THE ROLE OF JUNCTIONAL ADHESION MOLECULE-A IN CANCER CELL MIGRATION AND MORPHOLOGY

by

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ABSTRACT

The cell adhesion molecule (CAM) family of proteins is a large group of proteins that are typically transmembrane proteins used in the attachment of the cell to another cell or to the extracellular matrix (ECM). Junctional adhesion molecule A (JAM-A) is a member of the CAM family that has recently been identified to have a role in various cancers. Currently there is conflicting data for JAM-A in breast cancer concerning the role of JAM-A in cellular migration. Using the highly metastatic cell line MDA-MB-231, the aim for this study was to determine JAM-A's role in the migration of the MDA-MB-231 breast cancer cells and to start to determine the mechanism by which JAM-A may be contributing. Through transfection of the MDA-MB-231 cells with full length JAM-A, cytoplasmic deletion JAM-A (Δ257 JAM-A) and tyrosine to phenylalanine 261 JAM-A (Y-F^{261} JAM-A), this study shows that full length JAM-A decreased the migration of MDA-MB-231 cells and surprisingly Y-F^{261}JAM-A along with Δ257 JAM-A further decreased migration in transfected cells. Using immunofluorescence it was determined that JAM-A localized at the cell-to-cell adhesions between the transfected MDA-MB-231 cells and contributed to a more epithelial like morphology overall. Also through immunofluorescence, it was shown that transfection with full length JAM-A, Δ257 JAM-A and Y-F^{261} JAM-A increased

the cell-to-cell adhesions. These findings make JAM-A an interesting protein to examine in the migration and metastasis of metastatic breast cancer.

Chapter 1

INTRODUCTION

Cancer Metastasis

Cancer cell metastasis is the process by which cancer cells break away from the primary tumor and spread to another area in the body. These cells can then form secondary and tertiary tumors called metastatic tumors (National Cancer Institute 2011). In order for the cells to be able to form a metastatic tumor they need to be able to navigate the stroma, intravasate the vasculature, survive in the vasculature, extravasate the vasculature and then be able to survive in the new location (Yilmaz & Christofori, 2010). This is outlined in Figure 1.1. Without being able to perform all of these functions, a metastatic tumor will not form and the cells will most likely die along the way.

To detach from the primary tumor, cancer cells need to dissolve their cell-to-cell adhesions and lose their cell specific morphology(Yamaguchi, Wyckoff, & Condeelis, 2005). In the most common type of cancer (adenocarcinoma), epithelial cells often lose their epithelial morphology and transition to a more mesenchymal morphology. This is called epithelial to mesenchymal transition (EMT). During the process of EMT a common occurrence is for the cells to reduce or even completely stop their expression of cellular adhesion molecules such as E-cadherin, while up

regulating other proteins. While E-cadherin expression is being down regulated, N-cadherin is being upregulated aiding the cells to detach from their neighboring cells. The exchange of cadherin expression is just one marker for EMT and the loss of cell-to-cell adhesions (Cavallaro & Christofori, 2004; Onder et al., 2008). After the cells lose their cell-to-cell adhesions, they can now break away from the primary tumor and continue on the pathway to forming a metastatic tumor.

While dissociating from the surrounding cells in the primary tumor, the cancer cells need to be able to break through the basement membrane and migration through the extracellular matrix. In order to break through the basement membrane, the cancer cells secrete proteins such as matrix metalloproteinases (MMP) (Chambers & Matrisian, 1997; Zhang, Ma, & Fan, 2010). This large family of proteinases, which include the collagenases, stromelysins, elastases, gelatinases and membrane bound MMPs, degrade the ECM proteins and have been shown to contribute to angiogenesis and ECM remodeling to facilitate collective mass movement of cancer cells (S. A. Brooks, Lomax-Browne, Carter, Kinch, & Hall, 2010; Deryugina & Quigley, 2006; Friedl & Wolf, 2008). By secreting these proteinases the cancer cells are able to create a path from the primary tumor and begin their journey towards a secondary location.

In order to continue the process of metastasizing from the primary tumor, cancer cells need to be able to migration through the stroma. These cells can migrate in a multitude of different ways depending on the cell type and microenvironments. In broad categories cells can either migrate as single cells or as groups of cells. In single

cell migration there is further categorization such as mesenchymal migration and amoeboid migration. Mesenchymal migration is characterized by a five-step migration cycle (Friedl & Wolf, 2003; Polette et al., 1998). The first step in this five-step process is the protrusion of the leading edge by the polymerization of actin filaments. The small GTPases Rac, Cdc42, Rho and Ras regulate the process of actin polymerization (Kaibuchi, Kuroda, & Amano, 1999). This leading forms focal adhesions through integrins binding with ECM proteins. Then the integrins are connected to the actin filaments through adaptor proteins such as focal adhesion kinase (FAK) and talin (Burridge & Chrzanowska-Wodnicka, 1996). Once focal adhesions are formed, surface proteases such as MMPs and the pro-MMPs are localized to the focal adhesions in order to form soluble MMPs (Friedl & Wolf, 2003; Mueller et al., 1999). These soluble MMPs initiate focused proteolysis of the ECM. Now that a path through the ECM is being carved out for the cell to fit through, the cells need to contract through the actomyosin process. This process allows the cell to pull itself through the ECM using an actin myosin interaction controlled by Rho and Rho kinases (Fukata, Amano, & Kaibuchi, 2001). Finally the trailing edge of the cell needs to detach from the ECM by cleaving the focal adhesion protein complexes and integrins are internalized in vesicles to be recycled to be used again in future focal adhesions (Bretscher, 1996; Friedl & Wolf, 2003). Cell lines such as the metastatic breast cancer line MDA-MB-231 utilize this type of migration (Friedl & Wolf, 2003). Unlike mesenchymal migration amoeboid migration does not form strong connections with the ECM. These cells glide over the ECM utilizing weak binding interactions that is integrin independent (Fukui, 2002). Lymphomas and small cell lung

carcinomas are usually observed to move in an amoeboid fashion (Friedl & Wolf, 2003).

Groups of cells can collectively migrate as sheets, strands, or clusters of cells. Through this type of migration many cells can move from the primary tumor at the same time. Studies have shown that this type of migration is required for proper embryogenesis, but it is also seen often in many types of carcinomas (Friedl & Wolf, 2003; Theveneau & Mayor, 2013). With this type of migration cell to cell adhesions are not broken within the moving cluster unlike in the single cell migration methods. Proteins such as N-cadherin, Wnt and β-catenin can help to promote collective migration (Aman & Piotrowski, 2008; Cui & Yamada, 2013). Collective migration may have advantages over single cell migration due to the ability to secrete high levels of autocrine signaling of pro-migratory molecules, ECM proteinases and the ability to protect inner cells from the body's immune response (Friedl & Wolf, 2003).

Intravasation, survival in the vasculature and extravasation are all important steps for metastasis to occur. Intravasation occurs when the cancer cells migrate through the vascular basement membrane and the endothelial layer in blood vessels or the endothelial layer of the lymphatic vessels. Intravasation possibly occurs through two different theories (Bockhorn, Jain, & Munn, 2007; van Zijl, Krupitza, & Mikulits, 2011). The first is active migration into vessels. This involves the cells secreting MMPs that allow the cells to digest the ECM proteins in the vasculature basement membrane in blood vessels along with cell-to-cell connections formed between endothelial cell layer of blood vessels or lymphatic vessels (Freije et al., 2003). These

cells are actively moving towards a vessel and invading into it. The second possible method is passive shedding of tumor cells into the lumen of vessels. This type of intravasation comes from the leaky tumor vessels derived from tumor angiogenesis (Chang et al., 2000; Yilmaz & Christofori, 2010). In this method cells from the growing tumor are pressed against the leaky vasculature created by tumor angiogenesis and are shed into the vasculature as the tumor grows in size (Chang et al., 2000). Regardless of method, once the cells have entered the vasculature they need to survive. Platelets can adhere to invading cancer cells expressing glycoproteins that are properly sialylated through selectins and possibly protect them from the natural killer cells or possibly secrete growth factors such as vascular endothelial growth factor and aid in attachment to the endothelial cells of the vessel (Nash, Turner, Scully, & Kakkar, 2002; Stone & Wagner, 1993). Along with platelets, cancer cells can also interact with leukocytes potentially aiding in vasculature survival and extravasation (Konstantopoulos & Thomas, 2009; Slattery & Dong, 2003).

Finally once the cells have survived the vasculature and extravasate from the vessel, they need to have the ability to survive in the new environment. Many types of cancer have shown a propensity metastasize to certain distal organs more frequently than others. This is exemplified by breast cancer metastasizing to bone or melanoma to the lungs (Zhang et al., 2010). This preferential metastasis indicates that certain organs express growth factors or proteins that are required for the cancer to survive. Research has recently come out suggesting that the cancer cells are influencing the microenvironment in order to increase survivability (Guise et al., 1996; Weilbaecher, Guise, & McCauley, 2011). Through this microenvironment influence the metastatic

cells can now proliferate and form the secondary tumors. From here the cancer cells can repeat the entire metastatic process and metastasize to new locations throughout the body.

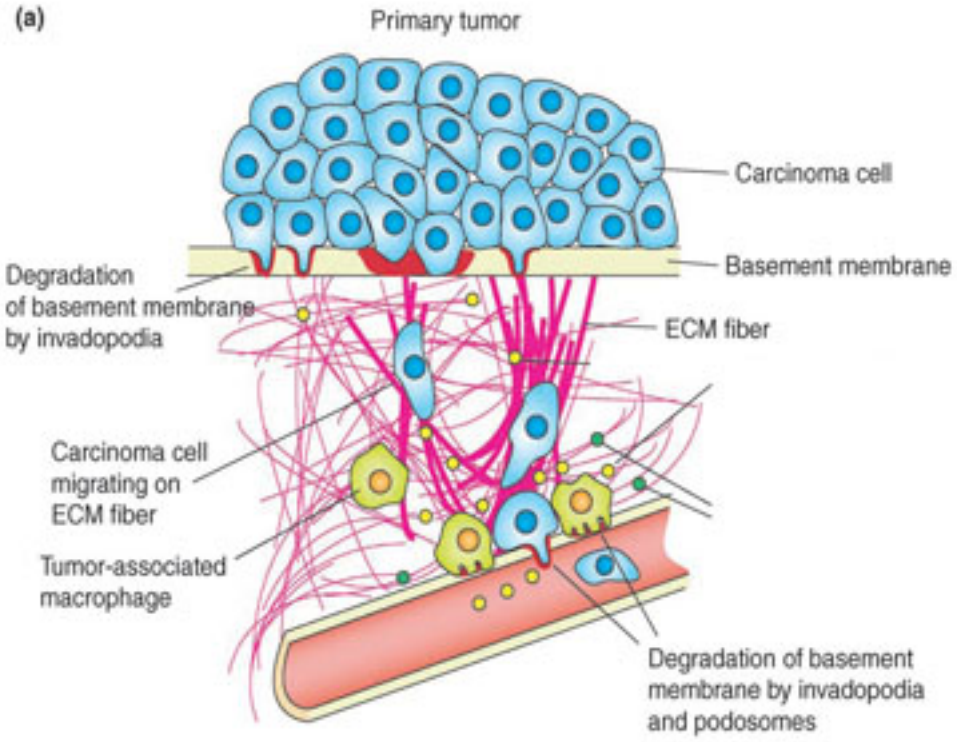

Figure 1.1: Brief schematic of the intial steps of metastasis
A summary of the first steps of metastasis consisting of breaking through the initial basement up until intravasion of the vasculature. Adapted from Yamaguchi et. al 2005.

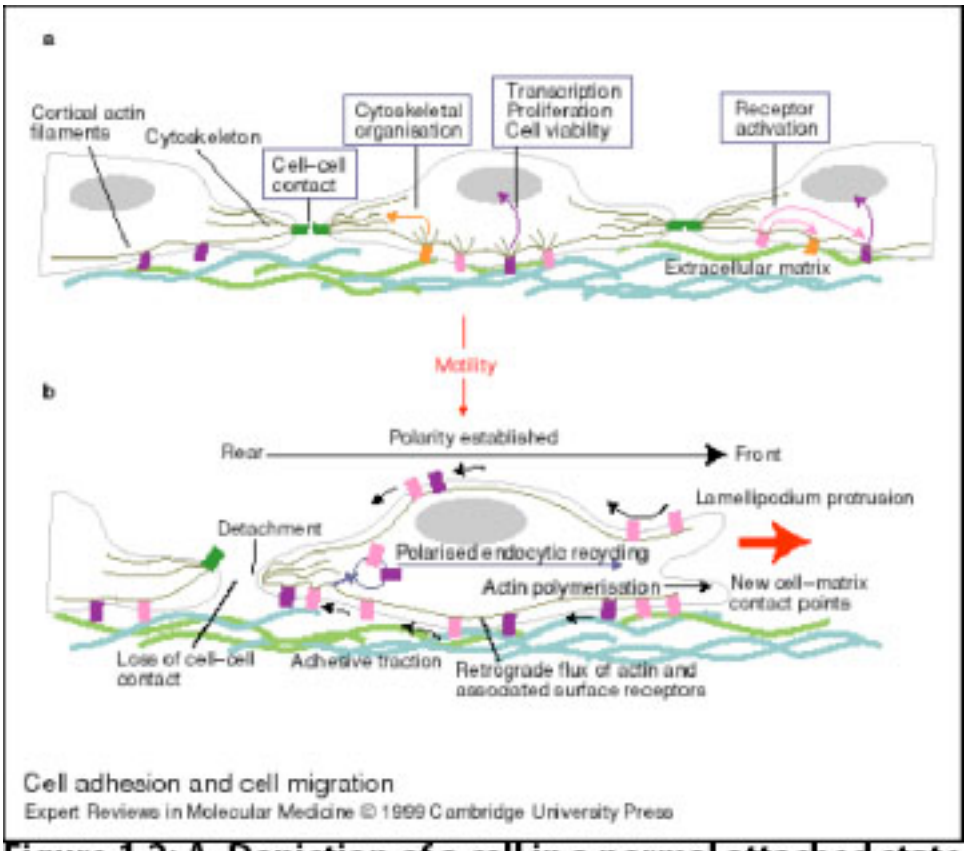

Figure 1.2: A. Depiction of a cell in a normal attached state before the migration process begins. Cells show attachment to the extracellular matrix and cell-to-cell contacts. B. Depiction of a cell in the process of migration over its extracellular matrix. There is a distinct front to rear polarity and actin tread milling is occurring.

Cell Adhesion Molecules in Cancer Metastasis and Migration

The family of proteins called cell adhesion molecules (CAM) are typically transmembrane proteins used to bind other CAMs on adjacent cells or ECM proteins. The typical CAM has an extracellular domain, transmembrane domain and a cytoplasmic tail. This family of proteins contains four predominate sub families: cadherins, selectins, integrins and the immunoglobulin superfamily. Each one of these families has been implicated in cancer migration and metastasis in some fashion.

In the cadherin family, research has shown that in carcinomas E-cadherin expression is absent or a mutated non-functional version of the protein is expressed (Cavallaro & Christofori, 2004; Perl, Wilgenbus, Dahl, Semb, & Christofori, 1998). Once E-cadherin is depleted or non-functional, proteins such as β-catenin are free to signal in other pathways such as the wnt pathway (Gottardi, Wong, & Gumbiner, 2001). The increase in wnt signaling from depletion of E-cadherin has been shown to increase proliferation of the metastatic cells (Gottardi et al., 2001). In some carcinomas in the place of E-cadherin, N-cadherin is upregulated and can enhance metastatic cell signaling (Hazan, Phillips, Qiao, Norton, & Aaronson, 2000). N-cadherin has the ability to bind fibroblast growth factor receptor family members and

other proteins increasing MAPK signaling leading to increased motility and invasion (Suyama, Shapiro, Guttman, & Hazan, 2002). Other members of the cadherin family such as VE-cadherin which is normally expressed in endothelial cells, have also shown expression in metastatic cancers leading to increased survival or increased motility (Cavallaro & Christofori, 2004).

The selectin family of CAM proteins bind sialylated proteins on adjacent cell membranes. As cancer progresses and becomes more metastatic, the expression of sialylated proteins on the cell surface increases (Kannagi, Izawa, Koike, Miyazaki, & Kimura, 2004). This increase in sialylated proteins allows the cancer cells to take advantage of cells in the blood stream expressing different selectins. P-selectin on platelets has been identified as a key protein in the binding of platelets to metastasizing cancer cells in the blood (Borsig et al., 2001). Aggregation of platelets on metastasizing cancer cells in mice deficient in P-selectin was almost non-existent providing evidence that P-selectin expression is key to platelet-carcinoma binding (Borsig et al., 2001). E-selectin expression also shows a correlation with cancer metastasis. E-selectin is expressed on activated endothelial cells and it is upregulated in certain types of cancer (Borsig et al., 2001). Expression of E-selectin could possibly allow cancer cells to "roll" across the endothelial layer of the vessels and then selectively extravasate (Laubli & Borsig, 2010).

Integrins are a heterodimeric protein composed of an α and a β subunit capable of binding extracellular matrix protein along with a variety of proteins on the intracellular in order to produce focal adhesions and attach cells to the ECM. As previously mentioned, integrins are an integral part of the cell migration process, but the integrins contribution to the metastasis of cancer is not simply limited to binding of ECM proteins and being a part of migration. Integrins are also capable of signaling within the cell. The intracellular domain of integrins has the ability to associate with a variety of proteins and lead to survival signaling and pro-migratory signaling such as MAPK, small GTPase proteins such as Ras and protein kinase C (Hood & Cheresh, 2002). Along with signaling integrins have been shown to be involved in MMP activation and recruitment to the leading edge of the migrating cancer cell (P. C. Brooks et al., 1996). This allows for focused ECM protein degradation, aiding the cancer cell in migrating through the stroma.

The last major family of CAM proteins is the Ig super family. The Ig cell adhesion molecules are distinguished by the presence of one or more Ig-like domains in the extracellular portion of the protein (Cavallaro & Christofori, 2004). These characterized Ig-like domains can be divided into two categories, variable domain or constant domain (Barclay, 2003). The Ig domains are characterized by a conserved di-sulfide bond that binds two beta sheets (Barclay, 2003). Variable Ig domains are typically classified as having between 65 and 75 amino acids whereas the constant Ig domains typically have 55 to 60 amino acids (Williams & Barclay, 1988). These Ig-

like domains are recognized by a wide variety of other proteins allowing one protein from the Ig super family bind multiple other proteins (Barclay, 2003).

More and more of the members from the Ig super family are being identified in cancer. Members such as neural cell adhesion molecule (NCAM) are being identified as down regulated or having different isoforms expressed in certain cancers (Cavallaro & Christofori, 2004; Perl et al., 1999). Down regulation or loss of NCAM leads to decreased adhesion to ECM proteins and eventual metastasis through decreased beta 1 integrin (β_1) activation (Cavallaro, Niedermeyer, Fuxa, & Christofori, 2001). Another member melanoma cell adhesion molecule (MCAM) has increased expression in more metastatic cancers by increasing invasiveness and metastasis in those cell lines (Wai Wong, Dye, & Coombe, 2012; Zeng, Cai, & Wu, 2011). This member of the Ig superfamily increases invasiveness and metastasis through increased survival signals such as pAkt pathway and increased pro-angiogenic proteins such as VEGF (Zeng et al., 2011). Another protein in the Ig superfamily recently identified as having a role in cancer cell migration is junctional adhesion molecule A (JAM-A) (M. Naik, 2008). JAM-A was shown to be decreased in more metastatic cancer cell lines (M. Naik, 2008). Despite showing decreased JAM-A in more metastatic cell lines, there has been conflicting evidence when JAM-A is transfected into these cell lines (McSherry, Brennan, Hudson, Hill, & Hopkins, 2011; M. Naik, 2008). More study of this protein is needed in order to determine its role in cancer cell migration and the exact function of the protein in terms of cancer cell migration and metastasis.

Junctional Adhesion Molecule A

Junctional Adhesion Molecule A (JAM-A) is a 299 amino acid, 32kDa protein in the Ig-superfamily of cell adhesion molecules located on Chromosome 1 at q21.2-q21.3 (Figure 1.3) (U. P. Naik, Naik, Eckfeld, Martin-DeLeon, & Spychala, 2001; Theveneau & Mayor, 2013). JAM-A is a type 1 transmembrane protein with primary localization at the tight junctions of cells such as endothelial and epithelial cells. It has two Ig loops linked by cysteine disulfide bonds at amino acid 50 and 109 for the variable Ig loop (D1) and at 153 and 212 for the constant Ig loop (D2). There are two N-glycosylation sites located on the D2 Ig loop. At amino acids 61 and 63, glutamic acid and lysine respectively, JAM-A can form cis homodimers. After a single pass transmembrane domain, JAM-A has a short cytoplasmic tail. This tail contains a type II PSD-95, discs large, ZO-1 (PDZ) binding domain at the end of the cytoplasmic tail along with an actively phosphorylated serine at 285 and a tyrosine at 280 (U. P. Naik et al., 2001) The amino acid sequence of JAM-A can be seen in Figure 1.4 and a graphical representation can be seen in Figure 1.5.

The extracellular region of JAM-A has been shown to be involved in homophilic cis and trans binding (Kostrewa et al., 2001; Mandell, McCall, & Parkos, 2004; U. P. Naik et al., 2001). Disrupting the cis homophilic binding of JAM-A

shows disruptions in function leading to increased junction permeability and loss of function (Mandell et al., 2004; Severson et al., 2008; Severson & Parkos, 2009). Along with homodimerization, the extracellular regions have been shown to interact with integrins such as β_3, β_1, and β_2 (M. U. Naik & Naik, 2006; M. U. Naik, Stalker, Brass, & Naik, 2012; Ostermann, Weber, Zernecke, Schroder, & Weber, 2002).

Although the cytoplasmic domain is relatively small, it has been shown to be important in allowing JAM-A to function in its roles in cell adhesion, monocyte transmigration, cell motility, proliferation and thrombosis (Bazzoni et al., 2005; Martin-Padura et al., 1998; M. U. Naik et al., 2012; U. P. Naik & Eckfeld, 2003; Nava et al., 2011; Ozaki et al., 1999). Through its PDZ binding domain, JAM-A has been shown to bind proteins such as ZO-1, Afadin, and PAR-3. Along with the PDZ domain, the phosphorylation sites at 284 serine and 280 tyrosine have been shown to be involved in modulating JAM-A's activity in different cell types (Iden et al., 2012; U. P. Naik, Ehrlich, & Kornecki, 1995; Ozaki et al., 2000; Ozaki et al., 1999).

JAM-A has been shown to have a wide distribution throughout a variety of cell types. JAM-A can be found in endothelial cells, epithelial cells, platelets, sperm neural cells, and leukocytes (Aravindan et al., 2012; U. P. Naik et al., 1995; Stelzer, Ebnet, & Schwamborn, 2010). JAM-A is primarily localized at the tight junctions in cells such as epithelial cells and the reduction or ablation of JAM-A has been shown to increase permeability of the cells and reduced JAM-A has been associated with diseases such as inflammatory bowel syndrome caused by increased permeability in cell junctions (Iden et al., 2012; Kucharzik, Walsh, Chen, Parkos, & Nusrat, 2001;

Mandell et al., 2004). In JAM-A knockout mice the loss of JAM-A also leads to increased intestinal permeability (Laukoetter et al., 2007).

In epithelial cells JAM-A is localized to the tight junctions and associated with proteins such as ZO-1, PAR3 and Afadin. Loss of JAM-A or decreases in JAM-A in epithelial cells disrupts junction integrity. Along with this, loss of JAM-A in epithelial cells increases proliferation through an Akt and β-catenin pathway (Nava et al., 2011). While JAM-A has been identified as primarily a tight junction protein in epithelial cells and endothelial cells, its function is not solely limited to junction integrity. In endothelial cells JAM-A has been shown to function in leukocyte transmigration and migration of endothelial cells (M. U. Naik, Vuppalanchi, & Naik, 2003; Ostermann et al., 2002). JAM-A levels correspond positively with migration in FGF stimulated endothelial cells and endothelial cells plated on vitronectin (M. U. Naik & Naik, 2006; M. U. Naik et al., 2003). This was shown to be through association with $\alpha_v\beta_3$ allowing downstream signaling leading to activated focal adhesion kinase and mitogen activated protein kinase.

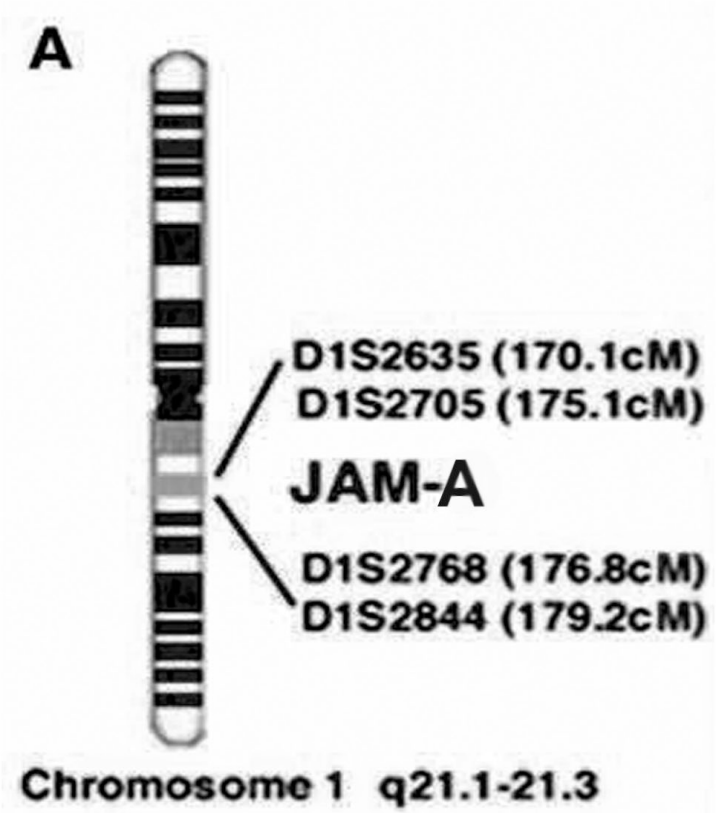

Figure 1.3 Chromosomal location of JAM-A. Junctional Adhesion Molecule A (JAM-A) is located on the long arm of chromosome 1 at location 21.1-21.3. Adapted from Naik et al. 2001.

MGTKAQVERKLLCLFILAILLCSLALGSVTVHSSEPEVRIPENNPVKLSC **50**

AYSGFSSPRVEWKFDQGDTTRLVCYNNKITASYEDRVTFLPTGITFKSVT **100**

REDTGTYTCMVSEEGGNSYGEVKVKLIVLVPPSKPTVNIPSSATIGNRAV **150**

LTCSEQDGSPPSEYTWFKDGIVMPTNPKSTRAFSNSSYVLNPTTGELVFD **200**

PLSASDTGEYSCEARNGYGTPMTSNAVRMEAVERNVGVIVAAVLVTLILL **250**

GILVFGIWFAYSRGHFDRTKKGTSSKKVIYSQPSARSEGEFKQTSSFLV **299**

Figure 1.4 Protein sequence of Junctional Adhesion Molecule A.
Shown above is the amino acid sequence of human JAM-A. Total length of the protein is 299 amino acids. The 27 amino acid signal sequence is underlined.

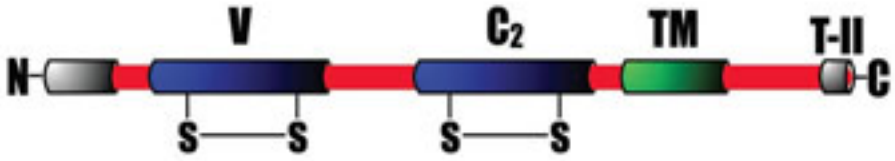

Figure 1.5 : Representation of Junctional Adhesion Molecule A. This figure shows the section arrangement of JAM-A protein. JAM-A is a type I transmembrane protein with two IG loops, a Variable loop depicted by V and a Constant loop depicted by C_2 in the extracellular domain. The short intracellular domain follows the transmembrane region (TM) and contains a PDZ binding domain.

Junctional Adhesion Molecule A in cancer

JAM-A has been shown to have a role in various cancers such as breast cancer, clear cell renal cancer, endometrial cancer and pancreatic cancer (Fong et al., 2012; Kojima & Sawada, 2012; M. Naik, 2008). In many cases JAM-A is involved in the potential migration of the cells or overall patient survival. In pancreatic cancer Fong, D. et al. show that low levels of JAM-A correspond to poor survival. While the current mechanism by which JAM-A functions is not known, pancreatic cancer patients were shown to have a decreased progression free interval along with a lower overall survival rate (Fong et al., 2012). Another type of cancer, endometrial cancer, also shows a decrease in overall patient survival rate and a decrease in their progression free intervals (Koshiba et al., 2009). Furthermore Koshiba et al. 2009 showed that poorly differentiated endometrial carcinoma cells had significantly decreased JAM-A compared to well differentiated endometrial carcinoma cells.

In melanoma cell lines, increased JAM-A levels correspond to decreased transendothelial migration(Ghislin et al., 2011). This mechanism is not fully understood, but Ghislin et al. 2011 propose that JAM-A decreases transendothelial migration because JAM-A of its role in cell polarity and junction formation. The study by Gutwein et al. 2009 looks at a different type of cancer, clear cell renal cancer and proposes a similar reason for reduced migration. Gutwein et al. look at the biopsy's of patients with clear cell renal cell carcinoma and find that JAM-A expression is significantly decreased compared to normal human kidney tissue. Using

tumor necrosis factor (TNFα) and interferon-γ (INFγ) on proximal and primary tubular cell, Gutwein et al. show that JAM-A is cleaved from the cell membranes in a matrix metalloproteinase manor. From here Gutwein et al. knocked JAM-A expression down in the RCC4 cell line, which had highly expressed JAM-A previously. The group found that when JAM-A was decreased, there was an increase in migration. They conclude that the downregulation of JAM-A may be due to the shedding of matrix metalloproteinases in the clear cell renal cell carcinoma(Gutwein et al., 2009).

In breast cancer cell lines and in breast cancer tumor the data on JAM-A is conflicted. In the paper from Naik et al. 2008 the group shows that increasing JAM-A in a highly metastatic cell line, MDA-MB-231, decreases the cells migration on collagen while decreasing JAM-A expression in a non-metastatic cell line, T47D, through siRNA increases the cells migration. This group also shows that JAM-A is decreased in breast cancer tumor tissue. In opposite, McSherry et al. 2009 show that increased amounts of JAM-A in breast cancer cell lines leads to increased migration. The group also shows that patients with tumors that have increased JAM-A have reduced survival.

Because of the conflicting data for JAM-A in breast cancer, there needs to be more research done to show its role in breast cancer cell migration and the potential mechanism. This study attempts to show that increased JAM-A in the breast cancer cell line MDA-MB-231 decreases cell migration on a collage matrix and that increased JAM-A increases the cell-to-cell connections. Along with this, the study

will show that JAM-A still decreases cell migration in the absence of the cytoplasmic portion of the protein while also still increasing the cell-to-cell connections.

Chapter 2

MATERIALS AND METHODS

Cell Culture and Transfection

All experiments were performed on a breast cancer cell line, the MDA-MB-231 cell line. The cell line was obtained from Dr. Kenneth van Golen at the University of Delaware. This cell line was originally obtained from a metastatic site, a pleural effusion, of a 51 year old woman. These cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum with 1% 100 units/mL penicillin and 300μg/mL streptomycin. All cells were maintained at 37°C in a humidified environment at 95% air with 5.0% CO_2.

All cells were stably transfected with either an empty pcDNA 3.1 vector or constructs containing full length JAM-A, JAM-A lacking cytoplasmic domain (Δ257 JAM-A) or JAM-A in which the tyrosine 261 is substituted with phenylalanine (Y-F^{261} JAM-A). To transfect the cell line, FUgene HD 6 (Roche) was used. To prepare for transfection MDA-MB-231 cells were plated on 6 well plates at a density of 300,000 cells per well over night. The following day the transfection complex was made by adding 3μg of construct or empty vector DNA and 9μL of FUgene HD 6 to

150μL of Opti-MEM medium. Medium was refreshed on the cells plated the previous day and then the transfection complex was added in a drop-wise manner to each well. Cells were kept for 48 hours and then the medium was changed to a high G418 solution containing RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin and 500 μg/mL G418 (Invitrogen). Cells were kept in this medium for 15 days and then the medium was changed to a low G418 solution containing only 300 μg/mL G418 (Invitrogen). This method was done according to the protocol established by Roche. Expression of the empty vector and the constructs was determined by western blotting and flow cytometry using the Accuri C6 flow cytometer.

Cell Migration Assay

Cells transfected with mock, JAM-A full length, Δ257 JAM-A and Y-F^{261} JAM-A were serum starved over night in RPMI 1640 supplemented with 0.5% bovine serum albumin. An 8 micron pore membrane from Neuroprobe had its underside coated with 30μg/mL collagen and was stored at 4°C over night. The following day the migration assay apparatus from Neuroprobe was assembled using the 8 micron pore membrane coated with collagen from the previous night. The bottom well contained 28μL of serum free medium that was used to serum starve the cells. The stably transfected cells that were serum starved the previous night were detached using sterile EDTA. Cells were counted with a hemocytometer and 5,000 cells in 50μL

were added to the top well of the migration assay apparatus. This apparatus was stored in 37°C with 5% CO_2 for 5 hours in order to let the cells migrate. After 5 hours the membrane was removed from the apparatus and the top was cleared of the non-migrated cells. Cells on the bottom of the membrane were fixed using 4% paraformaldehyde and stained using the Diff Quik reagents. Every cell in each well was counted using a microscope with a 40X objective. Protocol was derived from Naik et al. 2008 with only changes to the membrane system used for the cells to migrate through.

Time Lapse Microscopy

Cells stably transfected were detached using sterile EDTA and counted using a hemocytometer. 5,000 cells were added per well of an 8-chambered cover glass slide. Cells were allowed to attach to the 8 chambered cover glass slide chamber over night in a humidified environment at 37°C with 5% CO_2. The following day the 8 chambered cover glass slide was placed on an inverted microscope with Hoffman 40X objective (Zeiss Axiovert 200) equipped with a 37° heating chamber supplied with 5% CO_2. 4 different, non intersecting areas in each chamber were randomly chosen in each of the cardinal directions to provide an adequate representation of the average length of migration on the slide per cell. Each area was each photographed by the charge-coupled camera every 5 minutes for 12 hours using AxioVision 4.3 software. Cell paths were tracked using ImageJ software from NIH with the plugins Manual

Tracking and Chemotaxis Tool from Ibidi. This protocol was derived from Naik et al. 2008 with some minor changes.

Immunofluorescence

Cells stably transfected were detached using sterile EDTA and counted using a hemocytometer. 10,000 cells were plated on 18mm round cover slips coated with 30µg/mL collagen type I. The cells were placed into an incubator at 37°C and 5% CO_2 for 48 hours. After 48 hours the medium on the cells was removed and the cells were fixed with 4% paraformaldehyde and permeablilized with 0.2% Triton X. The cells were blocked with 3% BSA in 1X PBS for 30 minutes followed by overnight 4°C incubation with the primary antibody, human monoclonal F11r from BD Pharmagen, at a concentration of 0.5µg in 200µL of 3% BSA in 1X PBS. After the overnight incubation, the cells were washed with 1X PBS and incubated with Phalloidin 488 (Invitrogen) and Alexa Fluor 568 (Invitrogen) at a concentration of 2µg in 200µL of 3% BSA in 1X PBS for 1 hour at room temperature. Finally the slides were washed with 1X PBS and mounted on glass slides. Protocol was a modified version from Naik et al. 2008.

Western Blotting

To prepare the lysate, 500,000 cells were lysed by adding 1% NP40 lysis buffer containing protease inhibitors for 30 minutes on ice. Protein estimation was

done using the BCA protein assay kit from Pierce. 20µL of 20µg/mL protein lysate in Laemelli sample buffer was added to each well of a 10% acrylamide gel. Samples were run at 100V in an XCell II Blot Module from Invitrogen. The samples were then transferred to a PVDF membrane at 30V for 1.5 hours. Each membrane was blocked for 1 hour with 5% non fat dry milk and then incubated overnight at 4°C with a monoclonal human JAM-A antibody from BD Pharmagen at a concentration of 0.15µg in 3mL of 5% non-fat dry milk solution. After the overnight incubation the membranes were washed with 1X TBST and then incubated with anti mouse secondary conjugated with HRP at a concentration of 1:5000 in 5% non-fat dry milk for 1 hour at room temperature. After the incubation, the membranes were washed with 1X TBST and developed on Kodak film using LumiGLO from Cell Signaling.

Flow Cytometry

Stably transfected cells were detached using EDTA and counted using a hemocytometer. 100,000 cells were collected and incubated in 1mL of cold 0.5% BSA, 0.2mM EDTA in 1X PBS for 30 minutes. After 30 minutes cells were centrifuged and resuspended in 100µL of 0.5% BSA, 0.2mM EDTA in 1X PBS containing either a 1 to 10 dilution of anti JAM-A antibody conjugated with phycoerythrin from BD Pharmingen or 0.1µg of rat IgG conjugated with phycoerythrin. Cells were incubated in this solution for 1 hour on ice and then

centrifuged. After centrifuging the cells were washed two times with 1mL of cold 0.5% BSA, 0.2mM EDTA in 1X PBS. Then the cells were centrifuged and resuspended in 1mL of cold 1X PBS. Finally each sample was read on an Accuri C6 Flow Cytometer until 30,000 events were detected. Gating was set up so that only epithelial cells would be detected. This protocol was adapted from the protocol from Accuri Flow Cytometers and Naik et al. 2008.

Chapter 3

RESULTS

Cells Transfected with JAM-A and JAM-A Mutants Show Increased JAM-A Protein and Increased JAM-A Surface Expression

pcDNA 3.1 vector or JAM-A full length, Δ257 JAM-A or Y-F^{261} JAM-A constructs were transfected into MDA-MB-231 cells in order to determine the effect of JAM-A on MDA-MB-231 cells along with the possible important regions for the function of JAM-A in the migration and morphology of the cells. The expression of each construct was first determined by western blotting as can be seen in Figure 3.1. Each of the constructs expressed more JAM-A protein than the pcDNA 3.1 transfected cells. The Δ257 construct showed a decrease in protein size to around 25kDa, which is a decrease of around 6 to 10 kDa compared to full length JAM-A, while Y-F^{261} JAM-A was seen at the same kDa as full length JAM-A. From here surface expression of JAM-A was determined through use of flow cytometry. Cells were not permeablized so that the antibody could not bind intracellular proteins allowing for only the JAM-A on the surface of the cell to be recorded. Each of the constructs showed a higher mean fluorescent intensity when compared to the pcDNA 3.1 transfected cells when using a phycoerythrin-conjugated antibody to JAM-A (Figure 3.2). Immunohistochemistry was performed with a JAM-A antibody and fluorescent phalloidin on each of the cell lines in order to determine the localization of JAM-A

when the cells were placed on a surface coated with collagen type I (Figure 3.3). In the cells tranfected with only the pcDNA 3.1, there is no JAM-A staining on the cell surface and the cells are spindle shaped with distinct lamellipodia and filopodia. In the cells transfected with JAM-A full length, Δ257 JAM-A and Y-F^{261} JAM-A, there are cells spreading out and making connections to neighboring cells. JAM-A staining can be seen at the connections between the cells.

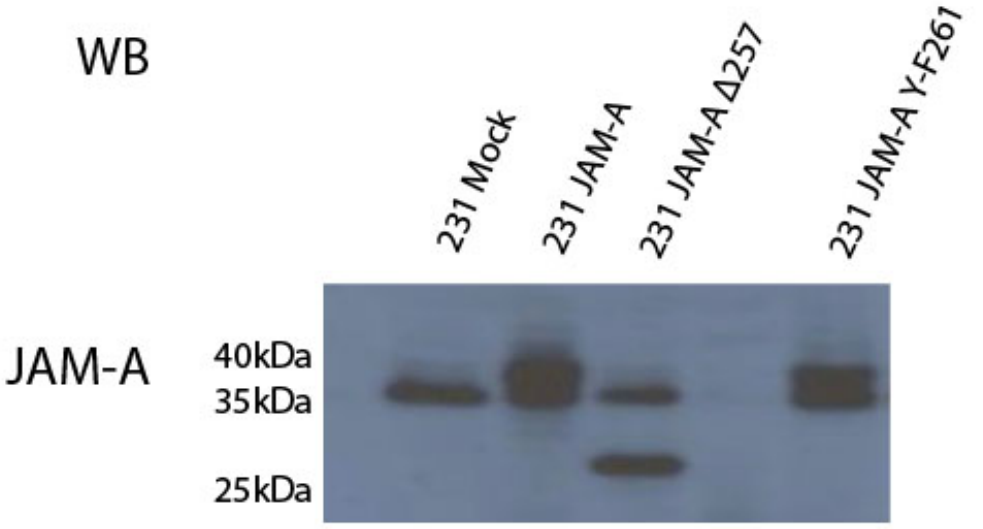

Figure 3.1: JAM-A Protein levels in each transfected sample. This western blot depicts the amount of JAM-A protein in MDA MB 231 cells transfected with either pcDNA 3.1 (Mock), JAM-A construct, Δ257 JAM-A construct or Y-F261 JAM-A construct. MDA MB 231 cells transfected with each of the constructs show more JAM-A protein being made.

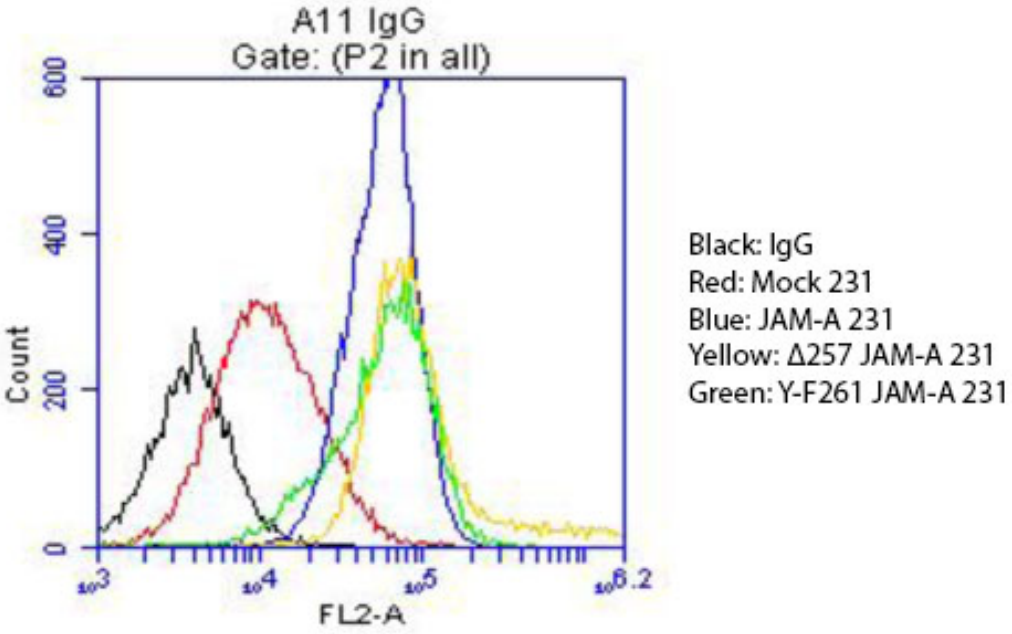

Figure 3.2: JAM-A protein surface expression in each transfected sample. Levels of JAM-A protein surfacely expressed on transfected MDA MB 231 cells determined by flow cytometry with a JAM-A phycoerythrin antibody. Each transfected sample shows higher levels of surface JAM-A compared to the Mock 231 sample.

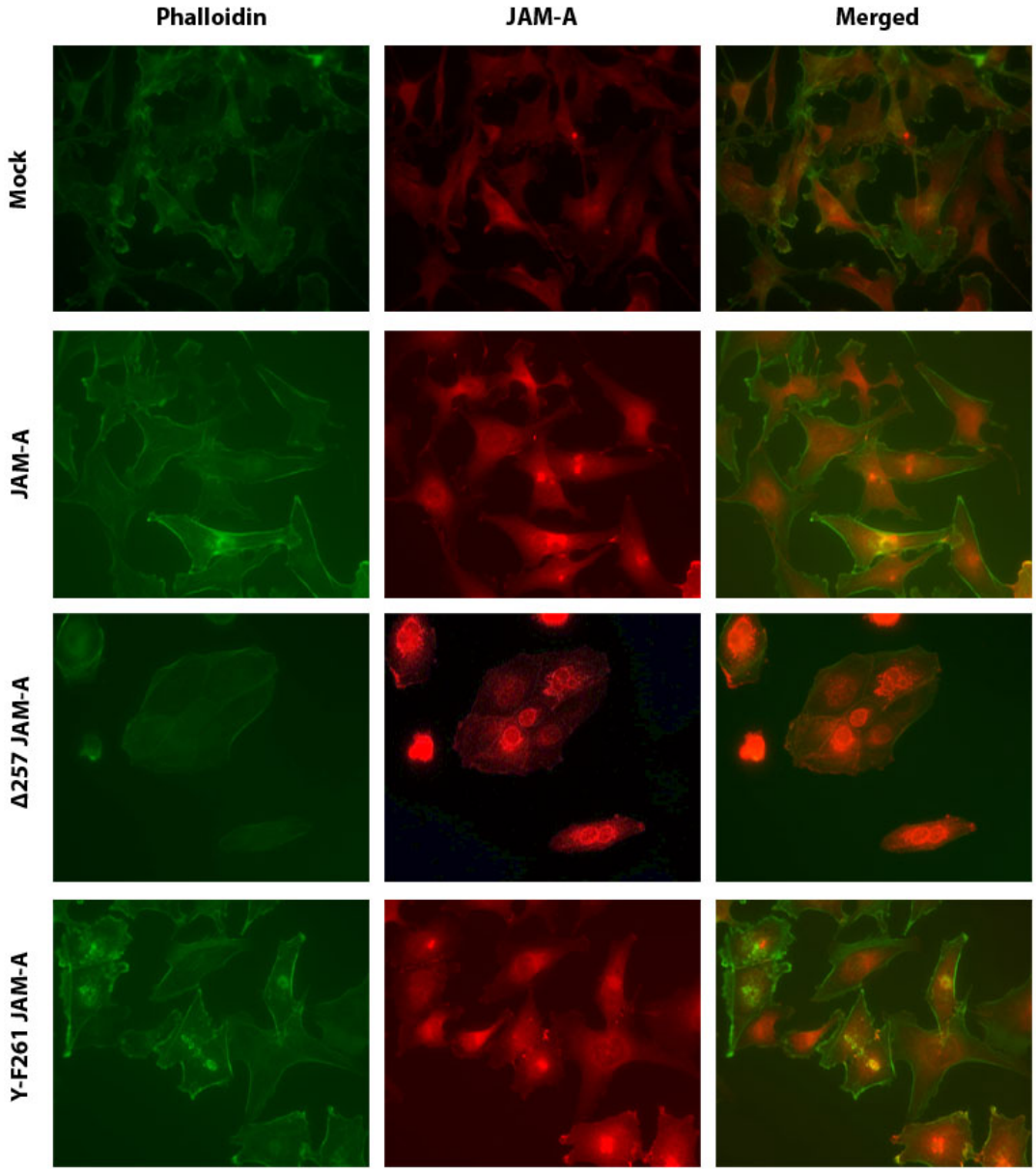

Figure 3.3: Immunofluorescent staining of JAM-A localization in transfected cells. Immunofluorescent staining using Phalloidin and JAM-A antibody to show JAM-A localization in transfected cells along with cytoskeletal arrangment. Cells transfected with JAM-A, Δ257 JAM-A or Y-F261 JAM-A show JAM-A staining on the cell surface at the contacts between cells.

Over Expression Of JAM-A or Expression Of JAM-A Mutants In MDA MB 231 Cells Shows Decreased Migration On Collagen Type I

To test the effect of JAM-A on MDA-MB-231 cell migration on collagen, cells that were transfected with pcDNA 3.1 or JAM-A full length, Δ257 JAM-A or Y-F^{261} JAM-A were allowed to migrate over the course of 5 hours on porous membranes (8μm pores) in the Neuroprobe apparatus coated with collagen type I. Cells that migrated through the pores and ended up on the underside of the membrane were fixed and counted. Images of the undersides after each cell line had migrated and was fixed and stained can be seen in Figure 3.4. Each cell was counted from each well of the samples and the data was recorded (Figure 3.5). Cells transfected with pcDNA 3.1 (Mock) migrated more than any other cell line at an average of 254.667±4.57 cells per well of the Neuroprobe 48 well apparatus. Cells transfected with JAM-A full length showed a significant decrease ($p=0.0004$) in the cells that had migrated to the underside of the membrane at an average of 34±2.2 cells per well while cells transfected with Y-F^{261}JAM-A also showed a significant difference ($p=0.00035$) with 35.333±5.45 cells per well. Δ257 JAM-A transfected cells showed the largest significant difference ($p=0.00089$) from the Mock cells with an average of 19.667±1.57 cells having migrated to the underside of the membrane. Δ257 JAM-A transfected cells also showed a significant difference ($p=0.028$) from the JAM-A full-length transfected cells. The difference between Δ257 JAM-A transfected cells and Y-F^{261} transfected cells was not significant ($p=0.104$). To visualize the difference in migration was happening, cells were plated on 8 well Nunc chambers coated with collagen type I. Images of the cells were captured every 5 minutes for 12 hours producing a time lapse of the migration of the cells. Cells were tracked using Imagej

and the plugins Manual Tracking with Chemotaxis Tool. Figure 3.6 shows a representation of a cell's migration path from each of the transfections. The Mock cells migrated much farther than the any of the JAM-A construct transfected cells, supporting the evidence provided by the previous experiment. Figure 3.7 shows the direction and the distance (in arbitrary units) of the cells recorded during the time-lapse microscopy. Each cell is measured from the origin of the cell from the first time-lapse image.

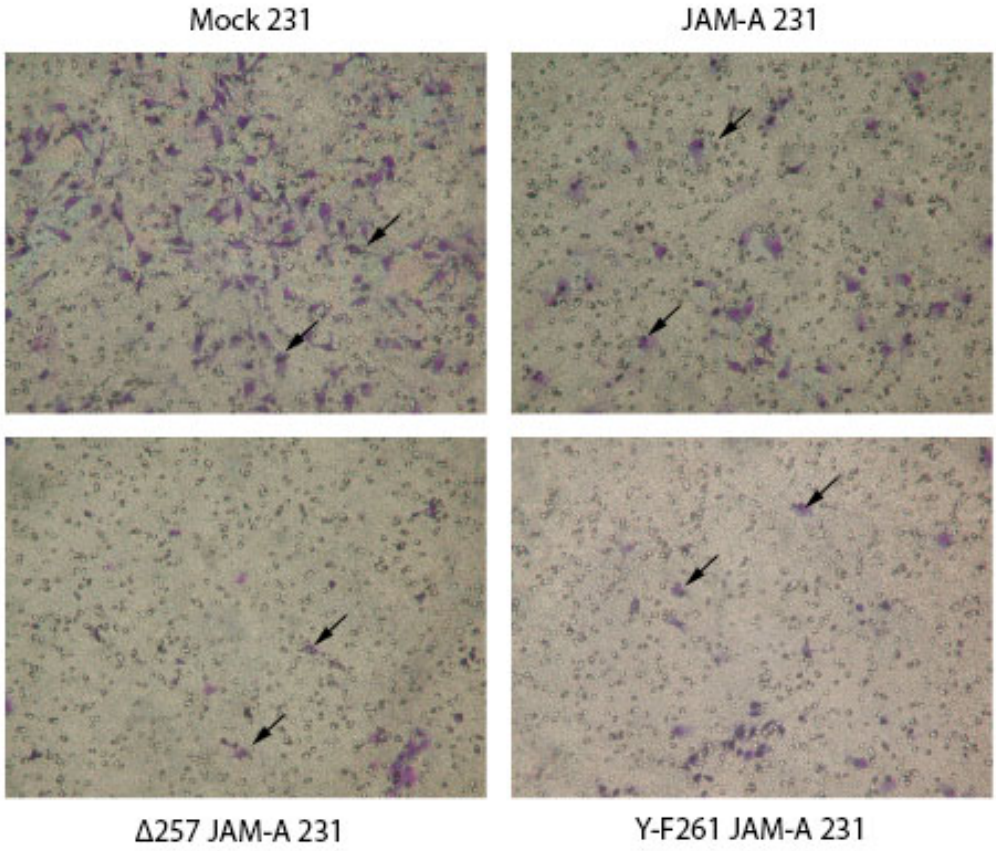

Figure 3.4: JAM-A and JAM-A mutation expressing MDA MB 231 cells show decreased migration on collagen type I. Migration results of cells transfected with the vector pcDNA 3.1, JAM-A, Δ257 JAM-A or Y-F261 JAM-A on collagen type I. Cells were stained with Diff Quik stain. Cells expressing JAM-A or a JAM-A mutation show decreased migration compared to the Mock cells.

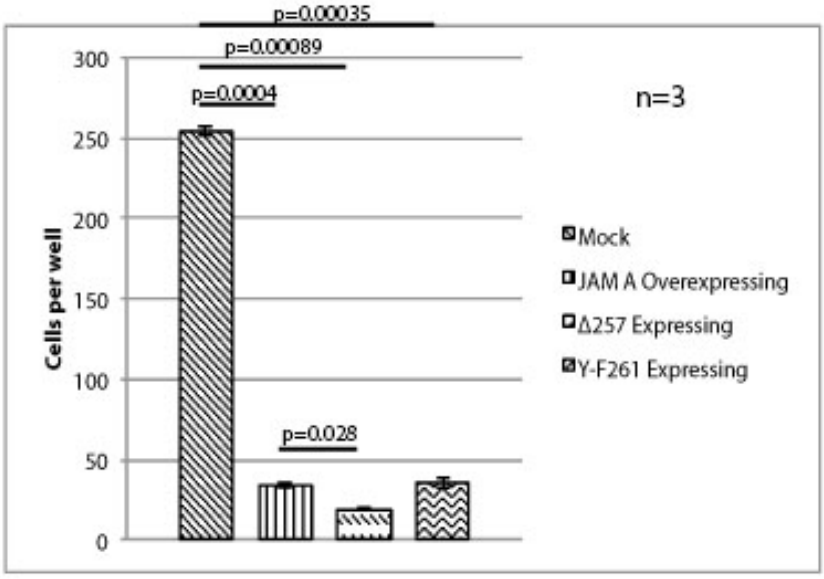

Figure 3.5 : JAM-A overexpression decreases migration in MDA-MB-231 cells. MDA-MB-231 cells transfected with either pcDNA 3.1, JAM-A full length construct, JAM-A Δ257 or JAM-A Y-F261 were allowed to migrate on an 8 micron pore membrane from Neuroprobe with the underside coated in collagen type I for 5 hours. Cells were stained with Diff Quik stain and counted using a hemocytometer. Transfection of MDA-MB-231 cells with any JAM-A constructs shows a dramatic decrease in cell migration. Cells transfectec with Δ257 JAM-A also shows a statistically significant difference from cells transfected with full length JAM-A.

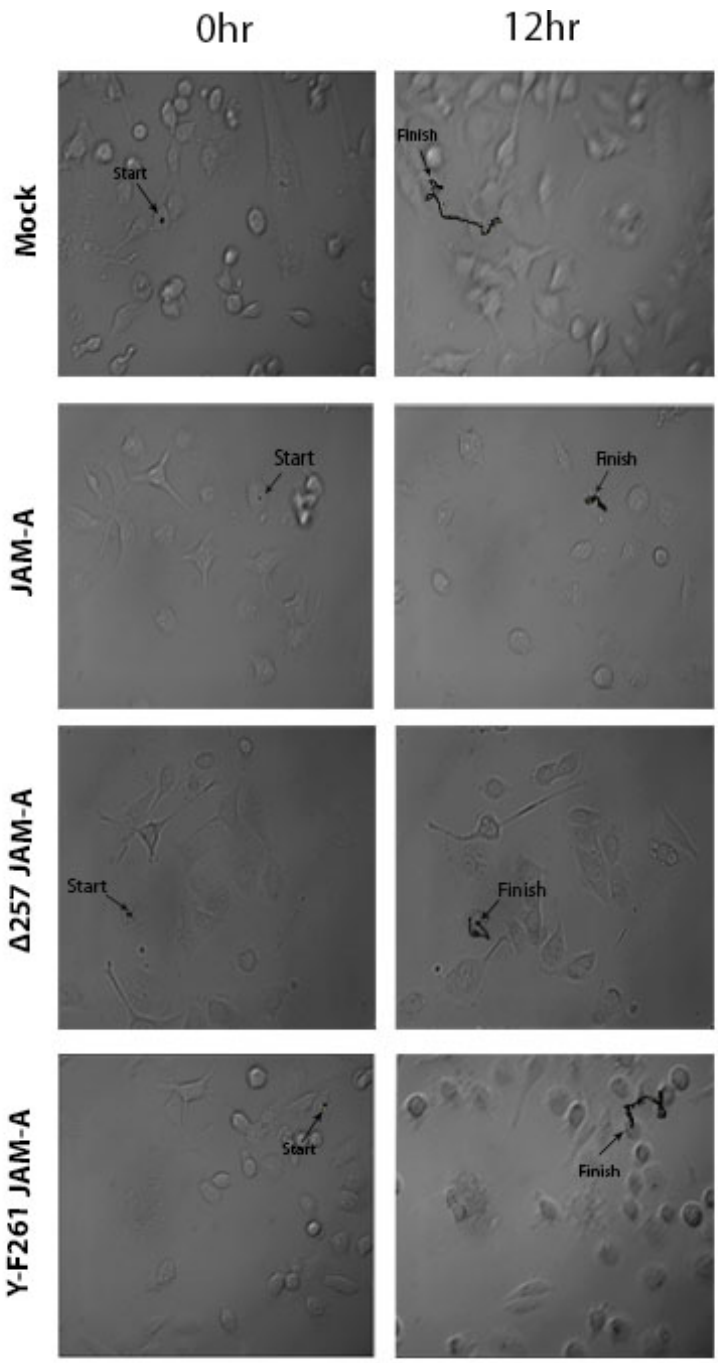

Figure 3.6: JAM-A overexpressing and JAM-A mutants show reduced migration on collage type I. Images were captured every 5 minutes for 12 hours of cells transfected with either pcDNA 3.1, JAM-A construct, Δ257 JAM-A construct or Y-F261 JAM-A construct. Using ImageJ with the Manual tracker plug in, cells were tracked for the full 12 hours. The black lines indicate the migration pathway for a cell that was tracked. The cells transfected with JAM-A construct or JAM-A mutation constructs showed decreased migration.

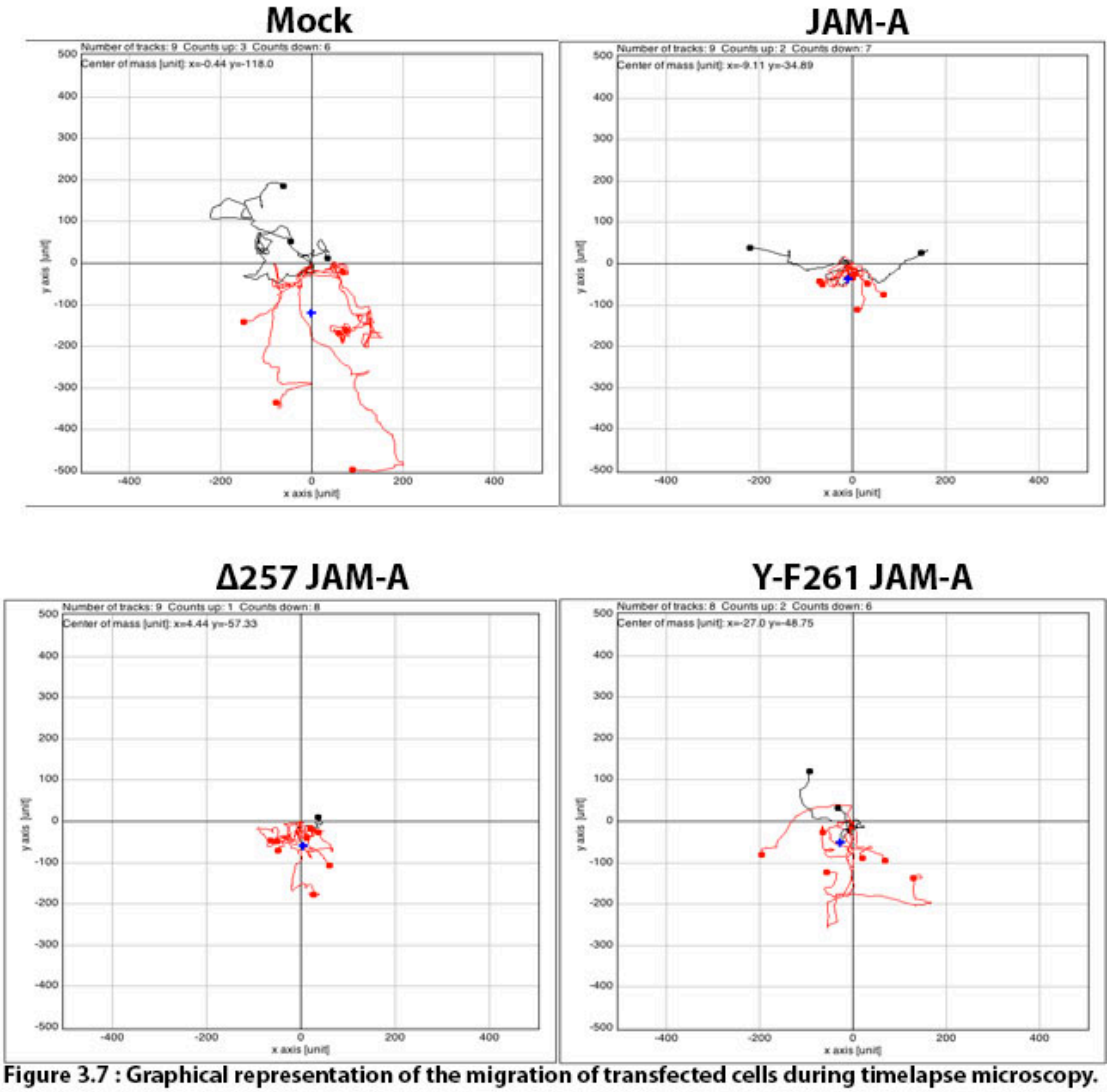

Figure 3.7 : Graphical representation of the migration of transfected cells during timelapse microscopy. This figure is a graphical representation of a view of each transfected sample during timelapse microscopy. Each sample had 9 individual cells tracked using ImageJ software with the plugin Chemotaxin. The cells were tracked a total of 144 times over the course of 12 hours of timelapse. Each line represents the distance (in arbitrary units) and direction of a cell during the timelapse from the origin of the cell. Mock cells are seen as the most migratory while each of the JAM-A transfected cell lines show a decrease in migration.

Over Expression Of JAM-A or Expression Of JAM-A Mutants in MDA MB 231 Cells Shows Increased Cell-To-Cell Connections with JAM-A Present

In order to see the morphological effects of transfecting JAM-A or JAM-A mutants on cell morphology and cell-to-cell connections, immunofluorescence using anti JAM-A and Alexa Fluor secondary were used with Mock, JAM-A, Δ257 JAM-A and Y-F^{261} JAM-A stably transfected MDA-MB 231 cells. Each of these cells were observed under a 63X objective and the cell-to-cell connections between cells with JAM-A present were counted in 10 positions on the slide as seen in Figure 3.8. This was normalized to the cell count per slide and a final cell-to-cell connections with JAM-A present per cell total was achieved. Stably transfected Mock MDA-MB-231 cells showed extremely few cell-to-cell connections with JAM-A present compared to the other cell lines. JAM-A, Δ257 JAM-A and Y-F^{261} JAM-A stably transfected cells all showed a significant increase in cell-to-cell connections with JAM-A per cell over the Mock stably transfected cells as seen in Figure 3.9. The Mock cell line showed only 0.07 cell-to-cell connections with JAM-A present per cell compared to 0.42, 0.40 and 0.42 of JAM-A, Δ257 JAM-A, and Y-F^{261} JAM-A respectively. There was no significant difference between JAM-A, Δ257 JAM-A or Y-F^{261} JAM-A transfections. Although one observation of note was that the Δ257 JAM-A cell line had less concentrated JAM-A at the cell to cell connections compared to the JAM-A and Y-F^{261} JAM-A transfected cell lines.

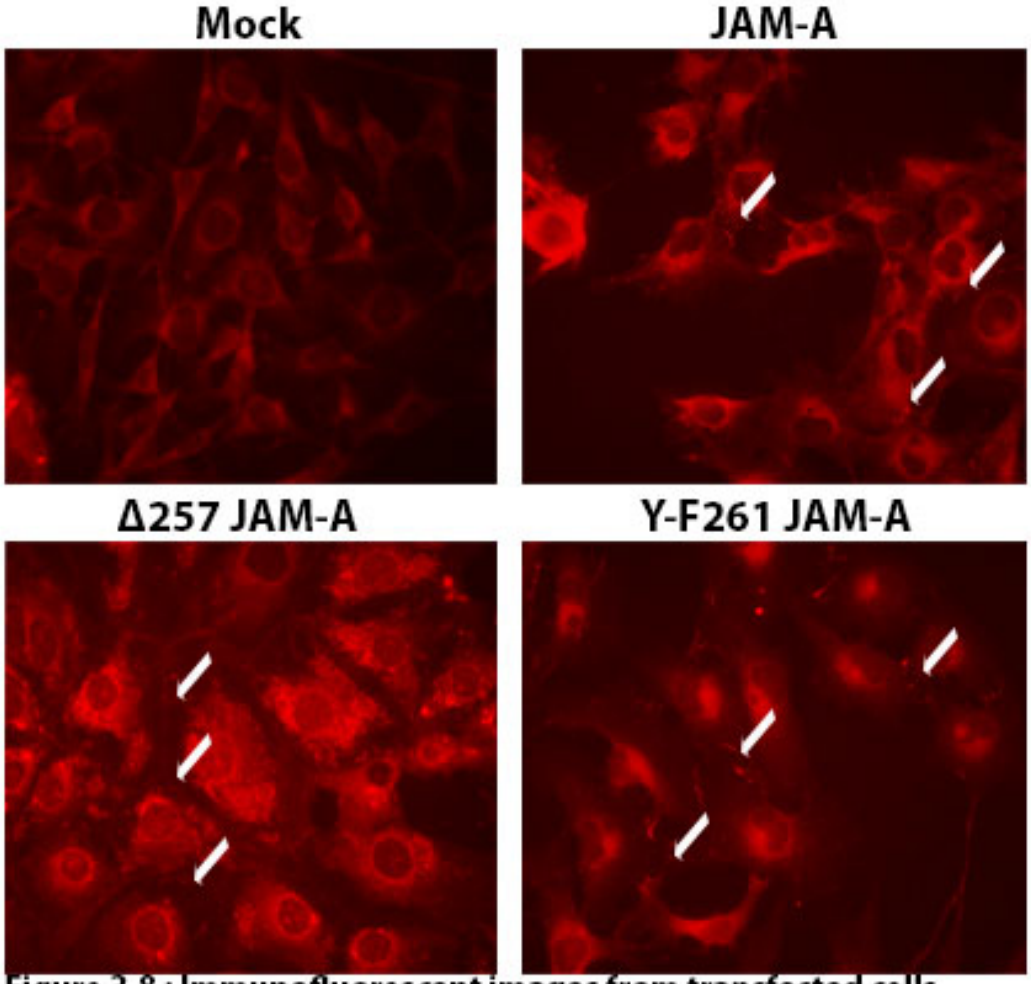

Figure 3.8 : Immunofluorescent images from transfected cells emphasizing cell to cell connections with JAM-A present.
This figure shows JAM-A immunofluorescence of transfected cells under 63x objective with an emphasis on the cell to cell connections with JAM-A present. Cells transfected with JAM-A, Δ257 JAM-A or Y-F261 JAM-A show significantly more cell to cell connections with JAM-A present compared to the Mock (pcDNA 3.1) transfected cells.

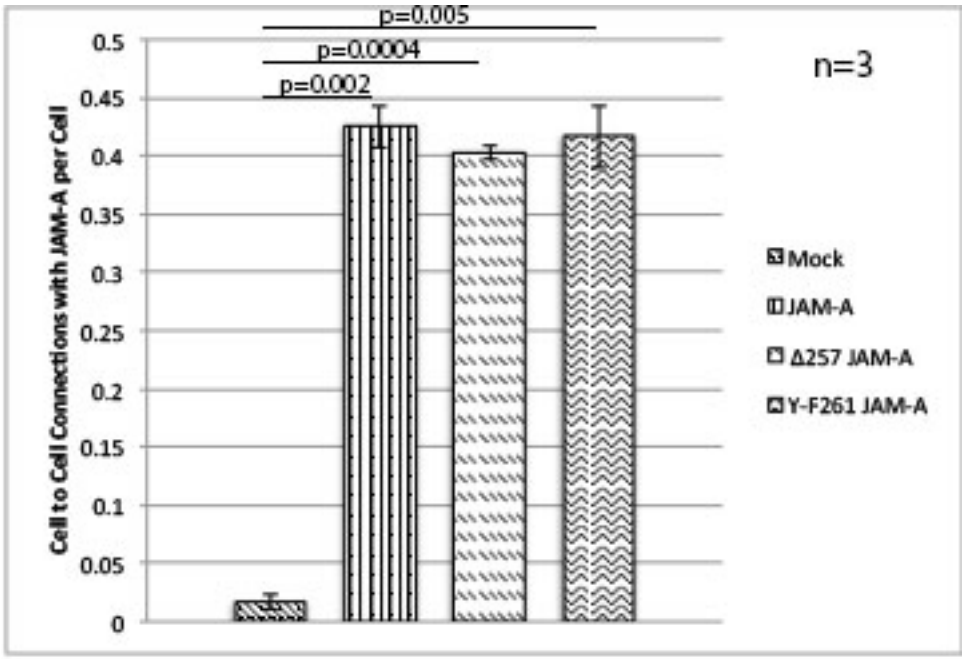

Figure 3.9: Cell to cell connections with JAM-A present per cell. This figure is a graphical depiction of the number of cell to cell connections per cell when transfected cells were stained with an anti-JAM-A antibody followed by an Alexa Fluor conjugated anti-mouse antibody. Each slide had the number of cells counted and then the number of cell to cell connections with JAM-A present counted in 10 predetermined arbitrary views under a 63x objective. A cell to cell connection with JAM-A present was determined as a connection between two cells were JAM-A staining was present as indicated by the arrows in Figure 3.7. The average number of connections with JAM-A present was then divided by the average number of cells in a view to give the final results.

Chapter 4

DISCUSSION

JAM-A Decreases Migration of MDA-MB-231 Cells and Increase the Number of Cell To Cell Connections with JAM-A per Cell

JAM-A has been shown to have a role in a variety of different cancer types. Here it is shown that when JAM-A is upregulated in a highly metastatic breast cancer cell line (MDA-MB-231), the migration of the cells decreases and the cells show increased cell-to-cell adhesions. These results agree with Naik et al. 2008, which also show that increased JAM-A in highly metastatic breast cancer cell lines decreases migration of the cells and pushes the cells to a more epithelial like morphology. Referring to the migration assay data on collagen type I (Figures 3.5), the data show that cells transfected with JAM-A, Δ257 JAM-A or Y-F^{261}JAM-A have a significant decrease in migration. To further support this observation, the time-lapse data show traces cells moving on a collagen type I ECM which visually indicates that cells with JAM-A, Δ257 JAM-A or Y-F^{261}JAM-A all have decreased migration when compared to the trace of the Mock transfected cells (Figures 3.6 and 3.7).

Along with decreased migration, this study also shows that cells transfected with JAM-A, Δ257 JAM-A or Y-F^{261}JAM-A display increased cell-to-cell

connections with JAM-A present compared with the Mock transfected cells. This again agrees with the data from Naik et al. 2008. Referring to the immunofluorescence data (Figure 3.3), the transfected cells all show JAM-A localized at the cell surface and at cell-to-cell connections. This agrees with previous data from other groups, indicating that JAM-A functions as a tight junction protein and is found at the cell adhesions in epithelial cells.

First and foremost, this study shows that increases in JAM-A decrease the migration and promote cell-to-cell adhesions in MDA-MB-231 cells. This finding is expected due to the location and proposed function of JAM-A in normal epithelial cells. In normal epithelial cells, JAM-A functions as a tight junction protein that maintains tight junction integrity and where loss of protein results in epithelial barrier break down. The data shown in this study provides that increasing JAM-A allows for the highly metastatic MDA-MB-231 cells, which normally do not form any cell-to-cell connections, to form cell-to-cell connections (See Figure 3.3 and 3.8).

Increased JAM-A may be decreasing the migration of the MDA-MB-231 cells in a few ways. The first mechanism may be that JAM-A is forming more trans homodimerizations between cells. In the study by Kostrewa et al. 2001, the group shows evidence that JAM-A may be able to form trans homodimerizations after forming a precursor cis homodimerization. While this study did use mouse JAM-A, the motif that the group examined and predicted for the dimerization, R(V,I,L)E, is conserved between human and mouse JAM-A. Increasing the amount of JAM-A in the MDA-MB-231 cells would increase the attachment strength between two cells and

would make migration increasingly difficult for cells as they keep attaching to one another. Through binding with other tight junctions proteins such as ZO-1, JAM-A could start the process of forming more mature junctions in the MDA-MB-231 cells, which normally do not form junctions at all.

Another possible mechanism through which JAM-A could be decreasing the migration of the MDA-MB-231 cells is through the sequestration of the β integrins through a complex of JAM-A and β integrins interacting directly or possibly in a complex with other proteins. In this study all experiments were done on a collagen type I matrix which is bound by the β_1 integrins. In the study done by Mandell et al. 2005, the group showed that JAM-A colocalized with β_1 integrin in epithelial cells. Their study showed that JAM-A was required for proper morphology of epithelial cells and that β_1 integrins were colocalized with JAM-A on the cell surface, but when JAM-A was knocked down the β_1 integrins were located intracellular within vesicles. This study did not look the migration of the epithelial cells with decreased JAM-A, but their morphology with decreased JAM-A resembled the MDA-MB-231 cells more closely than normal epithelial cells (Mandell, Babbin, Nusrat, & Parkos, 2005). This internalization of β_1 integrins shown could also be signs of trend milling of integrins that is required for migration of cells (Bretscher, 1996).

These results support the theory that increased JAM-A decreases migration of metastatic breast cancer cells from Naik et al. 2008. This agrees with other works from other groups in different forms of cancer such as clear cell renal cell carcinoma, melanoma, and pancreatic cancer (Fong et al., 2012; Ghislin et al., 2011; Gutwein et

al., 2009). In the McSherry et al. 2009 paper, discussed above, the group states that they were not able to get surface expression of transfected JAM-A in MDA-MB-231 cells so they were not able to test how increasing JAM-A in a metastatic breast cancer cell line affects cellular migration. Instead they used the MCF7 breast cancer cell line and reduced the expression of JAM-A through targeted short hairpin RNA constructs. Their results showed that reduced JAM-A decreased migration of the MCF7 cells, which is in stark contrast to the results of this thesis, but they also so a decrease in β_1 interin. This reduction of β_1 integrin was not seen by Naik et al. 2008 when they decreased JAM-A through siRNA in MDA-MB-231 cells. It is possible that in the poor migratory cell line, MCF7, JAM-A may be involved in downstream regulation of β_1 integrin, but in the highly migratory cell line, MDA-MB-231, JAM-A expression levels have no effect on β_1 expression levels. The McSherry results may differ from the other literature due to the reduction of β_1 integrin. However, it is important to note that a study by Wang et al. 2012 showed that a decrease in JAM-A expression in MCF7 cells through the TGF-β_1 pathway activation induced cell invasion (Wang & Lui, 2012).

Deletion of The Cytoplasmic Tail of JAM-A Further Decreases Migration of MDA-MB-231 Cells Compared to Full Length JAM-A

One unexpected finding of this study was that deleting of the cytoplasmic tail region of JAM-A further decreased the migration of the transfected MDA-MB-231

cells compared with MDA-MB-231 cells transfected with JAM-A alone. Findings from the migration assay performed on collagen showed that when the cytoplasmic region of JAM-A was deleted the cells had further deletion from the full length JAM-A (Figure 3.5), but they did not have a significant increase in cell to cell adhesions formed (Figure 3.9). While surprising at first, this type of finding is not too surprising for a protein in the Ig Superfamily.

In the study done by Chien et al. 2007, they demonstrate that the biphasic binding of cadherin molecules is independent of the cytoplasmic region. They postulate that while the cytoplasmic region is undoubtedly used to increase the long term binding strength of the cadherins, it is not used during the initial biphasic binding steps (Chien et al., 2008). While JAM-A is not a cadherin, the molecules share some similarities and JAM-A may still be able to trans homodimerize without the cytoplasmic region. With the ability to trans homodimerize JAM-A would still be decreasing the migration of the MDA-MB-231 cells and the Δ257 JAM-A may be able to cis homodimerize with some of the little native full length JAM-A, thus allowing for ZO-1 and other PDZ binding tight junction associated proteins to bind. This starts the formation of mature junctions in cells that normally do not form them, decreasing the migration of the cells.

Along with still being able to trans and cis homodimerize, in the study by Mandell et al. 2005 the group showed that the D1 region of the extracellular domain was the region of importance for the colocalization of the β_1 integrins with JAM-A. Since the D1 region is still intact, the Δ257 JAM-A would still be able to sequester the

integrins and decrease the migration through that mechanism. As mentioned previously, this sequestration of the β integrins would not allow the cell's filapodia or lamellipodia to attach the extracellular matrix and would not all the cell to migrate.

While all of this may explain why the Δ257 can still decrease migration in the MDA-MB-231 cells, it does not explain why it further decreases migration compared to upregulation of the full length JAM-A. It has been shown that the phosphorylation of JAM-A at the 285 serine in mouse models is required for tight junction formation with a serine to alanine mutation (Iden et al., 2012). I believe that the phosphorylation state of JAM-A determines its location on the cell membrane and its function. When full length JAM-A is serine phosphorylated it may be tightly clustered to areas where cell to cell adhesions are, but in another phosphorylation state, possibly tyrosine 280 or another tyrosine, JAM-A may move from the tight junction or possibly bind with other proteins. In adherins this phosphorylation switch is not uncommon and occurs when cells have the need to move and break cell to cell adhesions (Bertocchi, Vaman Rao, & Zaidel-Bar, 2012). In cells like the MDA-MB-231 cell line, there are many mutations to the DNA and many proteins have their expression greatly decreased or increased. Because of this I believe that when full length JAM-A is transfected into the MDA-MB-231 cells that some of the protein that is made is not properly phosphorylated for tight junction formation. That is the serine at 284 is either dephosphorylated by a phosphatase that may be upregulated due to the mutations to the cells DNA or that another phosphorylation site is phosphorylated due to a kinase being upregulated. This may change how JAM-A binds to other proteins and may

allow it to bind to other proteins that it previously did not. I believe that because the Δ257 JAM-A does not have its cytoplasmic tail it cannot be manipulated by the cancerous cell. Even normal epithelial cells need to break their cell-to-cell connections for various reasons so it would seem normal that cells would have a way to manage the binding of an adhesion molecule such as JAM-A through an intracellular mechanism (Kojima & Sawada, 2012).

Significance of Findings

In order to prevent metastasis and ultimately increase survival rates of cancer patients, first the mechanisms of migration of the cancer cells must be determined. This study shows evidence that JAM-A, a tight junction protein, decreases migration of the highly metastatic breast cancer cell line, MDA-MB-231, and increase the number of cell to cell connections with JAM-A present. This increase in cell-to-cell connections with JAM-A present is consistent with more epithelial like morphology compared to the original MDA-MB-231 morphology. Further this study starts to examine the important domains of JAM-A and concludes that the cytoplasmic region of JAM-A is not required for JAM-A to decrease the migration of the MDA-MB-231 cells and actually decreases the migration further when compared to either full length JAM-A or a tyrosine location mutation, $Y\text{-}F^{261}$. This finding starts to decipher the possible functioning domains for JAM-A to decrease migration and increase the cell-to-cell connections with JAM-A present.

Chapter 5

FUTURE WORK PROPOSED

In order to further the study presented here, I propose the following experiments to determine the mechanism by which JAM-A decreases the migration of MDA-MB-231 cells:

- Transfect JAM-A with mutations that delete either the extracellular domain D1 or the extracellular domain D2. From here determine if either of these decrease migration compared to the full length JAM-A or the Δ257 JAM-A.
- Using the cells transfected with mutations in JAM-A, determine if the beta 1 integrin can bind to or colocalize with each mutant. Finding the region that beta integrins bind JAM-A or the region that is required for colocalization will aid in determining if JAM-A binding to beta integrins and sequestering their function is the mechanism by which JAM-A decreases migration or even if it is a contributing factor.
- Determine how deleting the cytoplasmic region of JAM-A further decreases the migration of the MDA-MB-231 cells by examining the tyrosine and serine phosphorylation sites on the cytoplasmic region. It has been shown that at what phosphorylation site JAM-A is phosphorylated may determine where it is in the cell respective to the cell adhesion sites (Iden et al., 2012).

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