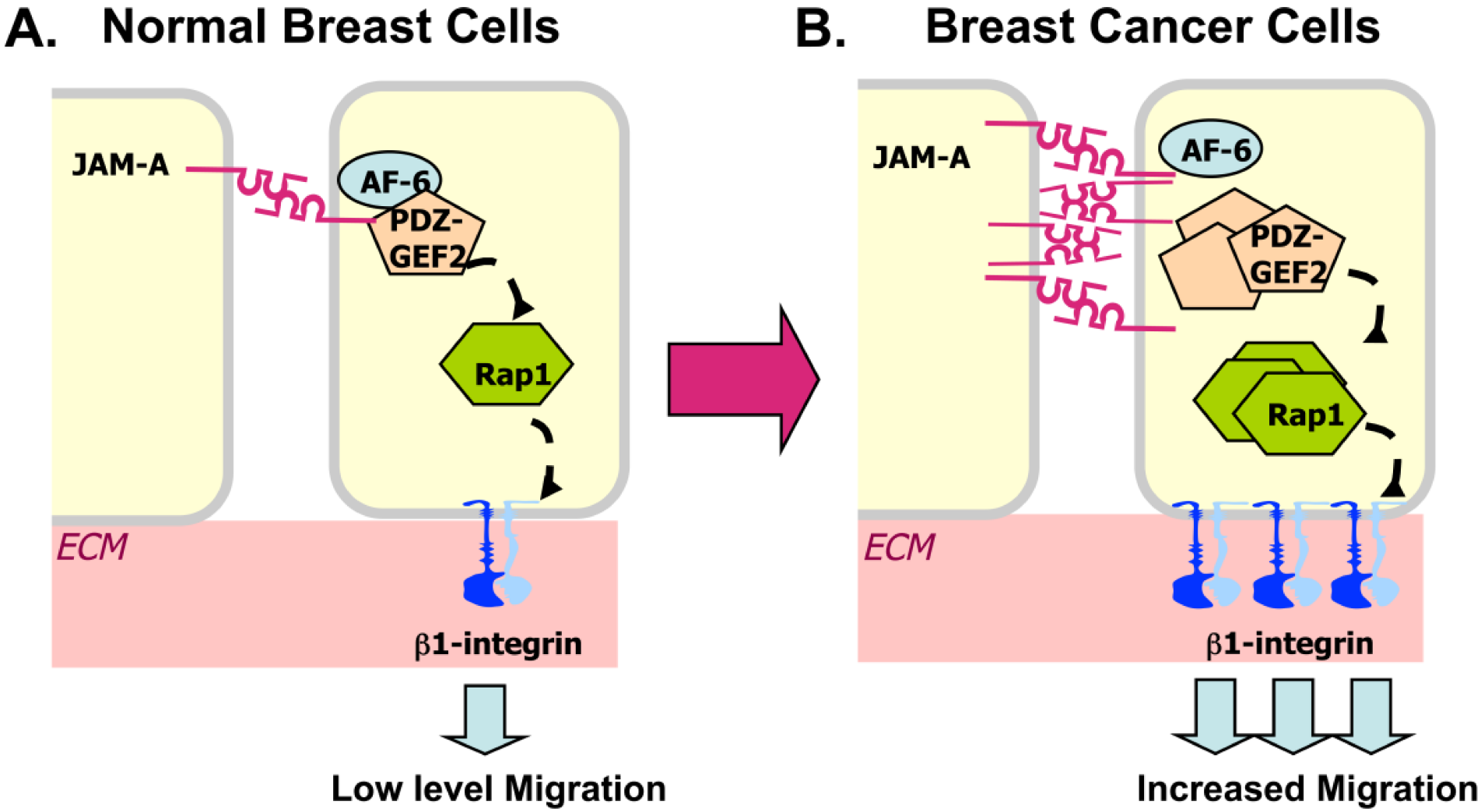
A. Normal Breast Cells
JAM-A
AF-6
PDZ-GEF2
Rap1
ECM
β1-integrin
Low level Migration
B. Breast Cancer Cells
JAM-A
AF-6
PDZ-GEF2
Rap1
ECM
β1-integrin
Increased Migration

Figure 6

Additional files provided with this submission:

Additional file 1: suppfig1.TIF, 1289K
http://breast-cancer-research.com/imedia/3011571152705447/supp1.tiff
Additional file 2: suppfig2.TIF, 996K
http://breast-cancer-research.com/imedia/1727992118527054/supp2.tiff
Additional file 3: suppfig3.tif, 111K
http://breast-cancer-research.com/imedia/1982297965527054/supp3.tiff