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TMIGD1 regulates epithelial cell polarity and morphology

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TMIGD1 REGULATES EPITHELIAL CELL POLARITY AND MORPHOLOGY

by

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DEDICATION

I would like to dedicate this thesis to my uncle, Detective Mike Doty. You illustrated a life worth living and your intrinsic goodness reminds me of the importance of endlessly offering kindness and generosity to those around me.

Secondly, I would like to dedicate this to my grandmother, Judith Goff. You showed me the power of perseverance and being a strong woman, and you taught me how to depend on and trust myself.

Finally, I would like to dedicate this to my parents, without whose love, understanding, and constant support, I would never have made it to Boston.

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TMIGD1 REGULATES EPITHELIAL CELL POLARITY AND MORPHOLOGY

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ABSTRACT

Epithelial cells are unique for their ability to strongly adhere to one another and coordinate communication across an asymmetrical, polar plasma membrane. These properties are necessary for carrying out normal epithelial function, such as absorbing/secreting molecules, repairing wounds, lining organs, etc. Cadherins, claudins, and occludins are major players of epithelial cell adhesion and polarity.

Previously, transmembrane immunoglobulin domain containing-1, TMIGD1, was identified as a novel cell adhesion molecule, whose expression is downregulated in human renal carcinomas. Re-expression of TMIGD1 in renal tumor cells resulted in altered cell morphology and inhibition of tumor growth.

In this study, we examined the hypothesis that TMIGD1 activity is associated with epithelial cell polarity. We demonstrated that TMIGD1 regulates actin stress fibril formation. A 3-dimensional (3D) cell culture assay was developed to examine the role of TMIGD1 in cell morphology and polarity. Our results demonstrate that TMIGD1 regulates actin fibril formation in Madin-Darby Canine Kidney (MDCK) cells, as blocking TMIGD1 activity by blocking antibody inhibited actin fibril formation in 3D cell culture system. Moreover, ectopic expression of TMIGD1 in rectal carcinoma cells, (RKO) , significantly inhibited filopodia formation. Taken together, our data identifies TMIGD1 as a possible regulator of epithelial cell morphology and polarity.

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LIST OF ABBREVIATIONS

AJ …….. Adhering Junction

AJC …….. Apical Junctional Complex

aPKC …….. Atypical Phosphokinase C

BU ..... Boston University

C/EBPs ……...CCAAT/enhancer-Binding Proteins

CRB ……. Crumbs

DAPI ……4’,6-diamidino-2-phenylindole

Dlg ……Discs Large

DMEM ……. Dulbecco’s Modification of Eagle’s Medium

E-Cadherin …… Epithelial Cadherin

ECM …… Extracellular Matrix

EMT ……..Epithelial Mesenchymal Transition

ERM ……. Ezrin Radixin Moesin

FBS …….. Fetal Bovine Serum

HUVEC …….Human Umbilical Vein Endothelial Cell

IF…….Immunofluorescence

IGPR1 …….. Immunoglobulin and Proline-rich Receptor-1

JAM ……. Junctional Adhesion Molecule

Lgl …… Lethal Giant Larvae

MDCK…….Madin-Darby Canine Kidney

PAE ……Porcine Aortic Endothelial

PALS1……Protein-Associated with Lin-7 1

PATJ ……...PALS1-associated Tight Junction Protein

Par3 ……Partitioning Defective-3

Par6 ……Partitioning Defective-6

PEI ……Polyethylenimine

PIP2 ……Phosphatidylinositol 4,5-Bisphosphate

RCC …… Renal Cell Carcinomas

RPMI…… Roswell Park Memorial Institute

Scrib …… Scribble

shRNA ……. Small Hairpin Ribonucleic Acid

TGF-β ……. Transforming Growth Factor β

TJ ……..Tight Junction

TMIGD1 ……. Transmembrane Immunoglobulin Domain Containing-1

Wnt ……. Wingless Int

ZO ……. Zonula Occludin

ZONAB …… ZO-1-Associated Nucleic Acid Binding Proteins

INTRODUCTION

Epithelial cells are sheets of interconnected cells that line our organs as well as our respiratory, gastrointestinal, urinary, and reproductive tracts, and when these cells begin dividing and altering their morphology in an unregulated manner, they are termed carcinomas. Carcinomas represent the most common type of human cancer (Jemal et al., 2011); therefore, studying the cellular dynamics and signal transduction pathways in normal epithelial cells as well as carcinoma cells is instrumental to understanding carcinogenesis and possible treatment.

Epithelial cells are fundamental in two distinct manners. First, they separate the inside, a hollow tube called that lumen, from the outside. This is crucial for absorbing or secreting specific molecules and is seen within the kidney's tubules, the small and large intestine, the trachea, vasculature, etc. The section of plasma membrane facing the lumen is termed the apical membrane; while the membrane facing neighboring cells or the underlying extracellular matrix (ECM) is termed the basolateral membrane. Secondly, to form a hollow lumen or participate in normal function, the epithelial cell must have a distinct polarity, meaning an asymmetry across the plasma membrane. The proteins and lipids located at the apical membrane must be distinct from the proteins and lipids at the basolateral membrane, and this is accomplished through vesicular trafficking of different polarity proteins and junctional complexes along the actin cytoskeleton. Both lumen formation and coordinated cell polarity are accomplished or seemed to be accomplished almost simultaneously, according to the present literature, and I will attempt to address each below as well as illustrate how it pertains to tumor development.

Epithelial cell polarity is established through polarity complexes and the formation of the apical junctional complex (AJC). The AJC is composed of tight or occludin junctions (TJ), adhering or adherens junctions (AJ), as well as an array of scaffolding proteins, kinases, phosphatases, and GTPases that basically allows epithelial cells to connect to one another (Quiros and Nusrat, 2014). RhoGTPases, including Rho, Rac, and Cdc42, are key regulators of AJC development and maintenance. To assemble an AJC, cell-cell contact is made, and actin polymerization is initiated through three cell signaling pathways, where Rac1 and Cdc42 are involved in activating the WAVE/WASP-Arp2/3 actin polymerization pathway, and Rho stimulates fibril formation (Ridley & Hall, 1992). With this available actin transport system, initial TJs and AJs are established.

The TJ is a water-tight seal that restricts the flow of molecules and ions, directing them to the AJ for passage into or out of the cell. Also denoted the zonula occludens, the TJ is positioned at the apical membrane and is a physical barrier that separates the apical membrane from the basolateral membrane (Dragsten et al., 1981). The TJ is composed of occludin, claudin, junctional adhesion proteins (JAMs), and zona occludin proteins (ZOs), and are depicted in Figure 1.

Both claudin and occludin are transmembrane proteins with four transmembrane domains, two extracellular loops, and two intracellular loops (Furuse et al.,1998). Both proteins form cis-dimerizations with their respective protein on a neighboring cell (Vanitallie & Anderson, 1997), which facilitates strong cellular adhesion points between cells. However, claudins and occludins are two distinct families of proteins, and while

they work together for the TJ's barrier-fence role, the literature considers the claudin family of proteins to serve as the major structural backbone of the TJ (Tsukita and Furuse, 1999).

Nevertheless, both claudins and occludins bind to ZO proteins, specifically ZO-1. ZO proteins are fundamental in connecting the actin cytoskeleton and actin-regulating proteins to claudins and occludins (Fanning et al., 1998; Radgers and Fanning, 2011). Therefore, the presence of ZO proteins at the tight junction allows cell-cell adhesion and cytoskeletal communication to occur between epithelial cells, as they are associated with thick actin bundles. Furthermore, these ZO proteins serve as nuclear localization signals (Sheth et al., 1997) and through sequestering ZO-1-associated nucleic acid bindings proteins (ZONABs) to the TJ, ZO-1 can limit cellular proliferation and density (Balda et al., 2003). Metais et. al (2005) found that ZO-2 binds hScrib, a member of the Scribble polarity complex discussed below, and furthermore, these two polarity-driving proteins partially colocalize at cell-cell adhesion sites. ZO-3 was also found to interact with PATJ, a member of the crumbs polarity complex, and was necessary for the localization of PATJ to the TJ (Roh et al., 2002), and further, ZO proteins and PATJ colocalize at the PDZ-binding domain of claudin (Roh et al., 2002). Altogether, through the coordination between claudins, occludins, ZO, and polarity proteins, the TJ is formed and regulated, functioning as a barrier to molecules and ions and forming the boundary of membrane asymmetry.

Just underneath or basal to the TJ lies the AJ. The AJ is composed of epithelial cadherin (E-cadherin), a calcium-dependent transmembrane protein important to adhering

neighboring cells together. While similar to the TJ proteins in cell-cell contact, the AJ proteins do allow molecules and ions to enter from neighboring cells or the surrounding interstitium. E-cadherin connects to the actin cytoskeleton through binding β- and α-catenin (Vasioukhim et al., 2000). This is represented in Figure 2.

The catenins represent a group of four cytoplasmic proteins, involved in signal transduction pathways. β-catenin plays a role in the Wingless Int (Wnt) pathway. This pathway is involved in gene transcription and cell proliferation/migration (Komiya and Habas, 2008), and Wnt pathway mutations are heavily associated with several different tumor lines, including breast and prostate cancer. (Tojo et al., 2015, Morris et al., 2013, Fancy et al., 2014). The ability for E-cadherin to trans-dimerize with an E-cadherin on a neighboring cell allows for linked actin cytoskeletons, facilitating communication and cohesion between neighboring epithelial cells (Balda and Matter, 2009), and similar to the points of TJ contact, the points of AJ contact are associated with dense actin bundling.

While the TJ and AJ facilitate cell-cell adhesion and communication, the integrin family of proteins lies along the basolateral membrane, adhering the cell membrane to the underlying extracellular matrix (ECM). Integrins bind different elements of the extracellular matrix, including collagen, fibronectin, and laminin, to the actin cytoskeleton and take part in bi-directional signaling, where the outside ECM and inside cytoskeleton both influence each other (Hynes, 1992). Integrins are heterodimers and are heavily involved in cell migration, proliferation, morphogenesis, and cell survival.

On a broad scale, epithelial cells function to absorb or secrete ions/molecules from or into the lumen, respectively. Adhering proteins, whether along the apical or basolateral membrane, influence the structure and dynamics of the actin cytoskeleton while also connecting and coordinating with cells within the larger tissue. Therefore, development, regulation, and turnover of these proteins are crucial to the function and integrity of epithelial tissue. There are three different protein complexes that contribute to the establishment and maintenance of the AJC, and they are the Crumbs complex, the Par complex, and the Scribble complex. Interestingly, each polarity complex localizes to a specific location on the membrane. For example, the Crumbs and Par complex are found along the apical membrane while the scribble complex localizes to the basolateral membrane (Macara, 2004). Furthermore, increased expression of one polarity complex gives an expansion of that membrane at the expense of the other. This is seen when a member of the crumbs complex, CRB3's, expression is increased, the apical membrane enlarges while the basolateral domains shrink (Li et al., 2015).

The crumbs polarity complex consists of three major proteins, crumbs (CRB), protein-associated with Lin-7 1 (PALS1), and PALS1-associated tight junction protein (PATJ), and these core proteins are heavily involved in TJ formation at the apical membrane. Using breast cancer cell line, MCF10A cells, that expresses little endogenous CRB and simultaneously does not form a TJ *in vitro*, when CRB is introduced, TJ assembly is initiated (Xiaona et al., 2017). Each CRB protein has PDZ-, FERM-, and Z-binding domains, allowing this protein to bind PALS1/Par6, ERM proteins, and ZO proteins, respectively. (Herd et al., 2003; Lemmers et al., 2004; Makarova et al., 2003).

PALS1, also a member of the Crumbs complex, is a scaffolding protein that binds Par6, connecting the Crumbs and Par polarity complexes. PALS1 regulates TJ formation, along with CRB, and furthermore, it was found that knockout of PALS1 affected both TJ and AJ formation. Without PALS1, E-cadherin was unable to traffic to the plasma membrane and cell adhesion was lost (Wang et al., 2007). PATJ, the last core protein member of the Crumbs complex was found to bind ZO-3 and claudin-1 through its PDZ binding domains (Roh et al., 2002).

The Par complex contains three core proteins: partitioning defective-3 (Par-3), partitioning defective-6 (Par-6), and atypical PKC (aPKC). Par-3 has a necessary role in TJ assembly as well as recruiting other polarity protein members to the apical membrane (Kohjima et al., 2002). Par-6 activates aPKC (Yamanaka et al., 2001). Par-3 is localized to TJ; however, upon phosphorylation by activated aPKC, Par-3 disassociates with the TJ and travels to the AJ (Morais-de-Sa et al., 2010). Then, through phosphorylation and subsequent inhibition by ROCK at the basolateral membrane, Par-3 in inactivated, inhibiting par polarity complex function at the basolateral membrane (Nakayama et al., 2008). Par-6 also binds Lgl (lethal giant larvae, a member of the Scribble complex); however, through Lgl phosphorylation by activated aPKC, Lgl disassociates from Par-6 and travels to the Scribble complex on the basolateral membrane (Plant et al., 2003).

The Scribble complex localizes to the basolateral membrane, and includes scribble (Scrib), Lethal giant larvae (Lgl), and Discs large (Dlg). Scrib is a large scaffolding protein that through binding Lgl through its leucine-rich-repeats domain, localizes both itself and Lgl to the lateral membrane (Kallay et al., 2006). Interestingly,

recent studies on the scribble polarity complex with regards to tumor development have been conducted. Either down-regulation or the accumulation of cytoplasmic scrib is associated with several cancers, including colon, endometrial, and breast (Gardiol et al., 2006; Zhan et al., 2008). Kapil et al. (2017) also found highly disorganized and mislocalized scrib in colon cancer, where instead of presenting on the basolateral membrane, scrib was found clustered in both the cytoplasm and nucleus. In this case, scrib bound directly to ERK, preventing its activation and downregulating the expression of several oncogenes, including c-Myc and cyclinD1, in hepatocellular carcinoma (Kapil et al., 2017). In another study, it was found that Scrib was required for lung development and lung tube formation. In scrib-deficient mice, even the morphology of lung epithelial cells was perturbed, as well as lumen formation (Yates et al., 2013). Scrib is also implicated in preventing epithelial to mesenchymal transitions (EMT) in epithelial cells of the eye (Yamben et al., 2013).

These polarity protein complexes allow the cell to remain in this asymmetrical, polarized state, and recent research has shown that knockdown of these polarity proteins leads to disruption in the formation of TJs or AJS (Chen & Macara, 2005; Shin et al., 2005; Ivanov et al., 2010). Furthermore, many oncogenes have been found to interact with or contribute to the downregulation of polarity proteins (Suzuki et al., 1999; Okajima et al., 2008). Nevertheless, it is becoming evident that polarity proteins, along with junctional adhering proteins are fundamental in keeping single cells functional and "normal" within the larger epithelial tissue.

When cells begin losing this asymmetrical polarity in addition to their adhesion to surrounding cells, that is termed an epithelial to mesenchymal transition (EMT). EMT is necessary for normal processes such as embryogenesis or wound healing; however, when these epithelial cells adopt this phenotype in an unregulated manner, tumor development and metastasis can result. This process involves losing or downregulating the expression of epithelial characteristics, such as E-cadherin, claudin, and occludin, and in its place are the expression of mesenchymal genes, such as N-cadherin and vimentin, an intermediate filament protein (Islam et al., 1996). Moreover, this change in cell surface molecules elicits a change in phenotype, where a normal appearing epithelial cell evolves into a fibroblastic-appearing cell (Gilles et al., 1996). Further, the actin cytoskeleton is reorganized and transformed from thin cortical bundles, necessary for cell adhesion, to thick contractile fibers, needed for a mesenchymal phenotype (Haynes et al., 2011).

Ikenouchi et al (2003) found that through exogenous expression of Snail, a transcription factor known to promote EMT, E-cadherin, claudin, and occludin expression was significantly downregulated. TGF-β is also a known inducer of EMT, and Wang et al. (2008) found that TGF-β downregulates Par-3, contributing to a dysfunctional TJ. It also phosphorylates Par-6 at the AJ, contributing to Par-6 degradation and AJ dysregulation (Ozdamar et al., 2005). Studying a known oncogene, Rafl, Wang et al. (2006) found that Rafl inhibited occludin transcription, therefore promoting EMT characteristics. Conversely, the protective role of ZO proteins has also been studied in the context of tumorigenesis. ZO-2 relocates to the nucleus and binds to the E-box of cyclinD1, inhibiting its transcription, and initiating a cell cycle blockade (Gonzalez-

Mariscal et al., 2009). Nevertheless, it is apparent that there are multiple pathways that downregulate or dysrupt AJC genes and promote EMT.

Recently, ERM proteins, specifically moesin, have also been implicated in EMT. ERM proteins are a family of molecules, Ezrin, radixin, and moesin, that link the plasma membrane and transmembrane proteins to the actin cytoskeleton (Bretscher et al., 2002). These proteins have an N-terminus and a C-terminus, denoted the FERM domain and C-ERMAD domain, respectively. ERM proteins have been shown to bind phosphatidylinositol 4,5-bisphosphate (PIP2), CD43, CD44, and ICAM-1, 2 through their FERM domain and the actin cytoskeleton through the C-ERMAD domain (Tsukita et al., 1994; Hirao et al., 1996; Serrador et al., 1997, Heiska et al., 1998). However, these proteins are only able to adopt an active conformation and bind actin after they are phosphorylated at a site-specific spot (Hirao et al., 1996). In fact, without this phosphorylation, ERM proteins are bound in an inactive conformation, where the binding site for actin fibers is hidden (Gary and Bretscher, 1995). However, once phosphorylated, moesin, for example, can bind actin and initiate several signaling pathways, as shown in Figure 3.

Haynes et al. (2011) found that in the case of mammary epithelial cells treated with TGF-β to induce EMT, the expression of moesin increased and its localization on the membrane was altered, going from an apical localization to a dorsal/ventral localization, as compared to the control. Moreover, through moesin knockout experiments, it was also found that moesin small hairpin RNA (shRNA) cells had significantly less thickly bundled actin contractile fibers compared to the control, and the

fibers that were present resembled thin actin bundles (Haynes et al., 2011). DeSouza et al. (2013) found that knockdown of moesin in glioma cells also exhibited reduced cell migration in a hyaluronan-stimulated migration model.

Furthermore, moesin expression in the necessary phosphorylated form has been implicated in cellular mitosis, playing a role in mitotic cell rounding and rigidity (Kunda et al., 2008). Interestingly, Shigenobu et al. (1999) found that overexpressing integral membrane proteins, such as CD43 and CD44, was associated with elongated microvilli, possibly facilitated by an increase in phosphorylated moesin loca78

The Role of TMIGD1-family Proteins

A family of novel cellular adhesion molecules has recently been studied, with Immunoglobulin and proline-rich receptor-1 (IGPR-1) and transmembrane immunoglobulin domain containing-1 (TMIGD1) being members. IGPR-1 is a major cell adhesion molecule on endothelial and epithelial cells that regulates permeability and angiogenesis through its association with vascular endothelial cadherin, VE-Cadherin, and this molecule is composed of three domains: an extracellular immunoglobulin domain, a transmembrane domain, and an intracellular domain made of proline repeats. **(**Rahim et al., 2012). These proline repeats, also termed proline rich domains (PRDs), are involved in many signaling pathways through interactions with Src homology domain 3 (SH3) as well as others **(**Zarrinpar et al., 2003). Using quantitative polymerase chain reaction (qPCR), Rahimi et al. (2012) found high IGPR-1 expression in arteries, veins, and neural tissue, and moderate expression in the bone marrow, liver, and lung. In

overexpressing IGPR-1 in porcine aortic endothelial (PAE) cells, increased capillary tube formation was seen. This aligns with previous findings that adhesion proteins are associated with actin polymerization localized to their place on the plasma membrane (Gory-Faure et al., 1999). Furthermore, overexpression of IGPR-1 in these cells is associated with increased cell adhesion and decreased permeability, and this seems to take place through the trans-dimerization of neighboring IGPR-1 molecules at the AJ (Wang et al., 2016). Conversely, knockdown of IGPR-1 in human umbilical vein endothelial cells (HUVECs) was associated with decreased capillary tube formation, and alternatively, overexpressing IGPR-1 in B16F melanoma cells was associated with increased angiogenesis. (Rahimi et al., 2012).

TMIGD1 has also been identified and studied as a novel cell adhesion molecule, showing high expression primarily in kidney and colon epithelial cells. TMIGD1 is structurally similar to IGPR-1, except the intracellular domain of TMIGD1 is shorter and does not contain PRDs and the extracellular domain contains two immunoglobulin domains, whereas IGPR-1 only has one (Arafa et al., 2015). These two immunoglobulin domains self-dimerize and then trans-dimerize with neighboring TMIGD1 molecules. Overexpression of TMIGD1 in HEK293 cells was associated with large cell aggregates, reduced cell migration, and decreased cell contractility (Arafa et al., 2015). Lastly, Arafa et al. (2015) found that the adhesive role of TMIGD1 may also contribute to kidney cell survival when cells are presented with oxidative damage

Due to findings that TMIGD1 is expressed in healthy kidney and colon epithelial cells, and that this expression is associated with increased cellular adhesion, expression of

TMIGD1 across different renal cell carcinomas (RCC) was studied. Meyer et al. (2017) found that TMIGD1 expression is widely downregulated across many RCCs, and this downregulation was facilitated by basic leucine zipper transcription regulators, called CCAAT/enhancer-binding proteins (C/EBPs) that bind DNA. Using a RCC line, 786-0, actin assembly, cell adhesion, migration, and proliferation was studied when cells expressed TMIGD1 versus the empty vector (EV). Figure 4 illustrates that expression of TMIGD1 in 786-0 cells is associated with a redistribution of actin polymers to resemble a more polarized epithelium. Furthermore, TMIGD1 expression is associated with decreased cell proliferation, cell size, branching, and migration compared to EV (data not shown). Meyer et al. (2017) also found 786-0 cells expressing TMIGD1 had an upregulation of key cellular signaling pathways that might explain TMIGD1's anti-proliferative power, where phosphorylated p38, phosphorylated Rb, p21, and p27 were all increased compared to the empty vector and likely contributed to a cell cycle blockade (Meyer et al., 2017).

Using mass spectroscopy, it was found that TMIGD1's cytoplasmic domain binds moesin, through moesin's FERM domain. This interaction is similar to what is seen with other transmembrane proteins, as moesin connects cell membrane proteins to the actin cytoskeleton. However, it is very compelling that moesin is associated with tumor progression and cellular metastasis; however, expression of TMIGD1 and its binding of moesin somehow alters moesin's signaling capabilities, leading to a suppressed cell cycle.

Considering the potential biological role of TMIGD1 in renal epithelial adhesion and polarity as well as renal carcinogenesis, the overall goals of this project were to establish a cell culture assay and examine the role of TMIGD1 in renal cell morphology and polarity. The specific aims of this study are:

(1) Establish a 3D culture system to investigate the role of TMIGD1 in renal epithelial cell morphology and polarity

(2) Examine the hypothesis that TMIGD1 regulates renal epithelial cell polarity

(3) Examine the effects of actin distribution when co-expressing TMIGD1 and moesin

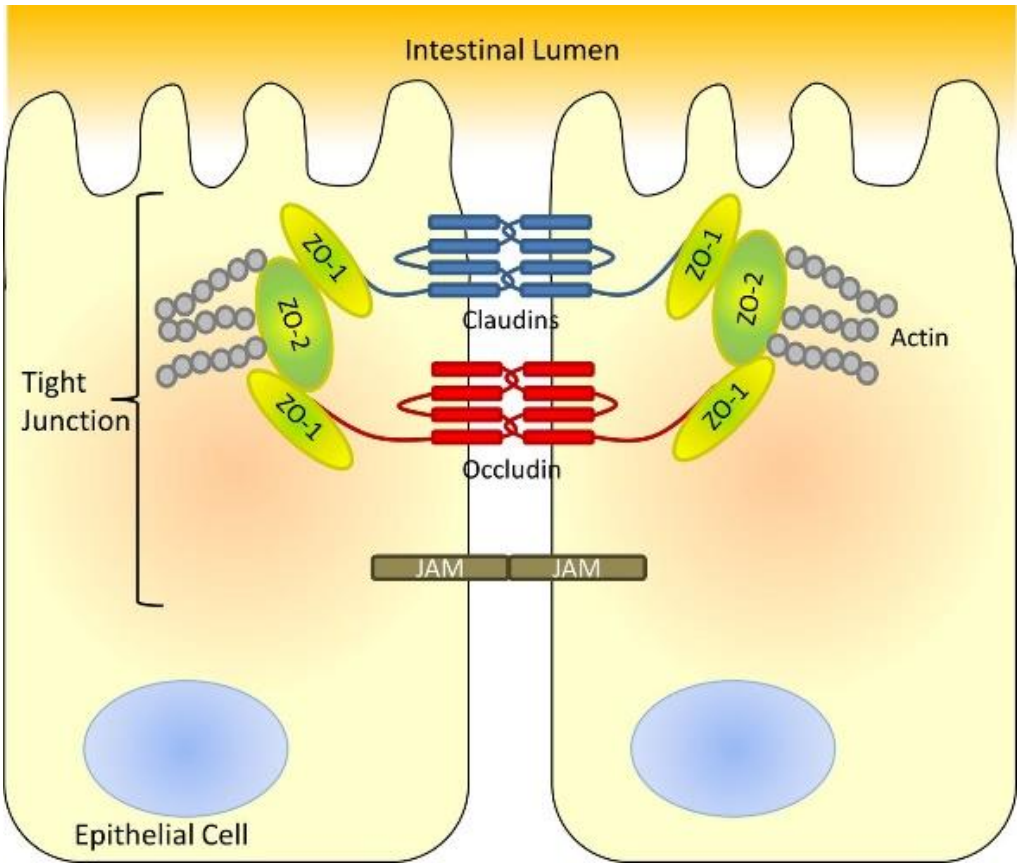


Figure 1: Tight Junction Protein Complex

Claudins and occludins are instrumental in forming the TJ. Both trans-dimerize with their respective neighbors, forming a tight seal and barrier to molecules and ions. These transmembrane proteins also interact with the actin cytoskeleton through association with ZO proteins. TJs define the apical membrane. Adapted from Collins et al., 2017

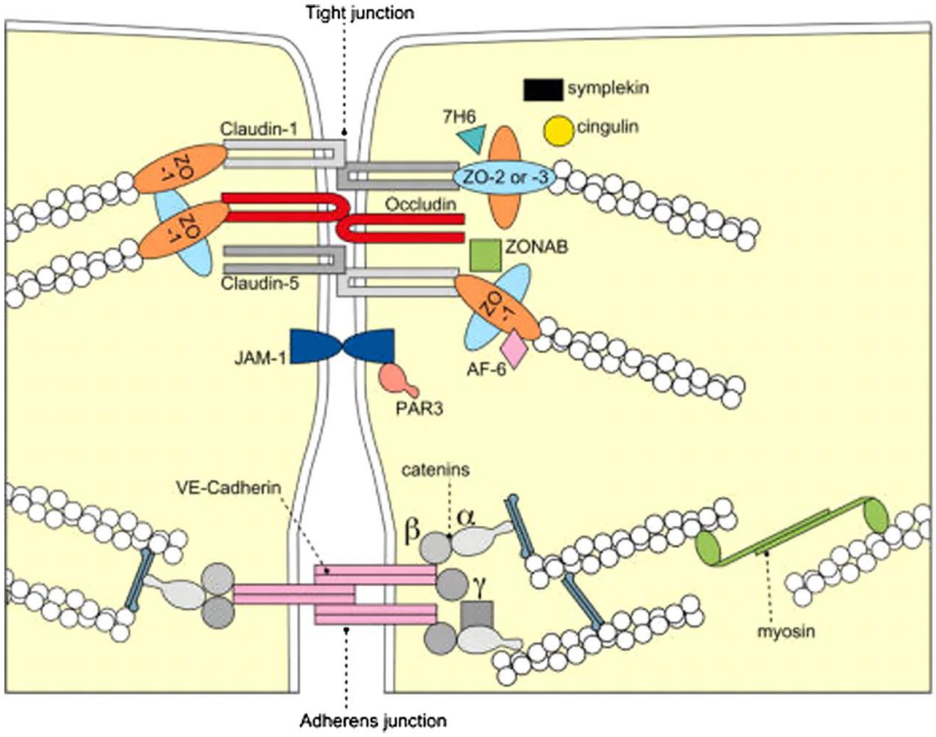


Figure 2: Adherens Junction Protein Complex

The AJ sits basal to the TJ and is composed of E-Cadherin, β-catenin, and α-catenin. Catenin proteins connect E-cadherin to the actin cytoskeleton. E-cadherins transdimerize with neighboring E-cadherins to allow for cell adhesion and cytoskeletal communication within the larger epithelial tissue. Adapted from Malaeb et al. (2012).

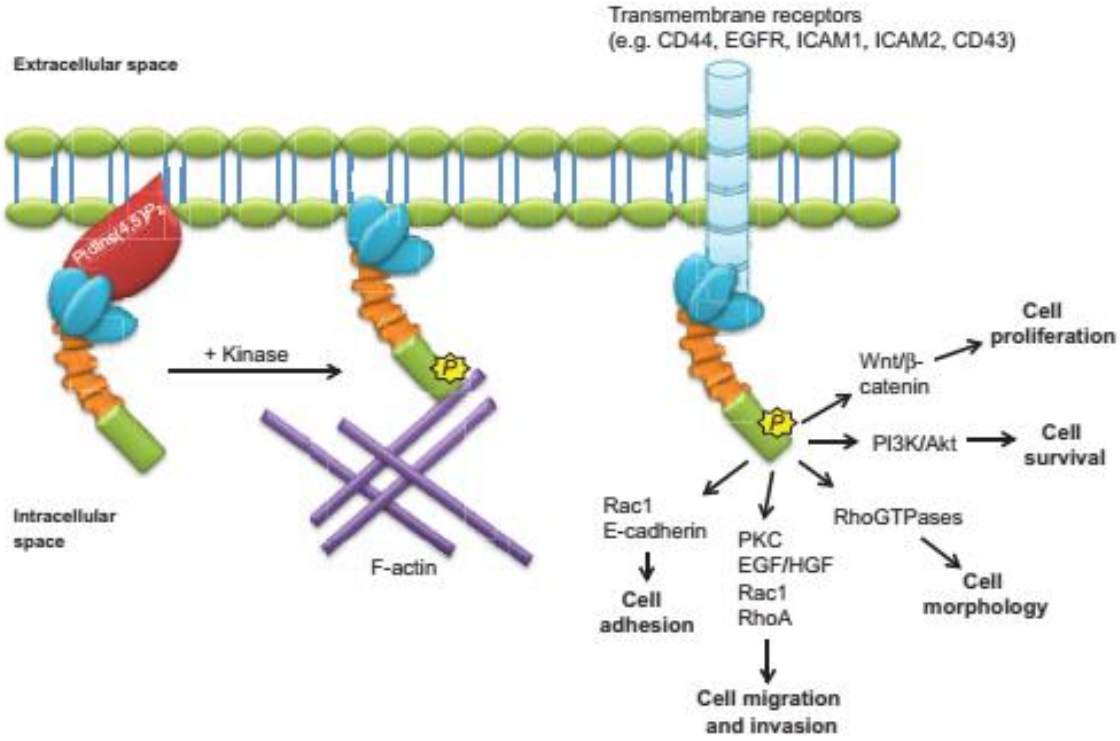


Figure 3: ERM proteins', specifically Moesin's, function in cell proliferation, migration, and invasion

Moesin, a member of the ERM protein family, binds PIP2 at the basolateral membrane, unfolding and unmasking a phosphorylation site. After phosphorylation, moesin binds to F-actin. Moesin can also bind to different transmembrane receptors and after phosphorylation, is able to influence several signaling pathways. These pathways are also implicated in cancer progression and metastasis. Adapted from Clucas et al. (2014).

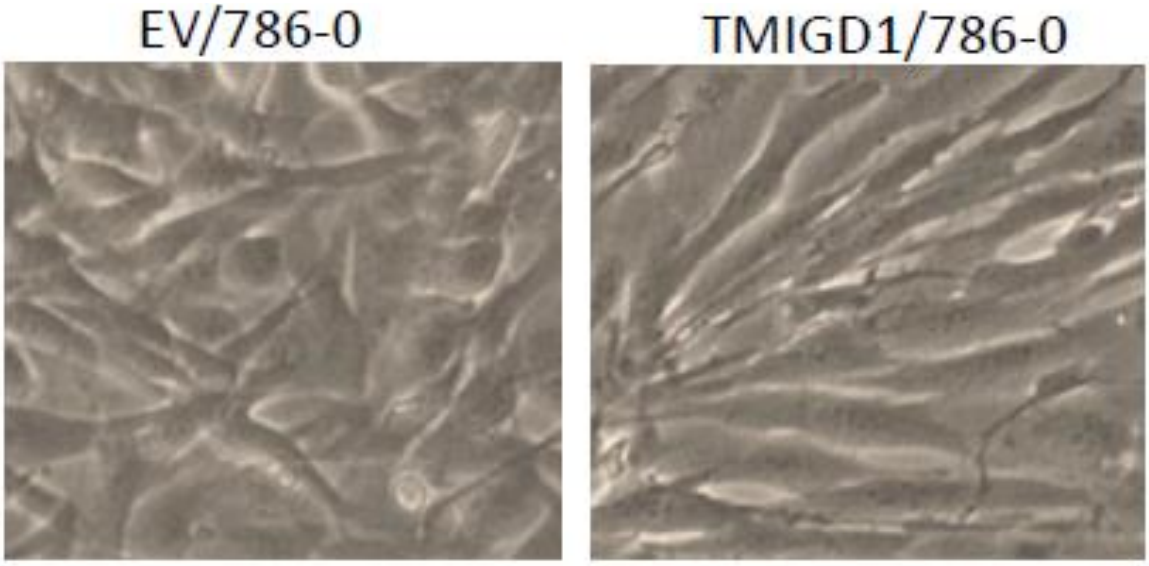


Figure 4: Actin arrangement in TMIGD1-expressing 786-0 cells compared to the Empty Vector

786-0 cells expressing exogenous TMIGD1 adhesion protein shows a significant redistribution of the actin cytoskeleton, with a noticeable polarity difference. Adapted from Meyer et al. (2017).

METHODS

Cell Lines, culture, and antibodies.

Human renal cell carcinomas, RKO were maintained in Roswell Park Memorial Institute (RPMI) cell culture media (HyClone, Logan, UT) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) plus 100 IU/100 μg/mL (1X) Penicillin-Streptomycin Solution (P/S) (Corning, Manassas, VA). All cells were contaminate-free and incubated in a humidified chamber at 37°C, 5% CO_2. RKO cells were made to express the TMIGD1 construct, as well as the empty vector (EV) construct, using the method previously described (Arafa et al., 2015; Meyer et al., 2017).

Rabbit polyclonal anti-TMIGD1 antibody was developed against peptides corresponding to the extracellular domain of TMIGD1 (Arafa et al., 2015). Anti-Moesin antibody, antibodies for phospholipase C (PLC) γ1, and Horseradish (HRP)-conjugated goat anti-rabbit antibody were purchased from Santa Cruz BioTechnology, Inc (Santa Cruz, CA). Anti-phospho-Moesin antibody was purchased from Cell Signaling Technology (Beverly, MA).

Transfection with GFP-Moesin/GFP-EV

RKO cells expressing TMIGD1 and EV were transfected with green fluorescent protein, GFP-moesin (mEmerald moesin-N14) and GFP-EV each (mEmerald EV N1) (Denville Scientific Holliston, MA), using polyethylenimine (PEI) and serum-free media for six hours. After that, RPMI culture media with 10% FBS was added.

2D Cell Fixation Method

RKO cells expressing TMIGD1 or EV were passaged onto coverslips for fixation and immunofluorescence (IF) microscopy. The protocol used was one established for IF in the Rahimi laboratory. Coverslips were incubated in 4% Paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) for 15 minutes to allow for fixation, followed by a three PBS+ washes. Coverslips were then incubated in a permeabilization solution of 0.25% Triton X-100 (diluted from 20% Triton X-100, Sigma Aldrich, St. Louis, MO) in Western Rinse for 10 minutes, followed by a three PBS+ washes for 1 minute each while gently shaking. Coverslips were then incubated for 10 minutes in rhodamine phalloidin antibody (Life Technologies, Eugene, OR) in PBS at a 1:400 dilution. This was followed by a two PBS washes, 1 minute each. Mounting the coverslips onto microscope slides was then accomplished using Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and any excess liquid was wiped away using a Kimwipe.

3D Cell Culture Assay

Madin-Darby Canine Kidney (MDCK) cells were cultured in a three-dimensional collagen assay as adopted from Elia et al. (2010) and described extensively in the Results section. 24-well cell culture plates and membrane inserts were purchased from Fisher Scientific Co, LLC (Nazareth, PA) and Becton Dickinson Labware (Franklin Lakes, UT), respectively. The collagen gel was made from glutamine 200 mM, sodium bicarbonate in deionized sterile water (American BioAnalytical, Natick, MA), minimum essential media

(MEM) 10X (without glutamine) (provided by Dr. Andrew Tilston-Lunel, Boston University), Rat tail collage type I (Discovery Labware, Bedford, MA), and phosphate buffered saline (PBS+) (PBS X1 supplemented with Ca2+ 0.9 mM and Mg2+ 0.5 mM) (Hyclone Laboratories, Logan, UT). A collagenase stock solution was prepared in PBS at a 1000U/mL PBS+ stock and stored in aliquots at -4°C. During collagenase treatment, a dilution of collagenase stock in PBS+ was made at a 1:10 ratio. 4% PFA was used in fixing the cell culture. A permeabilization solution was made new for each experiment using 10% FBS, 0.5% Triton-X-100 in PBS+. The secondary antibody permeabilization solution also contained a nuclear tag, using Vectashield with 4',6-diamidino-2-phenylindole (DAPI) and a rhodamine phalloidin tag to visualize actin. Vectashield with DAPI was also used as a mounting solution. Coverslips were used for mounting the gel onto a microscope slide.

Western Blot Analysis

Cells were grown to 90-100% confluence in adherent plates (Denville Scientific, Metuchen, NJ). Cells were then washed twice with chilled H/S buffer [20 mM Hepes (pH 7.4) and 150 mM NaCl] and lysed using lysis (EB) buffer [10mM Tris-HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100] containing 4 mM Na_3VO_4 and 1.5% 1X PIC (Protease Inhibitor Cocktail) [500 μM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, hydrochloride, 150 nM aprotinin, bovine lung, crystalline, 1 μM E-64 protease inhibitor, 0.5 mM EDTA, disodium, and 1 μM leupeptin, hemisulfate]. 5X sample buffer [3.8% Tris-base, 50% glycerol, 5% sodium dodecyl sulphate (SDS), 5% β-

mercaptoethanol, and .0025% bromophenol blue] was added to whole cell lysates, which were then incubated at 95°C for 10 minutes. Cell lysates were then resolved on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (cat # 88518; Thermofisher Scientific, Rockford, IL) previously activated by methanol. After the transfer, the membrane was placed on the shaker in Blotto [Western Rinse solution of 2% non-fat dry milk and 0.05% Tween-20] for 60 minutes. Membranes were washed for 5 minutes in Western Rinse and then incubated and rocked for 60 minutes with the primary antibody diluted in Block at a 1:5000 dilution for anti-TMIGD1, and 1:5000 for anti-PLCλ, used as loading control. After three 5-minute washes, membranes were incubated and rocked for 60 minutes with a HRP-conjugated goat anti-rabbit antibody diluted in Blotto (1:5000). The membranes were washed three more times for ten minutes and developed with enhanced chemiluminescence (ECL) (Made by Dr. Rahimi).

RESULTS

Exogenous Expression of TMIGD1 in RKO cells via Western Blot analysis

A western blot analysis of RKO cells expressing TMIGD1 versus EV was conducted. 786-0 cell lysate expressing TMIGD1, previously validated, was used as a positive control, and PLCγ1 was used as a loading control. This is illustrated in Figure 5.

TMIGD1 inhibits filopodia formation in RKO cells

RKO cells expressing TMIGD1 or EV were grown in 2-D cell culture with RPMI, 10% FBS culture media. Using immunofluorescence (IF), changes in actin distribution and cell morphology in RKO cells expressing TMIGD1 versus EV were observed. As seen in Figure 6, RKO cells expressing empty vector have hallmark characteristics of actin-assembled filopodial protrusions, as seen in migrating and often metastasizing cells (Mareel & Leroy, 2003). However, upon expression of TMIGD1, there is a noticeable decrease in filopodia density. The expression of TMIGD1 is associated with altered actin dynamics in the cell, in that there is a possible decrease in the signaling pathway for migration/invasion.

Previous data illustrated that the expression of TMIGD1 in kidney tumor cells (786-0) was associated with a decrease in cell migration, branching, and metastasis. This observation could be due, in part, by the decrease in actin-dense filopodia, seen here.

TMIGD1 expression is associated with changes in localization of GFP-moesin expression

RKO cells expressing TMIGD1 or EV were transfected with GFP-moesin and GFP-EV (used as a GFP control) and visualized via IF to examine possible changes in moesin expression in association with TMIGD1 expression. Since active, phosphorylated moesin has previously been found to localize to sites of filopodia formation, we hypothesized that since our data shows a decrease in filopodia formation when TMIGD1 is expressed, those same cells would also exhibit a decrease in moesin localization to filopodia. This hypothesis was tested in the experiment shown in Figure 7. RKO cells expressing EV and GFP-moesin appear to have a higher moesin density at the site of filopodia compared to RKO cells expressing TMIGD1 and GFP-moesin.

TMIGD1 is associated with a decrease in phosphorylated moesin

Since there seems to be a decrease in filopodia formation and an associated decrease in moesin localized to sites of filopodia, we hypothesized that the phosphorylated and active form of moesin would also be decreased when TMIGD1 was expressed. A western blot analysis confirmed this hypothesis (Figure 8).

Establishing 3-Dimensional Cell Culture Assay

After seeing the difference in cell morphology and actin redistribution in RKO cells expressing TMIGD1 versus EV in 2D, establishing a model to observe this effect in 3D was necessary. Using Elia et al. (2010) as a reference, we cultured one set of MDCK cells in a collagen matrix with DMEM, 10% FBS culture media (CM) and another set of

MDCK cells with a media that blocked the extracellular domain function of TMIGD1 (FC-TMIGD1). By blocking the extracellular domain function of TMIGD1, TMIGD1 molecules would be unable to transdimerize with neighboring molecules, and therefore, the adhesive role of TMIGD1 would be null. A 60-75% confluent plate of MDCK cells were trypsonized, counted, spun down, and then mixed in 1 mL of DMEM, 10% FBS media. Cells were then placed in a collagen mixture of purified collagen type I, sodium bicarbonate ($NaCO_3$), MEM 10X, hepes, and glutamine. Table 1 illustrates the proportions of these reagents. It is very important that the pH of the collagen concoction be neutral before adding cells to the mixture to allow for proper collagen polymerization.

Table 1: Final concentrations and volumes of reagents in 3D collagen concoction

	Stock Concentration	**Final Concentration**	**Volume**
Glutamax/Glutamine	200 mM	24 mM	240 μL
$NaHCO_3$	2.35 g/100 mL H_2O	2.35 mg/ml filtered H_2O	200 μL
MEM 10X		MEM 1X	200 μL
Hepes (1M pH 7.6)	20 mM	20 mM	40 μL
Collagen I stock	3 mg/mL	2 mg/mL	3 mL
PBS+			360 μL
Total Volume			~ 4000 μL

It is important to note that the volume of cells should not exceed 10% of the collagen concoction volume or else the collagen mixture will not polymerize properly. On the advice of Dr. Andrew Tilotson-Lunel, we used a cell density of $4x10^4$ cells per mL of collagen mixture. Sterilized membrane inserts were placed in a sterilized 24-well cell culture plate, and 100 µL of the cell/collagen concoction was then placed dropwise on the inside of a membrane insert, avoiding bubbles. Plated cells were placed in a 37°C CO_2 incubator for 30-45 minutes to allow the cell/collagen concoction to polymerize. After polymerization, 250 µL of culture media was placed dropwise in the inside of the membrane insert and 500 µL of media was placed on the outside. The cell culture was then incubated in a humidified chamber at 37°C, 5% CO_2, and media was replaced every 1-2 days.

After 9 days, preparation for microscope imaging was conducted. This involved treatment with collagenase, fixation, permeabilization, incubating in a primary antibody, secondary antibody, and loading onto a microscope slide. To begin with, the collagen gels were washed with PBS+ three times, inside and outside of the membrane insert. A dilution of collagenase-1/PBS+ (1000U/mL PBS) was prepared at a 1:10 collagenase/PBS+ in PBS. 250 µL was applied to the inside and 500 µL was applied to the outside of the membrane insert, and the inserts were incubated in 37°C for ten minutes. The inserts were then washed three times with PBS+.

Fixation involved applying 4% PFA, 250 µL and 500 µL to the inside and outside, respectively, of the inserts, followed by slow rocking of the inserts for 30 minutes at

room temperature (RT). Six washes of PBS+ were then conducted: three quick washes and three 10 minutes washes.

Permeabilization was the next step and very important for the ability of the primary and secondary antibodies to permeate through the collagen gel. A solution of 10% FBS in DMEM, 0.5% Triton-X-100 in PBS+ was used, and 250 µL and 500 µL of permeabilization solution was applied to the inside and outside of the inserts, respectively. Slow shaking of the membrane inserts was done at RT for 30 minutes.

Incubating the inserts in primary antibody solution was next. The primary antibody was diluted in the permeabilization solution in a 2:4000 µL ratio. 250 µL primary antibody solution was applied to both the inside and outside of the membrane inserts. The collagen gel was incubated overnight with the primary antibody/permeabilization solution at 4°C to allow for optimum antibody permeabilization.

The following day, the collagen gels were washed with more permeabilization solution six times: three quick washes followed by three 10-minute washes. A secondary antibody mixture was then prepared in permeabilization solution. This mixture contained alexa dyes (goat anti-rabbit, 488), nuclear staining dyes (DAPI), and actin-staining (rhodamine phalloidin) dyes at specific ratios and outlined in Table 2. (SIDE NOTE: We used Vectashield with DAPI as our nuclear staining dye. This is also a mounting gel. Therefore, we did not add a nuclear dye to the secondary antibody mixture, and instead only used it as a mounting medium). The collagen gel was incubated and slowly shaken in a light-proof container in the secondary antibody solution for 2-3 hours, followed by

six washes with permeabilization solution: three quick washes and three 10-minute washes.

Loading the gel on a microscope slide was next and was done in the dark. This involved placing a small drop of mounting solution onto the slide. In our experiment, we used Vectashield with DAPI. Very gently, the collagen gel was then placed on top of the mounting solution and a coverslip was placed on top of the gel. If the gel was not completely covered in mounting solution, a bit more was added as needed. The microscope slides plus collagen gel were placed in a sealed, dark container at RT overnight to allow drying and further stored at 4°C for up to six months.

Table 2: Concentrations in Secondary Antibody Dilution

	Ratio to Permeabilization Solution Volume
Goat Anti-Rabbit	1:400
Nuclear	1:1000
Actin (Rhodamine Phalloidin Stain)	1:300

Blocking TMIGD1 using FC-TMIGD1 affects actin distribution in MDCK cells cultured in 3D

In addition to establishing a system to study 3D cell polarity, the function of endogenous TMIGD1 in MDCK cells was examined. The control group was cultured in 3D as described above with DMEM, 10% FBS culture media. The second group was

cultured with a blocking antibody, FC-TMIGD1, in DMEM, 10% FBS. This blocking antibody has been previously tested and validated (data not shown). When cultured in normal DMEM media, actin distribution appears bundled in high concentrations around clusters of cells and at points of contact between cells. This aligns with the literature since adhesion proteins function to bind cells together and are associated with actin polymerization. Conversely, when cultured with the blocking antibody, actin distribution is less concentrated between points of cell contact and around whole clusters of cells. This is seen in Figure 9**.** In these experiments, a primary antibody against moesin was used; however, moesin was unable to be detected due to an incorrect antibody concentration or the fact that endogenous moesin has an undetectable concentration in MDCK cells cultured in 3-D.

Figure 5: Western Blot Analysis of TMIGD1 expression in RKO cells RKO cell lysates expressing EV (middle lane) and TMIGD1 (right lane) were analyzed for TMIGD1 expression using a western blot analysis. The left lane is a positive control (Previously validated 786-0 cells expressing TMIGD1. A loading control was used (bottom image).

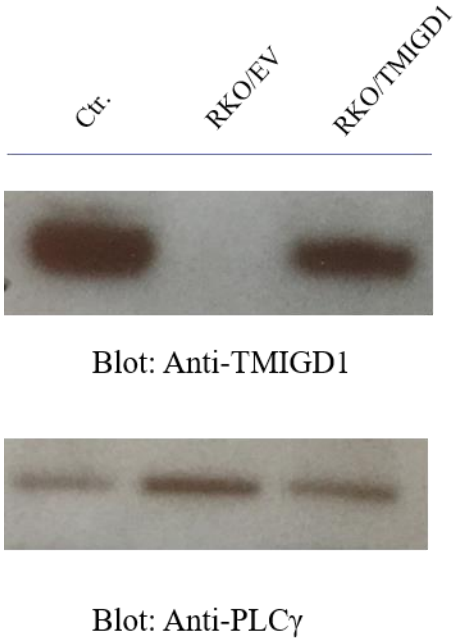


Figure 6: TMIGD1 expression is associated with decreased filopodia density in RKO cells. (A) RKO cells expressing EV (left column of A) appear to have a higher density of filopodia as compared to RKO cells expressing TMIGD1 (right column of A). Blue staining indicates DAPI-staining of nuclei. Magnification: 60X. (B) illustrates cell morphology of RKO cells expressing EV (left, bottom) or TMIGD1 (right, bottom). RKO cells expressing TMIGD1 appear to be more morphologically flatter than RKO cells expressing the empty vector. Magnification: 40X.

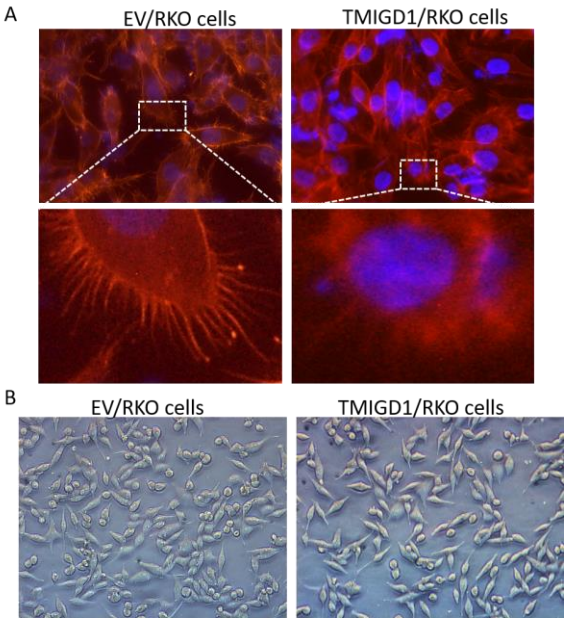


Figure 7: TMIGD1 expression is associated with altered moesin localization in RKO cells grown in 2D RKO cells co-expressing EV and GFP-moesin appear to have a higher density of moesin localization to sites of filopodia (middle column) compared to RKO cells expressing TMIGD1 and GFP-moesin (right column). Moesin's localization to the front or back pole of a migrating cell also aligns with previous literature. The left most column are RKO cells expressing EV and GFP-EV, used as a control.

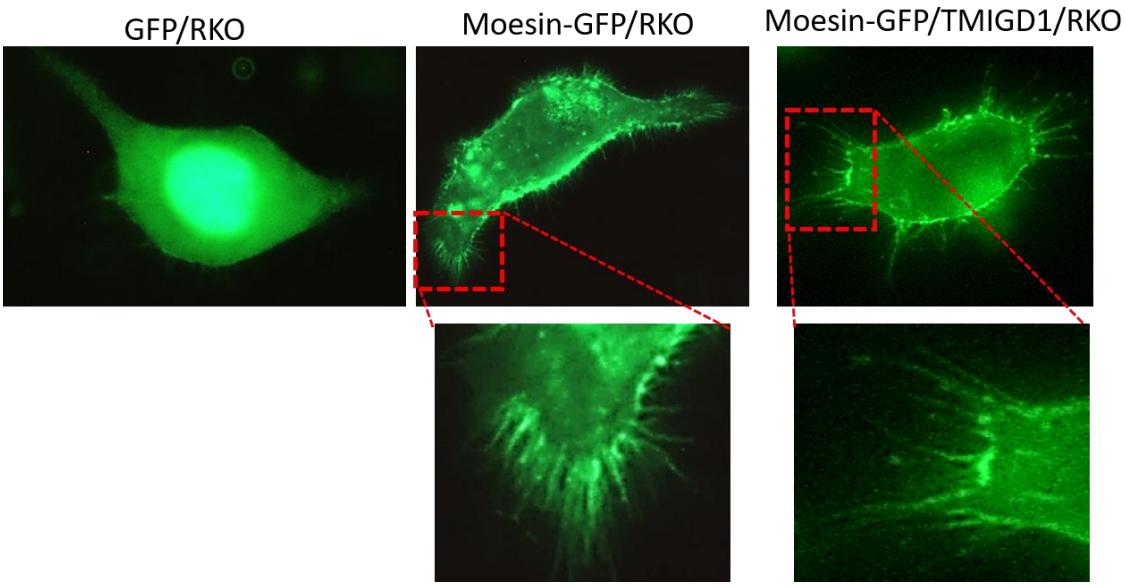


Figure 8: TMIGD1 is associated with decreased phosphorylation of moesin RKO cells expressing TMIGD1 and EV were analyzed for the phosphorylated form of moesin via western blot analysis. This preliminary data shows that expression of TMIGD1 is associated with a decrease in the phosphorylated and active form of moesin.

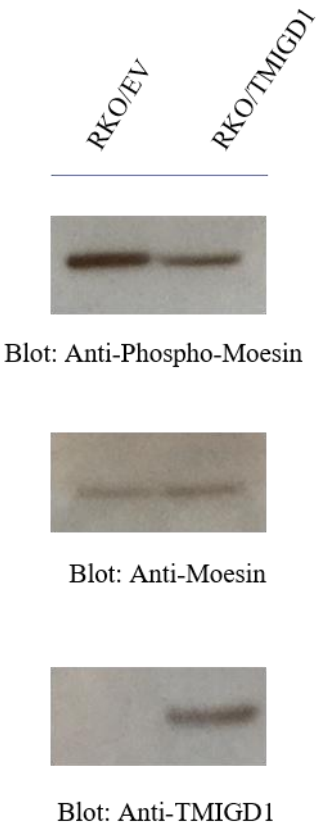
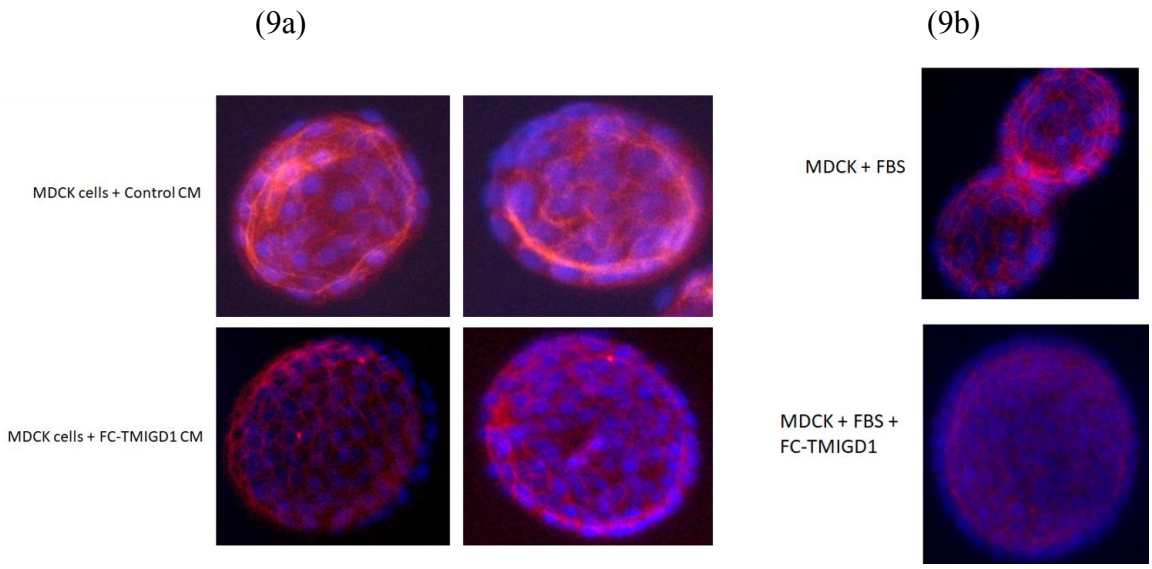


Figure 9: Blocking TMIGD1 function is associated with increased actin fibril formation in and around MDCK cell clusters Two groups of MDCK cells were grown in 3-Dimensional collagen culture media for nine days. (9a) The first group (top) was cultured in a control media (DMEM, 10% FBS). The second group (bottom) was cultured in a blocking media (FC-TMIGD1, DMEM, 10%FBS). (9b) A second experiment illustrates a similar point, where actin fibril formation appears more highly expressed (top image) versus blocking TMIGD1 and actin fibril formation appears decreased (bottom image). Red/pink color is rhodamine phalloidin, used for actin staining. The blue color is DAPI, used for staining the nuclei. Magnification: 60X.



DISCUSSION

Epithelial tissue lines our tracts and organs, allowing for a hollow lumen and the designation of inside versus outside. These cells maintain this function through adhesion to each other and a distinct polarity that separates apical membrane from the basolateral membrane. Therefore, cellular adhesion proteins, which maintain cytoskeletal connections and allow for intercellular communication are fundamental and necessary to the appropriate function of the entire tissue network. There are two distinct domains where specific cellular adhesion proteins are located. Defining the apical membrane, the TJ is composed of occludin, claudin, JAMs, and ZO proteins, along with many other kinases, phosphatases, and signaling molecules. Together, these proteins form a water-tight seal and function as a molecular and ionic barrier between neighboring cells, while also adhering these cells together through a dense actin cytoskeletal network. Sitting basal to the TJ lies to AJ. This adhesion complex is composed of E-cadherin, catenins, IGPR-1, kinases, phosphatases, and other signaling molecules, and further allows for strong points of cellular adhesion. However, it is at the AJ where appropriate flow of ions and molecules takes place. Altogether, the TJ and the AJ make up the Junctional Adhesion Complex, AJC, and for over two decades, it has been the proteins comprising this complex that have been studied in the context of cancer development and metastasis. More specifically, the downregulation of these adhesion proteins has been heavily implicated in the process of EMT, where a cell loses its defining epithelial characteristics, such as cellular adhesion, and adopts the morphological phenotype of a migratory mesenchymal cell.

In this study, we have identified another cellular adhesion molecule that is involved in maintaining that distinct epithelial phenotype. TMIGD1, a family member to IGPR-1, is a cell surface adhesion molecule found highly expressed in healthy kidney and colon cells. However, this protein is downregulated across many renal cell carcinomas and colon cancer lines, and its re-expression in kidney tumor line, 786-0, has previously been found associated with changes in cell phenotype, proliferation, and migration, in that is appears to act as a tumor suppressor, *in vitro*.

We were first able to visualize how cell morphology and actin distribution changes with expression of TMIGD1 using 2-D IF and staining for actin (phalloidin) and the nucleus (DAPI). Expression of TMIGD1 led to a decrease in filopodia formation in RKO cells, compared to the empty vector. As filopodia are actin-assembled protrusions of the cell membrane, used for probing and moving around within the environment, it is compelling that expression of TMIGD1 is associated with a downregulation of this phenotype. Furthermore, much of the literature cites that moesin is found at the site of filopodia formation, especially in carcinoma cells; therefore, we used exogenously expressed GFP-moesin to visualize the localization of the moesin in RKO cells expressing TMIGD1 or EV. As was predicted, moesin localized to filopodia regions, and expression of moesin was increased when TMIGD1 expression was absent. Noticing this decrease in moesin localization in association with a decrease in filopodia formation in cells expressing TMIGD1, we wanted to analyze the amount of active moesin in RKO cells. Moesin is active when phosphorylated at a cite-specific spot, and through western blot analysis, we found a decrease in phosphorylated moesin when cells were expressing

TMIGD1. With a decrease in active moesin, the signaling pathways moesin is involved in, especially with regards to tumorigenesis, is also decreased. This is depicted in the cartoon schematic below.

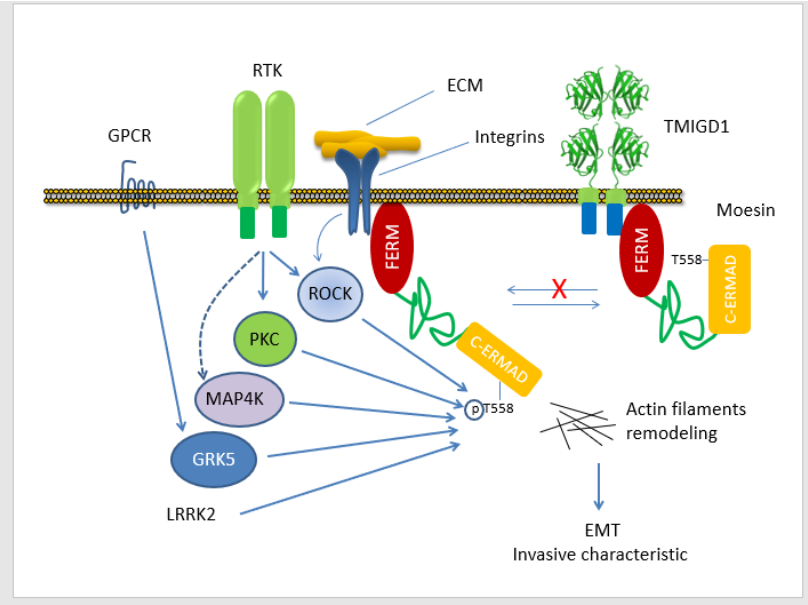


Figure 10: **Cartoon schematic of TMIGD1 binding moesin and inhibiting its phosphorylation**. Adapted from Dr. Nader Rahimi.

We hypothesize that TMIGD1 binds moesin similarly, however more strongly, than integrins or other transmembrane proteins, and this binding subsequently inhibits the phosphorylation of moesin at its site-specific spot. More research on this binding interaction and kinetics between TMIGD1 and moesin will prove compelling.

Next, we wanted to establish a culture system to study the morphological and phenotypic changes associated with functional TMIGD1. We used MDCK cells and two culture medias: a control media and a TMIGD1 blocking antibody, FC-TMIGD1, media. Effectively, this antibody would block transdimerization of TMIGD1 proteins on

neighboring RKO cells, therefore blocking the adhesive function of TMIGD1, and applying this, we were able to see a difference in actin distribution. Functional TMIGD1 is associated with points of cell adhesion, where actin staining is strong, and a bright band of actin polymers encircles the cell aggregate. Blocking TMIGD1 function appeared to decrease the points of actin-dense cell contact, and further, the band of actin encircling the cell aggregate appeared to be less dense. As we continue to use this model, we will culture different RCC lines in 3-D and examine the effects of actin distribution, while also possibly incorporating exogenous moesin, using the GFP-moesin transfection model.

Future research in the polarity and adhesion effects of TMIGD1 is needed. To begin with, using the blocking FC-TMIGD1 antibody, it would be interesting to study moesin's phosphorylation and localization in 3D. Secondly, overexpressing wild-type and mutant moesin in cells expressing TMGD1 and EV would prove interesting when examining for phosphorylation of moesin, morphological branching, and cell migration. Lastly, using preliminary data not shown, it is possible that TMIGD1 also binds scrib, a polarity protein part of the scribble family. It would be compelling to examine the relationship between the localization of scrib to the basolateral membrane and the expression of TMIGD1. If TMIGD1 is truly giving polarity back to renal carcinoma cells, it could be through its interaction with moesin and/or scrib.

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