15-9-2009

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Citation

McSherry EA, McGee SF, Jirstrom K, Doyle EM, Brennan DJ, Landberg G, Dervan PA, Hopkins AM, Gallagher WM. JAM-A Expression Positively Correlates with Poor Prognosis in Breast Cancer Patients. International Journal of Cancer. 2009 Sept 15;125(6):1343-51.

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JAM-A Expression Positively Correlates with Poor Prognosis in Breast Cancer Patients

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Short Title: JAM-A and breast cancer

Key words: junctional adhesion molecule-A; breast cancer; tight junction; migration.

Journal Category: Cancer Cell Biology

Statements: Our manuscript shows a novel association between high gene/protein expression of the epithelial tight junction protein Junctional Adhesion Molecule-A (JAM-A) and poor prognosis in primary breast carcinoma patients. Mechanistically we demonstrate that JAM-A knockdown decreases the motility of breast cancer cells, potentially resulting from a concomitant reduction in β1-integrin protein levels. We suggest that the impact of our finding lies in the future consideration of JAM-A as a target to prevent tumor cell dissemination in breast cancer patients.

Abbreviations:

ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; ECACC, European Centre of Animal Cell Cultures; ER, estrogen receptor; EV, empty vector; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; ILC, invasive lobular carcinoma; JAM-A, junctional adhesion molecule-A; NF, non-functioning; NHG, Nottingham Histological Grade; PR, progesterone receptor; shRNA, short hairpin ribonucleic acid; TJ, tight junction; TMA, tissue microarray; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2.

ABSTRACT

The cell-cell adhesion protein junctional adhesion molecule-A (JAM-A) influences epithelial cell morphology and migration. As migration is required for tumor cell invasion and metastasis, we sought to elucidate the role of JAM-A in invasive breast cancer. A breast cancer tissue microarray was analyzed for JAM-A protein expression, in parallel with analysis of JAM-A gene expression data from a breast cancer clinical dataset. Our data demonstrate a novel association between JAM-A gene and protein upregulation and poor prognosis in breast cancer. To mechanistically dissect this process, we used lentiviral technology to stably knock down JAM-A gene expression by shRNA in MCF7 breast cancer cells, which express high endogenous levels of JAM-A. We also antagonised JAM-A function in wild-type MCF7 cells using an inhibitory antibody which blocks JAM-A dimerization. Knockdown or functional antagonism of JAM-A decreased breast cancer cell migration in scratch-wound assays. Reductions in β1-integrin protein levels were observed following JAM-A-knockdown in MCF7 cells, suggesting a mechanism for reduced motility after loss of JAM-A. Consistent with this hypothesis, tissue microarray analysis of β1-integrin protein expression in invasive breast cancer tissues revealed a trend toward high β1-integrin protein levels being indicative of poor prognosis. 22% of patients were observed to co-express high levels of JAM-A and β1-integrin protein, and MDA-MB-231 breast cells stably over-expressing JAM-A showed an increase in β1-integrin protein expression. Our results are consistent with a previously unreported role for JAM-A over-expression as a possible mechanism contributing to progression in primary breast cancer, and a potential therapeutic target.

INTRODUCTION

Epithelial polarity is maintained by multi-protein adhesion complexes at cell-cell interfaces, including tight junctions (TJs) [1]. Dysregulation of individual TJ proteins (eg. claudins, ZO-1) have been implicated in the loss of tissue architecture and polarity associated with breast carcinoma and other malignancies [2, 3].

The junctional adhesion molecule (JAM) family of TJ proteins are type 1 transmembrane proteins of the immunoglobulin superfamily [4]. There are five characterised JAM family proteins, JAM-A, JAM-B, JAM-C, JAM-4, and JAM-L, which are expressed in various tissues throughout the body and on the surfaces of platelets, erythrocytes and leukocytes [5]. JAM proteins are known to have important regulatory functions in numerous cellular adhesive processes including platelet activation [6], leukocyte migration [7, 8], angiogenesis [9], intercellular junction assembly [10] and cell morphology [11]. Much evidence indicates an important role for JAM proteins in regulating and maintaining tight junctions through associations with proteins such as ZO-1, AF6, and PAR3 [5]. Interestingly, JAM-A has also been identified as the epithelial receptor for the pathogen reovirus [12].

As JAM family members form an important scaffold for assembly of other TJ proteins [5], it is plausible that loss of this key protein could facilitate cell-cell dissociation and promote invasive phenotypes in solid tumors. JAM-A expression has been previously shown to reduce spontaneous and random motility of endothelial cells [13]. Indeed, a recent publication reported for the first time an association between loss of junctional adhesion molecule-A (JAM-A) and the acquisition of invasive properties in breast cancer cells [14]. In this study Naik *et al.* hypothesised that JAM-A loss could potentially predict poor clinical outcome in breast cancer [14]. It is likely

that tight junction protein expression must be carefully balanced for normal cell function. However, it is also likely that numerous extrinsic signals from the tumour microenvironment may influence tight junction protein expression and function in tumour epithelial cells *in vivo*.

Thus to acknowledge the complexity of the *in vivo* breast cancer microenvironment, our current study has predominantly focused upon searching for associations between JAM-A expression and prognostic outcome in breast cancer clinical datasets. Analysis of a 270-patient invasive breast cancer tissue microarray (TMA) revealed a strong correlation between high JAM-A protein expression and reduced patient survival. Re-analysis of the independent van de Vijver breast cancer DNA microarray dataset (n = 295) [15] to focus on JAM-A gene expression supported our findings. Our mechanistic studies showed that knockdown or functional antagonism of JAM-A in MCF7 breast cancer cells significantly decreased cell migration. As β1-integrin protein levels were reduced in JAM-A knockdown cells, our data suggest that loss of β1-integrin may be one possible mechanism to explain observed reductions in cell migration downstream of JAM-A loss. In support of this, β1-integrin has been shown to regulate cell spreading [11] and the malignant phenotype in breast cancer cells [16], and antagonism of β1-integrin inhibits cell migration [17]. In fact our invasive breast cancer TMA showed a correlation between high expression of β1-integrin and poor prognosis; and JAM-A over-expressing MDA-MB-231 cells exhibited increased levels of β1-integrin protein. Therefore, we report for the first time a novel (and potentially important) link between increased JAM-A expression and poor clinical outcome in invasive breast tumors. Our results suggest that increased expression of JAM-A in tumor cells may ultimately facilitate integrin-mediated migratory events at the tumor cell-matrix interface; and warrants further exploration as a biomarker of poor prognosis in primary breast cancer or a drug target in the future.

MATERIALS AND METHODS

Cell culture. Breast epithelial cell lines were obtained from ATCC (MDA-MB-231, MCF7, Hs578T). Cell lines were grown under standard conditions (DMEM, 10% foetal bovine serum, 2mM L-glutamine, 50 IU/ml penicillin, 50μg/ml streptomycin).

Antibodies. Anti-human antibodies for Western blotting were sourced as follows: rabbit JAM-A (Invitrogen), rat β1- and mouse β4-integrin (BD Biosciences), rabbit E-cadherin (Cell Signaling Technologies), mouse actin (Abnova). Immunohistochemistry was conducted using mouse JAM-A (Abnova); immunofluoresence and inhibition studies using mouse J.10.4 JAM-A (kindly donated by Prof. Charles Parkos, Emory University, Atlanta). All JAM-A antibodies detected equivalent expression levels by Western blot (not shown).

Tissue microarray (TMA). Duplicate cores from consecutive breast cancer patients with invasive primary breast cancer in Malmo University Hospital (1988–1992) were used to construct a TMA (with ethical approval from Lund University Review Board) [18]. JAM-A and β1-integrin expression data was then determined by immunohistochemistry of this TMA of 270 patient specimens with survival data.

Immunohistochemistry. JAM-A and β1-integrin immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded TMA sections using standard protocols. Briefly, antigen retrieval was in citrate buffer pH 6.0 (10 min/95°C); primary antibody incubation (mouse JAM-A; Abnova or mouse β1-integrin; Novacastra) was for 60 min, secondary antibody incubation was for 20 min followed by DAB substrate for 10 min. Membranous expression of JAM-A or β1-

integrin in tumor cells was scored 0, 1+, 2+, or 3+ based upon staining intensity. JAM-A results were scored blind by 2 observers (one pathologist) and combined. β1-integrin results were scored by a pathologist according to the following scheme: 0 = 0% cells, 1 = <10% cells, 2 = 10-50% cells, 3 = >50% based upon membranous staining intensity of β1-integrin.

DNA microarray data analysis. The van de Vijver DNA microarray dataset [15] was obtained from Rosetta Inpharmatics (www.rii.com). In this breast cancer dataset, expression values for 25,000 genes had been determined. Normalised JAM-A and β1-integrin log-ratios were downloaded with survival and clinicopathological data. Tumor samples were classified according to JAM-A and β1-integrin mRNA expression based on absolute expression analysis p values (alpha level of 0.05) [18].

Statistical analysis. χ^2 test and Fisher's exact tests were used for comparison of JAM-A and β1-integrin expression with other clinicopathological parameters. Kaplan-Meier analysis and the log rank test were used to illustrate differences between recurrence-free survival and breast cancer-specific survival according to JAM-A expression. Calculations were performed with SPSS-v12.0.1.

Western blotting. Breast cells were harvested at 80% confluence in 100mM KCl, 3mM NaCl, 3.5mM $MgCl_2$, 10mM HEPES pH7.4, 1% Triton-X100, protease and phosphatase inhibitor cocktails (Sigma). Samples (25μg) were subjected to reducing SDS-PAGE, transferred to PVDF, and immunoblotted for JAM-A, E-cadherin, β1-integrin and β4-integrin.

Immunofluorescence. Confluent breast cell lines on chamber slides (Nunc, Denmark) were fixed in 3.7% paraformaldehyde for 20 min and immunofluorescently-labeled as described [19] using JAM-A antibody (5μg/ml), AlexaFluor568-coupled secondary antibody (2μg/ml) and DAPI (0.63μg/ml). Images were acquired in a single plane on a Zeiss LSM510-Meta confocal microscope.

JAM-A knockdown/over-expression. Three short hairpin (sh-)RNAs targetting JAM-A were designed using the siDESIGN tool (www.dharmacon.com). HEK293 cells were transfected with pLVTHm vector (Trono Laboratory) containing JAM-A shRNA or empty vector (for gene knockdown in MCF7 breast cells); or with LLCIEP vector containing full-length JAM-A or empty vector (for gene over-expression in MDA-MB-231 and Hs578T breast cells). Cells were also co-transfected with packaging plasmid and envelope plasmid (psPAX2 and pMD2G respectively; Trono Laboratory). Live virus was collected, filtered and used to infect 50%-confluent MCF7, MDA-MB-231 or Hs578T cells for 24 h. Cells were subcultured at 2-3 days.

Scratch-wound assay. Confluent breast cells were scratch-wounded with a sterile pipette tip and allowed to migrate in serum-free media (to ensure that the pro-migratory effects of serum did not mask detection of the effects of knockdown or antagonism of JAM-A). Images were acquired on a phase contrast microscope linked to a CCD camera and wound diameter was measured at 0, 2, 4, 6, 8 and 24 h using Scion Image (Scion Corporation, USA). For indicated experiments, monolayers were pre-incubated for 2 hr prior to wounding with anti-human JAM-A (J.10.4 antibody; 10μg/ml) or culture media for control cell monlayers.

Proliferation assay. Proliferation was assessed in breast cells grown on 96-well plates by incubation with MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 1.25mg/ml) for 4 h followed by solubilization with DMSO. Growth curves of absorbance at 540 nm versus time were plotted.

RESULTS

High JAM-A expression in breast cancer tissues is significantly associated with poor patient prognosis

Junctional adhesion molecule-A (JAM-A) has important functions in intercellular adhesion [5], and its loss in breast cancer cells has recently been observed [14]. To investigate the involvement of JAM-A in breast cancer patient survival, we analyzed JAM-A protein expression on an invasive breast cancer tissue microarray (TMA) of 270 tumor cores. Cores were scored 0 (negative), 1+, 2+ or 3+ according to the level of JAM-A membranous staining in tumor cells (**Fig. 1A**). Scoring was performed by 2 independent observers (one pathologist), and the results combined. Tumor membranous expression of JAM-A was classified as low (0 *(2 tissues*), 1+ *(62 tissues)* or 2+ *(122 tissues)*) in 186 tumors (69%) and high (3+) in 84 tumors (31%). Kaplan-Meier analysis and the log-rank test were used to relate JAM-A-expression to breast cancer-specific survival (time from diagnosis to death directly attributable to breast cancer burden) and recurrence-free survival. High JAM-A protein expression had a statistically significant association with reduced breast cancer-specific survival (**Fig. 1B**; $p<0.05$). In addition, patients whose tumors had high JAM-A protein expression were significantly more likely to develop a recurrence within 5 years compared to patients with low JAM-A expression (**Fig. 1C**; $p<0.05$). Examination of the relationship between JAM-A expression and other clinico-pathological variables (**Table 1**) revealed that high JAM-A expression was associated with high tumor grade ($p<0.05$) and increased expression of both VEGFR1 ($p<0.01$) and HER2 ($p<0.01$).

To confirm our findings, JAM-A mRNA expression was assessed in an independent dataset of gene expression information from 295 patients with primary breast cancer [15] (**Fig. 2**). All patients were younger than 53 years old, had stage I or II primary invasive breast cancer, with

approximately 50% being lymph node-positive tumors. Tumors were classified as high or low JAM-A expressers based on array probe-target cDNA hybridization levels. In this dataset, JAM-A gene expression was classified as low in 236 tumors (80%) and high in 59 tumors (20%). Kaplan-Meier analysis and the log-rank test were used to illustrate differences in overall survival (time from diagnosis to death) and metastasis-free survival (time from primary diagnosis to diagnosis of secondary cancer) according to JAM-A mRNA expression. High JAM-A mRNA expression was associated with reduced overall survival (**Fig. 2A;** $p<0.05$) and reduced metastasis-free survival (**Fig. 2B**; $p<0.01$). Differences in distribution of clinicopathological data and tumor characteristics between high JAM-A and moderate/low JAM-A expressing tumors were also evaluated using the χ^2 test and Fishers exact tests (**Table 2**). High JAM-A mRNA expression was associated with larger tumor size ($p<0.05$), higher grade ($p<0.001$), and ER negativity ($p<0.001$). Tumors in this study were also classified into molecular subtypes using the well-validated criteria described by Sorlie et al. [20]. This revealed that high JAM-A mRNA levels were associated with the basal subtype of breast cancer linked with poor prognosis ($p<0.001$). Finally, high JAM-A mRNA expression was also associated with poor prognosis using the 70-gene prognostic signature ($p<0.001$) derived by van't Veer *et al.* [21]. Together these data confirm that over-expression of JAM-A at both mRNA and protein level is associated with poor prognosis in breast cancer patients.

JAM-A knockdown or antibody-based inhibition reduces cell migration

We next reasoned that if JAM-A expression was increased in invasive breast cancer tissues, knockdown of high JAM-A expression levels in a cell line model may give clues to explain the *in vivo* mechanisms. To determine a suitable breast cell line for use in *in vitro* JAM-A knock-down studies, we first investigated the gene- and protein expression of JAM-A via RT-PCR, western

blot analyses and immunofluorecence localisation in a panel of breast cell lines. In agreement with recently-published work [14], we identified differences in JAM-A expression between breast cell lines (data not shown).

MCF7 breast cancer cells expressed high endogenous levels of JAM-A protein, which was shown by immunofluorescence confocal microscopy to localize to intercellular tight junctions (data not shown). Therefore we knocked down JAM-A protein in MCF7 breast cancer cells, using lentiviral technology to transduce cells with 3 different short hairpin (sh)-RNA constructs targeting JAM-A (shRNA1, shRNA2, shRNA3). Stably-transduced cell lines were prepared and JAM-A knockdown assessed at both gene- and protein expression level. We demonstrated by RT-PCR and western blot analysis that cells stably expressing the shRNA1 construct had similar JAM-A levels to empty vector (EV)-expressing control cells, and that shRNA3 mediated a 65% decrease in JAM-A expression relative to EV. **(Supplemental Fig. 1A & 1B**). Notably, shRNA3 showed comparable levels of JAM-A knockdown in another breast cancer cell line expressing high endogenous levels of JAM-A, BT474, indicating the specificity of shRNA3 (data not shown). Based on these results, MCF7 cells transduced with shRNA3 (henceforth designated JAM-A-shRNA) were selected for further study. Cells transfected with shRNA1 were chosen as a non-functioning (NF) control (henceforth designated NF-shRNA). NF-shRNA controlled for both lentiviral effects and also activation of RNAi machinery in transfected cells. Immunofluorescence microscopy for JAM-A revealed that its expression was reduced in cells transduced with JAM-A-shRNA relative to NF-shRNA, but residual protein in the former did still localize at intercellular junctions (**Supplemental Fig. 1C**).

As JAM-A had previously been shown to be crucial in adhesion or migration of epithelial cells[11] endothelial cells [22]and leukocytes[7], we used scratch-wound assays to assess the impact of JAM-A depletion on cell migration (**Fig. 3**). Migration was measured at short time intervals (2, 4, 6 and 8 hours post-injury) to exclude possible proliferative events. JAM-A-shRNA cells migrated significantly slower than NF-shRNA control cells, with respectively 6.9% and 9.4% wound closure at 4 hours (**Fig. 3A**; $p<0.05$). To further exclude the possibility that proliferative differences between NF-shRNA and JAM-A-shRNA cells could influence wound closure, we performed MTT assays on both cell lines (**Fig. 3B**). Identical growth curves were observed over a period of several days, indicating a specific effect of JAM-A knockdown in reducing MCF7 cell migration, rather than altering growth characteristics.

To confirm that reductions in cell migration resulting from JAM-A knockdown did not result from off-target effects of shRNA transduction, we also used an alternative method to antagonize JAM-A function. Scratch-wound migration assays were conducted in wild-type MCF7 breast cancer cells pre-incubated for 2 hours prior to wounding with an inhibitory JAM-A antibody which prevents dimerization of JAM-A (J10.4 [23]). MCF7 cells pre-incubated with J10.4 migrated significantly slower than control cells (**Fig. 3C**; $p<0.01$ at 4h). Experiments repeated on consecutive days showed identical trends, so a representative experiment is shown. Maximal differences in migration were seen at 6 hours post-injury, with 8% and 18.5% wound closure in J10.4- treated versus control MCF7 cells respectively ($p<0.05$). This supported our JAM-A knockdown data in MCF7 cells, confirming that knockdown or functional antagonism of JAM-A decreased the motility of breast cancer cells.

JAM-A knockdown reduces β1-integrin expression

We next sought to investigate possible mechanisms whereby JAM-A knockdown could decrease cell motility. Western blot analysis was conducted to determine whether JAM-A knockdown concomitantly affected the expression of other proteins important for cell adhesion or migration. Expression levels of the cell-cell adhesion protein E-cadherin and the cell-matrix adhesion protein β4-integrin were unaffected by JAM-A knockdown (**Fig. 3D**). However, expression of β1-integrin, a cell-matrix adhesion protein required for migration [17] , was dramatically reduced in JAM-A-shRNA cells relative to NF-shRNA controls (**Fig. 3D**). Our results suggest that downstream regulation of β1-integrin protein expression represents one possible mechanism for JAM-A to regulate cancer cell motility.

High β1-integrin protein (not mRNA) expression tends towards an association with poor prognosis in breast cancer

Results from our western blot analyses suggested that JAM-A over-expression exerts post-translational effects on β1-integrin expression. We therefore assessed β1-integrin mRNA expression in the dataset of gene expression information from 295 patients with primary breast cancer[15], which in **Fig. 2** had demonstrated an association between JAM-A mRNA over-expression and poor prognosis. As expected, there was no association between high β1-integrin mRNA levels and poor prognosis (**Fig. 4A**). We next immunostained our tissue microarray of 270 invasive breast cancer cases for β1-integrin protein expression. High β1-integrin protein expression was associated with reduced overall survival, but this was just outside the boundaries of statistical significance (p=0.08) (**Fig. 4B**). However the staining for β1-integrin on formalin-fixed paraffin tissues was always sub-optimal (despite trying 3 different antibodies and several optimization methods), so with better quality staining the association between poor prognosis and

over-expression of β1-integrin might be significant. We also saw that 22% of patients expressing high levels of JAM-A protein co-expressed high levels of β1-integrin (**Fig. 4C**).

To further explore the putative association between JAM-A protein over-expression and β1-integrin protein upregulation, we used lentiviral technology to stably-overexpress JAM-A in MDA-MB-231 cells, an invasive breast cancer cell line with very low endogenous levels of JAM-A. Western blot analysis revealed that cells overexpressing JAM-A expressed higher levels of β1-integrin protein relative to EV control cells (**Fig. 4D**). Unfortunately however, the vast majority of overexpressed JAM-A was seen to localize in the cell cytoplasm rather than at intercellular tight junction membranes (**Supplemental Fig. 2F**). This made it unfeasible to directly examine the effect of JAM-A overexpression on functional parameters (such as migration) in these cells; since they could not be correlated with observations from the breast cancer TMA in which only membranous JAM-A expression had been scored. Nonetheless it did suggest some parallel regulation between the total protein expression of JAM-A and β1-integrin; in a manner which we hypothesize might influence cancer cell migration.

DISCUSSION

The intercellular tight junction protein JAM-A has been shown to have important physiological functions in numerous cellular adhesive processes including leukocyte migration [7], intercellular junction assembly [10] and the regulation of cell morphology [11]. Re-expression of JAM-A in endothelial cells of JAM-A knockout mice has also been reported to prevent spontaneous motility, indicating that JAM-A may regulate effectors of motility [13]. Furthermore, it has recently been reported for the first time that loss of JAM-A may also play a pathophysiological role in breast cancer [14].

As we were interested in exploring the role of JAM-A in breast cancer patient survival, we analyzed the expression of JAM-A protein in a tissue microarray (TMA) of 270 invasive breast cancers. This is, to our knowledge, the first study that has examined patient outcomes in relation to levels of JAM-A expression. We found that high levels of JAM-A protein expression were associated with high grade tumors, significantly poorer breast cancer-specific survival and poorer recurrence-free survival.

Some key points in our study conflict with the recent findings of Naik et al.[14] who reported that *loss* of JAM-A expression correlates with breast cancer progression using a small commercially-available TMA and several breast cell lines. However our patient dataset was significantly larger than that of Naik et al. (270 vs 112 primary breast tumors), and had the additional advantage of associated clinico-pathological data and survival data. In particular, our survival data provide compelling evidence that JAM-A protein over-expression in invasive breast tumors is associated with an unfavourable patient outcome. Nonetheless, we also analyzed JAM-A gene expression in a publicly-available invasive breast cancer dataset of 295 invasive breast tumors [15]. In support of

our TMA data, analysis of this dataset revealed that high levels of JAM-A mRNA significantly correlated with reduced overall survival in breast cancer patients. In addition, these data indicated that patients whose tumors had high JAM-A gene expression levels were significantly more likely to develop metastasis.

It must be noted, however, that over-expression of JAM-A at the gene and protein level may be a *consequence* of breast tumorigenesis rather than a *cause* of tumor initiation or invasion. Therefore, we next sought to mechanistically dissect the functional significance of JAM-A over-expression in breast cancer via *in vitro* cell line studies. We utilised shRNA technology to stably knock down the expression of JAM-A in MCF7 breast cancer cells, which express high endogenous levels of JAM-A. In colonic epithelial cells, siRNA-mediated knockdown of JAM-A expression has been reported to decrease cell adhesion and spreading [11, 22, 24]. Knock-down of JAM-A expression has also been previously shown to decrease migration of endothelial cells[23, 25]. As migratory capacity is one index of tumor cell dissemination, we therefore assessed the impact of JAM-A loss on MCF7 cell migration in scratch wound assays. Again, in contrast to the breast cancer findings of Naik et al., [14] we found that knockdown of JAM-A expression significantly decreased the migration of MCF7 breast cancer cells. In support of our results, a previous report has shown that migration of kidney epithelial cells is reduced in the presence of an antibody which prevents JAM-A dimerization and downstream signalling [25]. The same antibody also reduced migration of wild-type MCF7 breast cancer cells in our hands, supporting data from our MCF7 cell line in which JAM-A had been stably knocked down using lentiviral technology.

In an attempt to account for reduced cell motility after JAM-A knockdown, we examined the expression of various proteins which function in either cell-cell or cell-matrix adhesion.

Knockdown of JAM-A in MCF7 cancer cells dramatically reduced the expression of the cell-matrix protein β1-integrin. This membrane protein links to cytosolic adaptor proteins such as paxillin, which in turn affiliate with F-actin fibres to drive cell migration[26] . However there is conflict in the literature as to the effects of JAM-A knockdown on levels of β1-integrin protein expression. In agreement with our results, JAM-A knockdown by siRNA reportedly reduces β1-integrin expression in colonic epithelial cells via the small GTPase Rap1 [11, 25]. However Naik et al. mention (as data not shown) that JAM-A downregulation in invasive MDA-MB-231 cells did not affect β1-integrin protein levels in their system [14]. This discrepancy may have arisen because levels of JAM-A are already very low in invasive MDA-MB-231 cells relative to either MCF7 cells (in our study) or colonic epithelial cells (as used by Mandell et al. [11]). Therefore it is a possibility that JAM-A levels must be above a certain threshold in order to impact β1-integrin protein levels. It must also be stated that migration is a fundamental cellular function, and that β1-integrin is only one of several proteins utilized by cells during migration. Therefore it is possible that different cell lines utilize alternative pathways of different relative importance for migration, or that other pathways may partially compensate for low levels of JAM-A and/or β1-integrin in order to influence cell migration.

To further explore the possible link between of JAM-A and β1-integrin in breast cancer, we analyzed the expression of β1-integrin protein in our TMA of 270 invasive breast cancers. High β1-integrin protein expression tended to associate with reduced overall survival ($p=0.08$). We found that 22 % of patients expressing high levels of JAM-A also expressed high levels of β1-integrin. However, this association was not statistically significant. One potential reason for this is that we observed very poor β1-integrin staining in most tissue cores. Only 4% of cores stained for β1-integrin were classified as score 2 or 3. This is in contrast to results for JAM-A

membranous staining, where 31% (84 of 270) were classified as score 3 alone. These low levels of β1-integrin staining suggest that IHC antibodies for detection of β1-integrin may not yet be of sufficient quality for this technique; potentially accounting for non-significant associations between poor survival and concomitant over-expression of JAM-A and β1-integrin. In fact there is little published work reporting the use of IHC to assess β1-integrin protein levels in formalin-fixed paraffin-embedded clinical tissues. It is likely that β1-integrin staining in fresh-frozen patient tissues, although more difficult to procure in large numbers, would be more informative.

In a parallel attempt to probe possible associations between over-expression of JAM-A and β1-integrin, we also performed stable over-expression of JAM-A in two breast cancer cell lines with low endogenous JAM-A expression (MDA-MB-231 and Hs578T; **supplemental Fig 2**). Although gene and protein levels of JAM-A increased significantly in both cell lines, JAM-A protein localisation was not, however, restricted to intercellular TJ membranes as in cell lines endogenously expressing JAM-A. Only a small minority of transfected cells showed increases in JAM-A in the cell cytoplasm. We therefore felt that performing functional assays (such as migration) with these lines would not be informative given that JAM-A should localize at tight junction membranes. In addition, our immunohistochemical analysis of JAM-A protein in patient TMAs scored only membrane-associated JAM-A; and therefore clinical data could not be directly compared to functional data from JAM-overexpressing cell lines. However, MDA-MB-231 cells over-expressing JAM-A did show an increase in β1-integrin protein expression, supporting our hypothesis that JAM-A and β1-integrin may be two components of a signalling pathway in breast cancer cells that culminates in increased migration.

As β1-integrin is required for cell migration in some cells [17], our study suggests that loss of β1-integrin downstream of JAM-A knockdown may contribute to observed reductions in migration in our cancer cell line models. Conversely, since over-expression of JAM-A appears to upregulate β1-integrin expression, this may be one potential mechanism driving enhanced cancer cell motility. As tumor cell motility is required early in the metastatic process, we speculate that increased JAM-A expression in invasive breast cancer tissues will in parallel upregulate β1-integrin protein expression and ultimately promote motility. In support of an important effector role for β1-integrin, this protein has long been implicated in breast cancer malignancy via effects on growth, apoptosis, migration and invasion [27]. *In vitro* experiments using 3-dimensional cultures of breast cancer cells show that inhibitory antibodies to β1-integrin induce disorganized aggregates of malignant cells to undergo a phenotypic reversion, ultimately recovering a structural organization *in vitro* which is reminiscent of normal breast acini *in vivo* [16, 28]. It will be interesting to investigate if JAM-A is an important upstream regulator of β1-integrin during migration.

We sum up our study on the potential role of JAM-A in breast cancer by considering at least two distinct possibilities. One possible explanation is that loss of JAM-based cell-cell adhesion in cancer facilitates cell dissociation and promotes motile behaviour (as recently reported by Naik *et al*. [14]). However our data suggest a second possibility, that over-expression of JAM-A in invasive breast cancer patient tissues promotes cell motility via downstream effects on β1-integrin. This may mean that in some breast cancers JAM-A has a more important role in regulating pathophysiological events at the cell-*matrix* interface rather than at the cell-*cell* interface. However a "balancing act" of JAM-A protein expression in normal versus tumor states is likely to crucial and could explain discrepancies between our *in vivo* and recently published *in vitro*[14]

results. Low JAM-A expression in breast epithelial cells is likely to impair cellular adhesion and polarity, favouring tumor initiation. Therefore increasing JAM-A expression in MDA-MB-231 cells, which express low levels of JAM-A, may account for reported reductions in cancer cell invasion [14]. Conversely, reducing high levels of JAM-A expression in breast cancer cells, as we have shown in MCF7 breast cancer cells, may decrease cell motility through downstream effects on β1-integrin. Given the complexity of this situation in relation to human breast cancer, further research is needed to elucidate the potential downstream influences of altered JAM-A signalling on β1-integrin function and tumor cell migratory events.

In conclusion, our data suggest for the first time a novel link between increased JAM-A expression and reduced survival of breast cancer patients. JAM-A may ultimately be a relevant breast cancer biomarker. Our results with a JAM-A inhibitory antibody suggest that JAM-A dimerization is required for downstream promotion of cancer cell migration. If so, it is tempting to speculate that JAM-A may be a promising future target for biological antibody therapies similar to those targeting breast tumors over-expressing HER2 [29].

ACKNOWLEDGEMENTS

We thank Prof. Charles Parkos for J.10.4 antibody and Mairéad Walsh for administrative assistance. This work was funded by Cancer Research Ireland, the Irish Research Council for Science, Engineering and Technology (National Development Plan of Ireland) and the Health Research Board of Ireland ("Breast Cancer Metastasis: Biomarkers and Functional Mediators").

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TABLES

Table 1: Clinicopathological features stratified according to JAM-A protein expression

Variable	*N*	High JAM-A (%)	Low JAM-A (%)	P value
Age (years)	272			0.741
n		84	188	
Median (Range)		63.8 (35 - 89)	64 (36 - 96)	
Tumor Diameter (mm)	272			0.262
T1 (1-20mm)		49 (58.3)	123 (65.4)	
T2 (>20mm)		35 (41.7)	65 (34.6)	
Histological Subtype	272			0.551
Indeterminate		10 (11.9)	11 (5.9)	
IDC		54 (64.3)	132 (70.2)	
ILC		11 (13.1)	26 (13.8)	
Tubular		6 (7.1)	10 (5.3)	
Medullary		2 (2.4)	4 (2.1)	
Mucinous		1 (1.2)	5 (2.7)	
NHG	200			**0.038 ***
I - II		47 (56)	129 (69)	
III		37 (44)	58 (31)	
Lymph Node Status	244			0.183
N0 Negative		43 (56.6)	110 (65.5)	
N1+ Positive		33 (43.4)	58 (34.5)	
ER Status	265			0.16
Negative (1-10%)		16 (19.8)	24 (13)	
Positive (11-100%)		65 (80.2)	160 (87)	
PR Status	216			0.325
Negative (1-10%)		29 (43.3)	54 (36.2)	
Positive (11-100%)		38 (57.7)	95 (63.8)	
Ki67 Status	264			0.081
Negative (1-10%)		25 (30.5)	76 (41.8)	
Positive (11-100%)		57 (69.5)	106 (58.2)	
VEGF	198			0.523
0-2+		55 (83.3)	105 (79.5)	
3+		11 (16.7)	27 (20.5)	
VEGFR1	243			**0.008 ****
0-2+		36 (48)	111 (66.1)	
3+		39 (52)	57 (33.9)	
VEGFR2	196			0.848
0-2+		54 (87.1)	118 (88.1)	
3+		8 (12.9)	16 (11.9)	
HER2	189			**0.003 ****
0-2+		46 (82.1)	127 (95.5)	
3+		10 (17.9)	6 (4.5)	

Values in parentheses indicate percentages. IDC = invasive ductal carcinoma, ILC = invasive lobular carcinoma, NHG = Nottingham Histological Grade, ER = estrogen receptor, PR = progesterone receptor, VEGF = vascular endothelial growth factor, VEGFR1 = vascular endothelial growth factor receptor 1, VEGFR2 = vascular endothelial growth factor receptor 2.

Table 2:
JAM-A mRNA expression in the van de Vijver microarray dataset according to various clinicopathological and molecular characteristics

	JAM-A High (%) (n = 59)	JAM-A Low/Negative (%) (n = 236)	P value (X^2 test)
Age, years			0.682
Median (range)	43.5 (28 - 52)	44 (26 - 53)	
< Median (26-44)	28 (47.5)	105 (44.5)	
> Median (44-53)	31 (52.5)	131 (55.5)	
Tumor Diameter, mm			**0.041 ***
T1 (1-20mm)	24 (40.7)	131(55.5)	
T2 (>20mm)	35 (59.3)	105(44.5)	
Grade			**<0.001 *****
I	7 (11.9)	68 (28.8)	
II	15 (25.4)	86 (36.4)	
III	37 (62.7)	82 (34.7)	
Lymph Node Status			0.727
Negative	29 (49.2)	122 (51.7)	
Positive	30 (50.8)	114 (48.3)	
ER Status			**<0.001 *****
Negative	30 (50.8)	39 (16.5)	
Positive	29 (49.2)	197 (83.5)	
Molecular Subtype [20]			
Normal	0 (0.0)	31 (13.1)	
Luminal A	9 (15.3)	79 (33.5)	
Luminal B	18 (30.5)	63 (26.7)	
Basal	20 (33.9)	25 (10.6)	**<0.001 *****
ERBB2	12 (20.3)	38 (16.1)	
70-Gene Signature[21]			**<0.001 *****
Good Prognosis	7 (11.9)	108 (45.8)	
Poor Prognosis	52 (88.1)	128 (54.2)	

FIGURE LEGENDS

Figure 1: High levels of JAM-A protein are associated with poor prognosis in breast cancer patients

A. 270 invasive breast cancer TMA cores were scored either 0, 1+, 2+ or 3+ based on levels of membranous JAM-A protein expression. Kaplan-Meier analysis comparing tumors scored 0, 1+ or 2+ (JAM-A low) to those scored 3+ (JAM-A high) for breast cancer-specific survival (**B.**) and recurrence-free survival at 5 years (**C.**) based upon JAM-A protein expression.

Figure 2: High levels of JAM-A mRNA are associated with poor prognosis in breast cancer patients

Kaplan-Meier estimate of overall survival (**A.**) and probability of metastasis as first event (**B.**) with low versus high mRNA expression of JAM-A in the van de Vijver *et al*. (2002) DNA microarray dataset, comprised of data from 295 primary invasive breast tumors.

Figure 3: JAM-A knockdown or antibody-based inhibition reduces migration of MCF7 cells

A. Migration of JAM-A-shRNA MCF7 cells and NF-shRNA cells were compared using scratch-wound assays photographed at 0, 2, 4, 6, and 8 hours post-injury. Error bars refer to standard deviation from the mean (S.D.) and represent triplicate values in a representative experiment. **B.** MTT proliferation assays were conducted on NF-shRNA and JAM-A-shRNA MCF7 cell lines. Error bars refer to S.D. and represent five internal replicates. **C.** Scratch-wound assays were used to assess the migration of wild-type MCF7 cells pre-incubated for 2 hours with 10μg/ml JAM-A inhibitory antibody (J.10.4) or for controls, media alone. Error bars refer to S.D. and represent triplicate values in a representative experiment. **D.** Western blot analysis of a panel of

proteins was conducted on NF-shRNA and JAM-A-shRNA MCF7 cell lines. Assessment of actin expression was performed to control for protein loading. *Note*: Unpaired Student's t-tests were performed to assess differences between cell lines in functional assays A, B and C.

Figure 4: β1-integrin expression in breast cancer

A. Kaplan-Meier estimate of overall survival with low versus high mRNA expression of β1-integrin in the van de Vijver *et al*. (2002) DNA microarray dataset, comprised of data from 295 primary invasive breast tumors. **B**. Kaplan-Meier estimate of overall survival with low versus high protein expression of β1-integrin in the TMA of 270 patients used to determine JAM-A expression in **Fig.1**. *Note: five patients cores were unsuitable for analysis therefore n = 265*. **C.** Number of tissues from invasive breast cancer TMA (n = 270) which overexpressed JAM-A *versus* JAM-A plus β1-integrin. **D**. Western blot analysis showing the expression of JAM-A and β1-integrin in MDA-MB-231 empty vector control and MDA-MB-231 JAM-A over-expressing cells.

Supplemental Figure 1: JAM-A knockdown in MCF7 breast cells

A. MCF7 cells were transduced with pVTLH containing JAM-A hairpins shRNA1, shRNA2 or shRNA3 or empty pLVTH vector (EV), and analyzed for levels of JAM-A gene expression by TaqMan RT-PCR. **B.** JAM-A protein expression levels in EV and shRNA-JAM-A MCF7 lines as determined by Western blot analysis. **C.** Immunofluorescent localization of JAM-A protein in MCF-7 cells expressing non-functioning shRNA1 (NF-shRNA) or JAM-A shRNA3 (JAM-A-shRNA).

Supplemental Figure 2: Over-Expression of JAM-A in Breast Cancer Cell Lines

Western blot comparison of JAM-A protein expression in Hs578T **(A.)** and MDA-MB-231 **(B.)** empty vector and JAM-A over-expressing cell lines. TaqMan RT-PCR results showing JAM-A mRNA expression in Hs578T **(C.)** and MDA-MB-231 **(D.)** empty vector and JAM-A over-expressing cell lines. Fold-normalized JAM-A gene expression in the over-expressing cell line relative to the empty vector control cell line is plotted on the Y-axis. Confocal immunofluorescence micrographs show JAM-A, and F-actin protein localization patterns in Hs578T **(E.)** and MDA-MB-231 **(F.)** empty vector and JAM-A over-expressing cell lines.

Figure 1: McSherry et al.

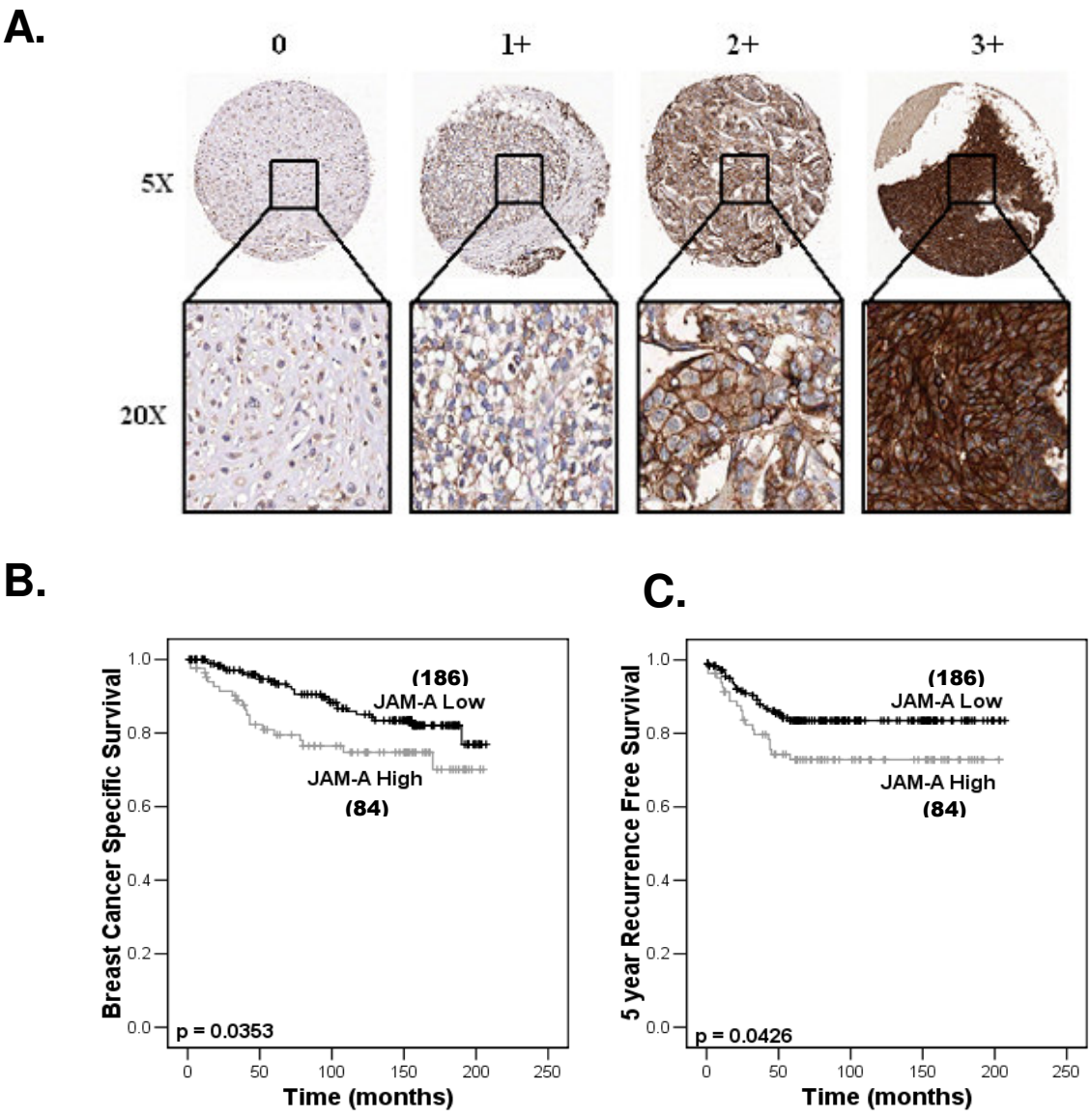
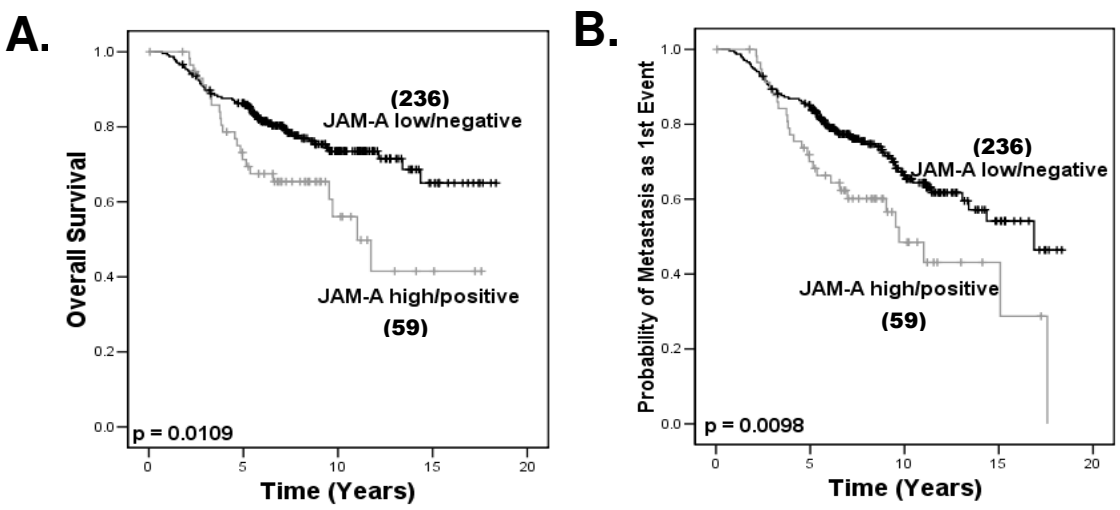


Figure 2: McSherry et al.



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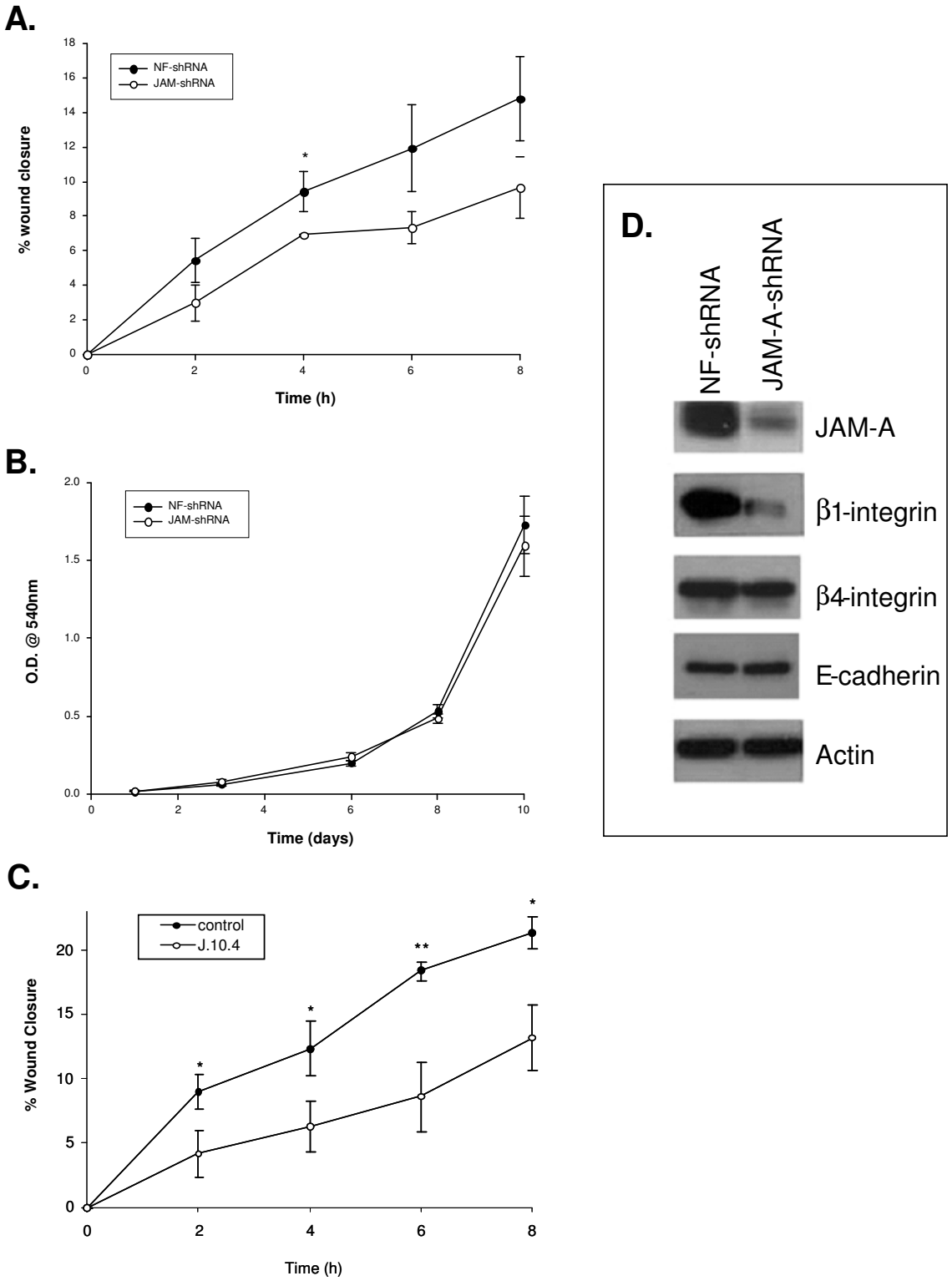
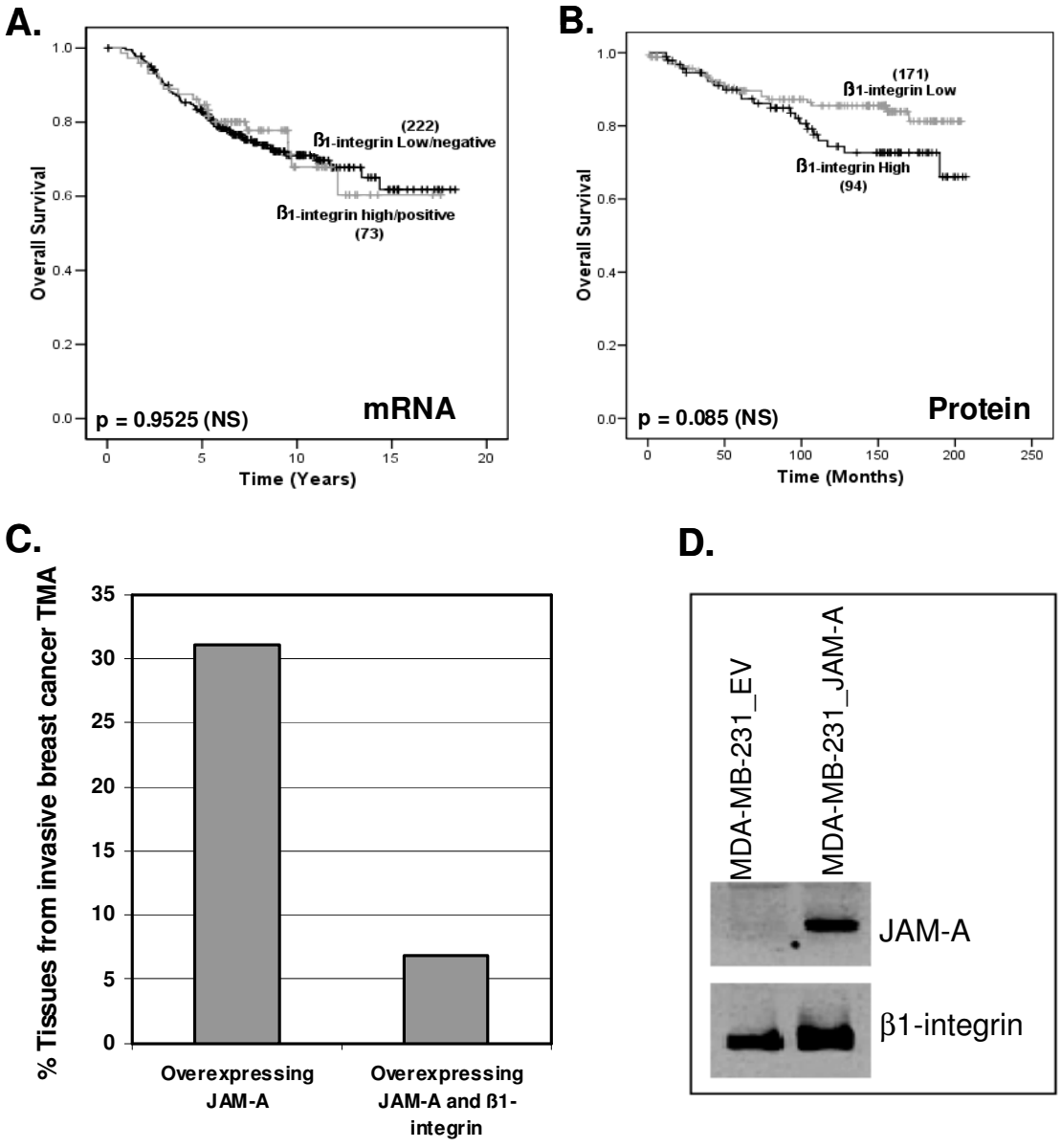
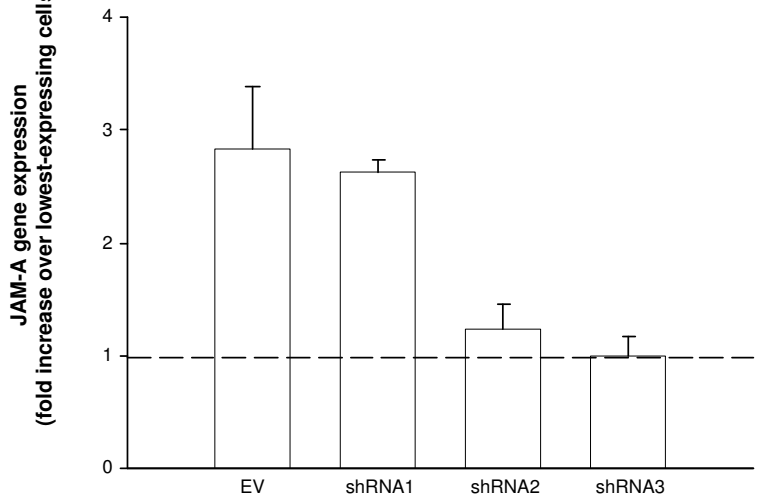


Figure 4: McSherry et al.



Supplemental Figure 1: McSherry et al.

A.



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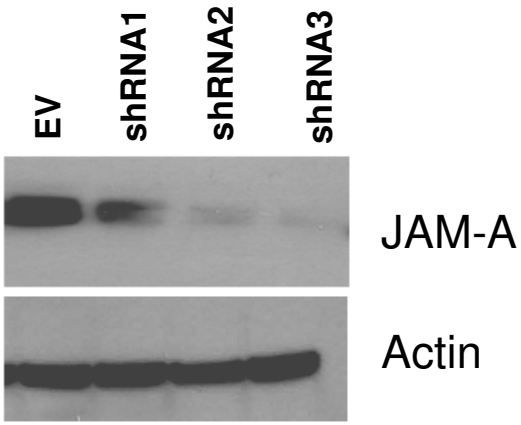
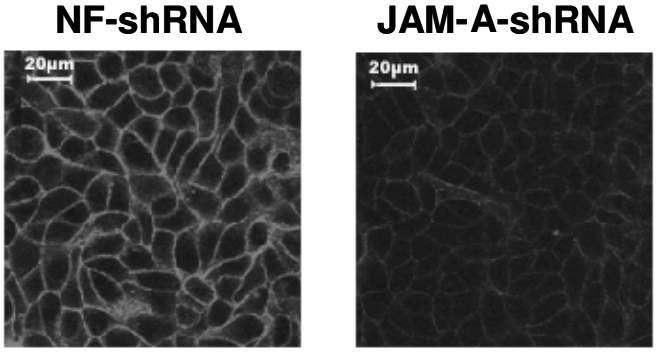


C.



Supplemental Figure 2: McSherry et al.