ABSTRACT

OTT, LAURA ELIZABETH. The Role of Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) in Cell Migration. (Under the direction of Dr. Samuel Jones and Dr. Jeffrey Yoder).

Neutrophils are a pivotal component of the host response to invading pathogens as they efficiently migrate into inflamed tissue to destroy pathogens and recruit other members of the immune response. However, exacerbated neutrophil migration has been linked to the pathophysiology of chronic inflammatory diseases. Further, fibroblast migration is crucial to the process of wound healing and like neutrophils; excessive fibroblast migration also contributes to various diseases, including cancer. Therefore, inhibiting the migration of these cells is the focus of many therapeutic strategies for the treatment of a variety of diseases.

Our purpose was to identify potential targets for combating neutrophil and fibroblast migration. Previously, a role for myristoylated alanine-rich C-kinase substrate (MARCKS) has been established in the regulation of neutrophil migration *in vitro*. A myristoylated peptide corresponding to the first 24 amino acids of MARCKS (MANS peptide) results in decreased neutrophil adhesion and migration, identifying the amino-terminus of MARCKS as a regulator of MARCKS function. Further, this data provides evidence for targeting MARCKS function as a therapeutic approach for combating diseases associated with excessive cell migration. Thus, herein we sought to (1) determine if fibroblast migration is regulated by MARCKS function by utilizing the MANS peptide, (2) determine what aspect(s) of the MANS peptide are involved in regulating cell migration, and (3) determine the role of MARCKS in regulating *in vivo* neutrophil migration.

Our results demonstrate that MARCKS is involved in the regulation of fibroblast migration as MANS peptide treatment results in decreased fibroblast migration and chemotaxis. A genetic structure function analysis in fibroblasts reveals that MANS is targeted to cell membranes, presumably through amino-terminal myristoylation, whereas unmyristoylated MANS is localized to the cytosol. However, myristoylation of the MANS peptide is not involved in MANS peptide mediated inhibition of fibroblast migration. Further, we identify that zebrafish express two MARCKS paralogs (Marcksa and Marcksb), both of which are homologous to MARCKS expressed in other vertebrate species. Both Marcksa and Marcksb are involved in the normal development of zebrafish, which supports previous research demonstrating a role for MARCKS in mammalian and *Xenopus laevis* development. Utilizing a transgenic zebrafish line (MPO:GFP), in which green fluorescent protein (GFP) is expressed under the neutrophil specific myeoloperoxidase (MPO) promoter, we demonstrate that MARCKS deficient zebrafish have a decreased number of neutrophils relative to control animals. However, the neutrophils of MARCKS deficient zebrafish are still capable of migrating, as determined by similar total distance and velocity traveled. Interestingly, we do observe a trend for decreased directed migration in Marcksb deficient animals, indicating that further investigation into the role of MARCKS during *in vivo* neutrophil migration is warranted.

Taken together, we demonstrate a role for MARCKS in regulating the migration of neutrophils and fibroblasts and provide evidence that MARCKS is a potential therapeutic target for the treatment of diseases associated with exacerbated cell migration, including inflammatory diseases and cancer.

The Role of Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) in Cell Migration

by
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DEDICATION

To my two favorite men: my husband and father

Captain Chad Ott, United States Army

Louis Neuder, DVM

For their love, support and protection

BIOGRAPHY

Laura Ott (née Neuder) is the daughter of Louis and Susan Neuder and older sister to Brad (wife Allison) and Julie. Laura grew up in St. Johns, Michigan and had a typical Midwestern upbringing: she was active in the marching band, the swim team and 4-H, where she showed her horse Bandit. Laura's love of biology was cultivated while spending numerous hours on farm calls with her father, a veterinarian, and for most of her childhood, she wanted to follow in his footsteps.

Laura graduated from St. Johns High School in June of 2001 and began her freshman year at Michigan State University that fall. Michigan State was the only University that she applied to given that she is a die-hard Spartan and it was a family tradition to attend Michigan State. While at Michigan State, she had two research opportunities that fostered her interest of immunology and research. During the summers, she was an intern at Neogen Corporation working in their R & D lab designing ELISAs for the detection of food allergens. During the school year, she worked in the Immunogenetics Lab in the Department of Animal Science under the direction of the late Dr. Jeanne Burton. In Dr. Burton's lab, she worked on a project where she transformed *Klebsiella* isolates from mastitic dairy cows for use in neutrophil phagocytosis assays to determine the efficacy of a commercially available mastitis vaccine. Laura graduated from Michigan State in May of 2005 and moved to Raleigh, NC in July of that year. In the August of 2005, she joined the lab of Dr. Sam Jones and began her PhD in Immunology at North Carolina State University, College of Veterinary

Medicine. While in Dr. Jones lab, her research focus was on mechanisms of inflammation and identifying novel targets for combating excessive inflammation. Further, her research interest focus also included mechanisms of neutrophil migration. Laura also became an honorary member of Dr. Jeff Yoder's lab, given the many hours she spent in his lab working with zebrafish.

In the spring of 2006, Laura met her future husband, Chad Ott, an Officer in the Army stationed at Fort Bragg. They became engaged after dating for two years and married a year later in July of 2009. They live in Sanford, NC and have two dogs, Ladybug and Kona.

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INTRODUCTION

Infiltration of neutrophils into tissue is essential for the processes of inflammation. Neutrophils efficiently migrate into inflamed tissue and kill invading pathogens by phagocytosis and oxidative burst, the release of bactericidal enzymes and neutrophil extracellular traps (NETs). However, during chronic inflammation, neutrophils play a role in tissue damage by migrating into tissues and releasing many of the toxic mediators involved in the killing of invading pathogens. Further, neutrophil transepithelial migration of mucosal epithelium occurs during chronic inflammation of the gastrointestinal and respiratory tracts resulting in decreased barrier function (54, 55, 61, 63, 67, 84). Fibroblasts are mesenchymal cells involved in the process of wound healing as they migrate into wounds and synthesize extracellular matrix proteins that are essential for tissue repair (112-115). However, like neutrophils, excessive fibroblast migration has been linked to the pathophysiology of various diseases, including cancer (119). Thus, given that the migration of neutrophils and fibroblasts has been shown to contribute to disease, identifying novel targets to inhibit their migration has become the focus for various therapeutic strategies.

One such target is myristoylated alanine-rich C-kinase substrate (MARCKS), a protein kinase C (PKC) and calmodulin substrate capable of regulating actin dynamics. MARCKS binds to cell membranes via its myristoylated amino-terminus and electrostatic interactions between the basic effector domain of MARCKS and the acidic phospholipids of the plasma membrane. PKC phosphorylation or calmodulin binding results in the complete

dissociation of MARCKS from cell membranes and subsequent localization to the cytosol. In the cytosol, MARCKS is dephosphorylated by various protein phosphatases, allowing for MARCKS to return to and associate with the plasma membrane. The exact mechanism by which MARCKS regulates actin dynamics is not completely understood, however MARCKS has been shown to cross-link filamentous actin (F-actin) as well as regulate phosphatidylinositol 4, 5-bisphosphate (PIP_2) signaling. Thus, MARCKS has been shown to be involved in various cell processes, including cell migration (133, 134).

Previously, we have demonstrated a role for MARCKS in the regulation of *in vitro* neutrophil migration by utilizing a cell permeable myristoylated peptide corresponding to the first 24 amino acids of MARCKS (MANS), which has been shown to disrupt MARCKS function (143). MANS peptide treatment results in decreased neutrophil chemotaxis towards a variety of chemoattractants as well as decreased β_2-integrin dependent adhesion (142). MANS peptide treatment also results in decreased leukocyte degranulation, mucin secretion in the airway epithelium and inhibited mesenchymal stem cell chemotaxis (141, 143, 256). These results suggest that the amino-terminus of MARCKS is involved in the regulation of MARCKS function, although the exact mechanism by which the MANS peptide regulates MARCKS function is not completely understood. Further, these results suggest that targeting MARCKS is a potential therapeutic approach for the treatment of various diseases in which excessive cell migration is involved.

However, to further confirm our previous results demonstrating a role for MARCKS in neutrophil migration, an *in vivo* model for cell migration is required. Unfortunately,

utilizing the typical mouse model to study MARCKS function *in vivo* is not feasible because targeted disruption of the MARCKS gene in mice is embryonic lethal (236). Recently, however, transgenic zebrafish models have been utilized for studying *in vivo* migration of neutrophils (100-102). With numerous options for the method to inhibit gene expression in zebrafish, they have become an excellent replacement for the mouse model to study the role of various proteins in cell migration *in vivo*.

Thus, the central hypothesis to be tested herein is that MARCKS function is required for fibroblast migration *in vitro* and neutrophil migration *in vivo*.

Specific Aims:

1. To determine if fibroblast migration is regulated by MARCKS.
2. To determine if amino-terminal myristoylation of MANS is involved in the regulation of fibroblast migration.
3. To determine if MARCKS is involved in the embryonic development of zebrafish.
4. To determine if MARCKS is involved in the migration of neutrophils *in vivo*.

CHAPTER 1: LITERATURE REVIEW

A. Neutrophil Migration

Neutrophils are to the immune system what the Special Operations forces are to the United States Military. Neutrophils are fast and efficient migratory cells and are thus first to be recruited into combat. Neutrophils begin the initial assault by engulfing pathogens and releasing strategic enzymes and mediators that weaken the enemy and recruit other members of the immune system to ultimately destroy the opposition. Given that neutrophils are short lived cells that die via apoptosis (or programmed cell suicide) and never return to the blood stream; some may rather compare neutrophils to Japanese kamikaze fighters during World War II. However, neutrophils are such a pivotal component of the immune system that without them the organism that they serve are at grave danger, similar to how the special operations forces are essential to the protection of the United States citizens.

1. Neutrophil Diapedesis

Diapedesis is a coordinated multi-step process in which neutrophils emigrate from circulating blood (usually postcapillary venules) into inflamed tissue. There are four steps to diapedesis: capture, rolling, arrest and transmigration. Two classes of cell adhesion molecules (CAMs), selectins and β2-integrins, are responsible for mediating neutrophil diapedesis (1-3).

Selectins are transmembrane glycoproteins expressed on inflamed endothelial cells (E- and P-selectin), platelets (P-selectin) and leukocytes (L-selectin). All three members of the selectin family recognize the sialylated Lewis X (sLe^x) antigen found on membrane glycoproteins as well as P-selectin glycoprotein ligand-1 (PSGL-1) (3-5). L-selectin is expressed throughout myeloid differentiation and is expressed by the majority of unstimulated neutrophils in circulation. Upon activation of neutrophils, L-selectin is shed by endoproteolytic cleavage, resulting in functional soluble L-selectin present at high levels in plasma and shedding of L-selectin results in the activation of additional adhesion molecules on the surface of neutrophils. Soluble L-selectin is thought to be a buffer to prevent neutrophil diapedesis at sites of subacute inflammation and elevated levels of soluble L-selectin have been found in the plasma of patients suffering from sepsis or Acquired Immune Defficiency Syndrome (AIDS) (4, 6). P-selectin is constitutively found within Weibel-Palade bodies of endothelial cells and various pro-inflammatory mediators (histamine, complement fragments and cytokines) mediate the mobilization of P-selectin to the surface of endothelial cells. P-selectin expression on the cell surface of endothelial cells is for a relatively short period of time (minutes) and thus involved in mediating early neutrophil-endothelial interactions. E-selectin expression is rapidly induced by stimulation of endothelial cells with pro-inflammatory mediators, including tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and lipopolysaccharide (LPS) and is involved in mediating later neutrophil-endothelial interactions (4).

After the initial capture event, which is mediated by selectins, neutrophils are activated and begin to roll along the endothelium. Both capture and rolling of neutrophils on the endothelium occur under the shear stress conditions of blood flow and studies have revealed that shear stress is required for both L- and P-selectin mediated neutrophil rolling (1, 7, 8). Activation of neutrophils is mediated by both selectin binding and chemoattractants, such as interleukin-8 (IL-8), platelet activating factor (PAF), and leukotriene B_4 (LTB_4) (9-11) which signal through seven-transmembrane G-protein-coupled receptors (GPCRs) (12). Activation of neutrophils through GPCRs and/or signaling through selectins result in the activation of intracellular signaling pathways that lead to β_2-integrin activation, specifically leukocyte function-associated antigen-1 (LFA-1 or $\alpha_L\beta_2$ or CD11a/CD18) and macrophage antigen-1 (Mac-1 or $\alpha_M\beta_2$ or CD11b/CD18). Neutrophil-endothelial interactions involving both L- and E-selectin activate β_2-integrins in a mechanism involving the p38 mitogen-activated protein kinase (MAPK) pathway (13, 14). The β_2-integrins, LFA-1 and Mac-1, are also involved in neutrophil rolling along the endothelium where LFA-1 is involved in earlier stages of rolling and Mac-1 is involved mediating slow rolling (1, 15).

Studies have revealed that neutrophil arrest on the endothelium is dependent on β_2-integrin mediated binding to its ligands. LFA-1 binding to inter-cellular adhesion molecule 1 (ICAM-1) is responsible for arrest of neutrophils on the endothelium. ICAM-1 is an immunoglobulin superfamily ligand that is constitutively expressed at low levels on endothelial cells with upregulated expression observed upon IL-1β and TNFα stimulation (1, 16, 17). Integrin activation is rapidly triggered by chemokine signaling through GPCRs,

which is an example of inside-out signaling. Briefly, endothelial bound chemoattractants activate the GPCR and signals to LFA-1 via Gβγ triggering of phospholipase C (PLC). PLC cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) into inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG), which activates guanine nucleotide exchange factors which target small GTPases. Eventually talin, a cytoskeletal protein, is activated and mediates a conformational change in LFA-1, in which it goes from its inactive, low-affinity folded conformation to its active, unfolded and extended high-affinity conformation. LFA-1 is then capable of binding to ICAM-1, which further signals integrin activation via outside-in activation. Force exerted by the ICAM-1/LFA-1 interaction as well as shear stress from blood circulation results in further cleavage of PIP_2 and activation of talin resulting in stabilization of activated LFA-1. The Src family kinases are also involved in mediating outside-in signaling during LFA-1/ICAM-1 mediated neutrophil arrest and LFA-1/ICAM-1 interaction results in increased filamentous actin (F-actin) expression in neutrophils (1, 17-19).

Once neutrophils have arrested on the endothelium, they then begin the transmigration process of diapedesis in a manner that is dependent largely on Mac-1. The first step in transmigration is neutrophil crawling along the endothelium, which is performed so that the cell can find a preferential site to transmigrate (1, 20). Neutrophils transmigrate the endothelium by one of two routes: paracellular or transcellular. For the paracelluar route, Mac-1 interacts with ICAM-1 during neutrophil crawling resulting in increased intracellular Ca^{2+} concentrations and the activation of p38 MAPK and Rho GTPases in endothelial cells.

This results in myosin contraction and the opening of the endothelial cell junctions. Once the endothelial cell junction is open, both LFA-1 and Mac-1 can interact with endothelial junctional molecules to transmigrate the endothelium. Specifically, LFA-1 can interact with junctional adhesion molecule A (JAM-A) and ICAM-1 and -2 while Mac-1 interacts with JAM-C and ICAM-1 and -2 (21-24). The immunologlobulin superfamily member platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and the glycoprotein CD99 are molecules expressed by both neutrophils and endothelial cells. PECAM-1 or CD99 on neutrophils interacts with PECAM-1 or CD99, respectively, that are present in the cell-cell junctions of the endothelium (2, 23). Therefore, during paracellular endothelial migration, neutrophil β_2-integrins, as well as other ligands, interact with endothelial cell junction molecules to migrate between the endothelial cells.

Until recently, paracellular migration was considered to be the only mechanism by which neutrophils transmigrate the endothelium. However, neutrophils can also transmigrate via a transcellular route, which was first demonstrated by Feng, *et al* (25). This study showed that adherent neutrophils project cytoplasmic processes into the endothelial cells in areas where the endothelial cells are thin. Electron micrograph studies revealed that the neutrophils were in fact transmigrating the endothelium via a transcellular route (25). Further *in vitro* studies have revealed that neutrophils utilize vesiculo-vacuolar organelles (VVOs) as a passage way to transmigrate the endothelium. VVOs are specialized endothelial organelles that form when ICAM-1 coated caveolae link together forming a channel for the neutrophil to transmigrate through (1, 26). Although evidence of *in vivo* VVO formation has yet to be

shown, it has been estimated that approximately 5-20% of neutrophils undergo transcellular migration in models utilizing cytokine activated human umbilical vein endothelial cells (HUVECs) (1).

Once neutrophils have transmigrated the endothelium, they have to transmigrate the endothelial cell basement membrane (a meshwork of laminins and collagen proteins) as well as pericytes before they can start migrating towards the inflamed tissue that they have been recruited to (1). Endothelial transmigration of neutrophils can trigger the activation of molecules that aid in neutrophil migration through the basement membrane and pericytes. An example is PECAM-1 mediated mobilization of $\alpha_6\beta_1$ integrins from intracellular stores to the membrane of neutrophils. The integrin $\alpha_6\beta_1$ is the main leuckoyte receptor for laminin and thus is key for mediating neutrophil migration through the endothelial basement membrane (27). Further, neutrophil membrane bound enzymes, such as matrix metalloproteinases and neutrophil elastase, have been hypothesized to aid in degrading the type IV collagen network of the basement membrane allowing the neutrophil to migrate (1, 28). Neutrophils then transmigrate the pericytes, which are cells that form a network around endothelial cells. The pericyte network is discontinuous and large gaps between pericytes exist, which are the regions where neutrophils migrate through (1). Once neutrophils migrate past the basement membrane and pericytes, they are able to migrate to the source of inflammation.

2. Neutrophil chemotaxis

Chemotaxis is defined as directed migration of a cell towards a chemical point source, which differs from chemokinesis or random cell migration. For neutrophils, the chemical point sources or chemoattractants signal through chemoattractant receptors, which are seven transmembrane GPCRs. The GPCRs responsible for chemotaxis are of the G_i and $G_{12/13}$ families which activate signals at the front and rear of the cell, respectively (29). These signals lead to pseudopod formation and polarization of the leading edge and contraction of the uropod or trailing edge of the cell, resulting in locomotion of the cell along the chemoattractant gradient being sensed (30).

For G_i chemokine receptors, chemoattractant binding results in a conformational change in the receptor leading to exchange of GDP for GTP in the α subunit and dissociation of the βγ subunits. The βγ subunit is responsible for activating the phosphoinositide 3-kinase (PI3K) pathway and PI3K localizes to the leading edge. The PI3K pathway is responsible for phosphorylating the membrane phosphoinostide PIP_2 generating phosphatidylinositol (3, 4, 5)-trisphosphate (PIP_3), which goes on to activate protein kinase B (PKB)/AKT and the GTPases Rac and Cdc42. PKB/AKT localizes to the leading edge while Rac and Cdc42 activate the Wiskott-Aldrich syndrome protein (WASP) family of proteins, which are F-actin nucleation promoting factors involved in activating the F-actin nucleation Arp2/3 complex. Ultimately, localized F-actin polymerization results in the extension of a leading edge pseudopod. Polarization occurs when multiple pseudopods form at the leading edge

resulting in extension towards the chemoattractant point source with simultaneous contraction of the sides and trailing edge of the cell. Trailing edge contraction occurs through the activation of Rho GTPases, which are downstream of $G_{12/13}$ GPCRs. The α subunit of $G_{12/13}$ directs Rho GTPases to the rear of the cell and results in the activation and rear localization of phosphatase and tensin homolog (PTEN). PTEN is a protein phosphatase responsible for the conversion of PIP_3 to PIP_2, thus inhibiting PI3K and PKB/AKT signaling. Further, RhoA activation of Rho-stimulated kinase (ROCK) and myosin light chain kinase (MLCK) results in activation of myosin II through phosphorylation of myosin light chain (MLC) and uropod contraction (1, 29-35).

Neutrophil chemoattractants include the chemokines IL-8 (CXCL-8), macrophage inflammatory protein-1a and -2 (MIP-1a or CCL3; MIP-2 or CXCL2) and CXCL-1 (Groα) as well as the non-chemokine chemoattractants PAF, LTB_4, fMLP and C5a (9-11, 36-41). A recent hierarchy for neutrophil chemoattractants has been established. Chemoattractants produced in the vicinity of infection, fMLP and C5a (termed end stage chemoattractants), dominate neutrophil chemotaxis compared to IL-8, MIP-2 and LTB_4 (intermediary chemoattractants) (42-44). The end stage chemoattractants induce a signaling event that is dependent on the p38 MAPK pathway in mediating neutrophil chemotaxis whereas the intermediary chemoattractants induce a signaling event dependent upon PI3K signaling (43). Both neutrophil chemokinesis and chemotaxis are regulated by type I PI3Ks (β and γ). Inhibition of PI3Kγ significantly reduces $LTB4_4$ and fMLP mediated β_2-integrin adhesion and neutrophil influx to the respiratory mucosa upon LPS exposure (45, 46). Further,

inhibition of either PI3Kβ or PI3Kγ results in the inability of neutrophils to polarize (46, 47). Pharmacological inhibition of the p38 MAPK pathway in neutrophils results in decreased neutrophil migration towards LTB_4 and fMLP as well as inhibited neutrophil polarization towards LTB_4 and PAF (48-50). Besides being involved in neutrophil migration, both the PI3K and p38 MAPK pathways are also involved in regulating IL-8 production (51-53).

Neutrophil's "job" is to migrate to the source of bacterial infection and kill the bacteria by phagocytosis, release of antimicrobial mediators, production of neutrophil extracellular traps (NETs), and to recruit and activate other immune cells (54, 55). However, neutrophils can also contribute to the pathophysiology of chronic inflammatory diseases. Neutrophils are quickly recruited to sites of inflammation such as to the kidneys of patients suffering from glomerulonephritis and to the joints of patients suffering from rheumatoid arthritis (56, 57). Recent studies have revealed that blocking CXCR2 may be suitable therapeutic for treating rheumatoid arthritis. CXCR2 is the chemoattractant receptor on neutrophils that recognize CXCL1, MIP-2α and CXCL5, all chemokines involved in neutrophil migration. In a model for autoantibody induced arthritis, $CXCR2^{-/-}$ mice have decreased neutrophil migration into the joints compared to littermate controls (57). Further, neutrophil migration to inflammatory sites can be induced by immune complexes through the activation of C5a (58) or by interacting with neutrophil Fcγ receptors (FcγRs; CD16) (59). In a mouse model of immune complex mediated disease, symptoms were diminished by administering a CD16-Ig decoy, blocking neutrophil FcγRs and resulting in decreased neutrophil migration (60). Neutrophils can also exacerbate intestinal disease by releasing the

enzymes elastase and proteinase-3. Both of these enzymes can activate protease activated receptor (PAR) -1 and -2 on epithelial cells, resulting in decreased epithelial barrier function (61). Although neutrophil migration into inflamed and/or damaged tissue is critical to combating the source of inflammation, excessive neutrophil migration into chronically inflamed tissue often exacerbates disease. Therefore, the identification of novel targets to combat neutrophil migration may offer future therapeutic options for the treatment of these diseases.

3. Neutrophil transepithelial migration

Neutrophil migration into mucosal tissue has been attributed to the pathophysiology of many diseases, including inflammatory bowel disease (IBD), Salmonellosis and ischemia reperfusion injury in the gastrointestinal tract (62-64) as well as chronic obstructive pulmonary disease (COPD) in the respiratory tract (65). During gastrointestinal inflammation, intestinal epithelial cells secrete IL-8 basolaterally, resulting in the recruitment of neutrophils to the sub-mucosa (66, 67). Subsequently, luminal fMLP guides neutrophils to undergo transepithelial migration (TEM) in the basolateral to apical direction (63, 67). Multiple studies have been performed by Charles Parkos, Sean Colgan and James Madara, who have elegantly described the molecular mechanisms of neutrophil TEM (63, 67-72). This group of researchers developed a method for studying neutrophil TEM using polarized monolayers of T84 cells, a human colonic intestinal cell line, that are grown in either a

conventional (apical to basolateral direction) or inverted (the physiologically relevant basolateral to apical direction) manner on permeable transwell inserts. The inserts are placed into the well of a tissue culture plate with a chemoattractant in the bottom chamber and cells in the upper chamber. This model is designed to study two aspects: neutrophil migration across the epithelial monolayer and the permeability and barrier function of the T84 monolayer by electrical parameters. Studies with this model have revealed a correlation between the number of transmigrated neutrophils and the barrier function showing in higher numbers of transmigrated neutrophils correlate directly with lower transepithelial electrical resistance and thus decreased barrier function (68).

The above model has also revealed a critical role for Mac-1 (CD11b/CD18) in mediating neutrophil TEM. CD11b and CD18 blocking antibodies interfere with the decreased transepithelial electrical resistance (TER) associated with neutrophil TEM. Furthermore, CD11b and CD18 blocking antibodies inhibit neutrophil migration across a T84 monolayer in the presence of an fMLP gradient (68). Studies have revealed that Mac-1 on neutrophils initially makes contact with fucosylated glycoproteins on the basolateral membrane of the epithelial cells (69, 70) and that this interaction increases the paracellular permeability of the intestinal monolayer independent of neutrophils undergoing TEM (71). This not only allows solutes and harmful bacteria to transverse the intestinal barrier but it also opens the epithelial tight junctions so that neutrophils can migrate in a paracellular manner. Between epithelial cells, neutrophil Mac-1 interacts with JAM-C, which is protein associated with the cell-cell junctions of epithelial cells (72). Further interaction between

neutrophil junctional adhesion molecule-like (JAML) and epithelial coxsackie and adenovirus receptor (CAR) is also involved in mediating neutrophil TEM (73). An additional contact involving signal regulatory proteins (SIRPs) have also been hypothesized in mediating neutrophil transepithelial migration. Antibody mediated ligation of SIRPβ1, a disulfide-linked homodimer expressed on neutrophils, results in enhanced neutrophil TEM in the presence of an fMLP gradient (74). Further, interaction between neutrophil SIRPα and epithelial CD47 is also hypothesized to be involved in mediating neutrophil TEM with CD47 being a membrane immunoglobulin superfamily member involved in mediating neutrophil TEM after the initial Mac-1 dependent basolateral adhesion event (67, 75). Once neutrophils have migrated through the epithelial junction, they can interact with the apical membrane of epithelial cells. Studies in the respiratory mucosa have revealed the requirement for ICAM-1 in mediating neutrophil transepithelial migration, as demonstrated by decreased TEM in the presence of an ICAM-1 blocking antibody (76). Inflammation of the intestinal barrier results in upregulation of ICAM-1 to the apical surface of epithelial cells and although there is no direct evidence, it is hypothesized that Mac-1 interacts with ICAM-1 on the apical surface of intestinal epithelial cells (67, 75). Also at the apical membrane, neutrophil FcRs can interact with apical membrane ligands that are upregulated in inflamed intestinal cells (77).

Neutrophil migration into mucosal tissue results in an assault on the source of the inflammation and during both *Salmonella typhimurium* and *Shigella flexneri* infections, neutrophils are signaled to undergo TEM of the intestinal epithelium (78). SipA is an effector protein of the type III secretion system of *S. typhimurium* that elicits a

proinflammatory response resulting in elevated IL-8 production and neutrophil TEM (62, 79, 80). Transepithelial migration of neutrophils enhances the killing of *S.typhimurium* in a mechanism involving IL-6 produced by inflamed intestinal epithelial cells. This enhanced killing is due to the neutrophil degranulation and extracellular release of myeloperoxidase and lactoferrin (81). However, in many cases, exacerbated neutrophil TEM is associated with an increase in disease pathophysiology. The tight junction protein occludin is downregulated in IBD patients, which may have a role in the increased paracellular permeability and neutrophil TEM associated with the disease (82). Further, an upregulation of CD11b and CD18 on neutrophils is observed in ulcerative colitis patients (83) and neutrophils are capable of injuring both the intestinal and respiratory epithelium by the release of oxidants, defensins, elastase and matrix metalloproteinases (84). Taken together, targeting the TEM of neutrophils into mucosal tissues during chronic inflammatory diseases is a therapeutic approach that many researchers and clinicians are interested in pursuing further for the treatment of inflammatory diseases of mucosal tissue.

4. The use of the zebrafish model to study neutrophil migration

A new *in vivo* model for studying neutrophil migration has been established in which transparent zebrafish embryos are utilized. Zebrafish (*Danio rerio*) are fresh water tropical fish belonging to the minnow family (*Cyprinidae*). Zebrafish are becoming an increasingly popular model organism for biological sciences given that they are inexpensive to maintain

and can be easily bred with embryos that are transparent, develop external to the mother and have a short generation time. Further, the zebrafish genome has been sequenced and techniques are available for easy gene manipulation and generation of transgenic lines. Although initially used as a developmental model, zebrafish are becoming increasingly popular as a model organism for the study of innate immune function (85, 86).

Vertebrate hematopoiesis involves common myeloid progenitor cells giving rise to two separate cell lineages; the granulocyte/macrophage lineage and the erythrocyte/megakaryocyte lineage (87). Transcription factors are key to regulating hematopoiesis in mammals and homologoues of many mammalian hematopoietic transcription factors have been identified in zebrafish (88, 89). In mammals, primitive hematopoiesis occurs outside the embryo in the blood islands of the yolk sac and moves into the aorta-gonad-mesonephros region and the fetal liver while adult hematopoiesis occurs in the bone marrow (90-93). In zebrafish, primitive hematopoiesis occurs in the intermediate cell mass (ICM), a region between the notochord and endoderm of the trunk (94). There is evidence that primitive hematopoiesis also occurs in the paraxial mesoderm and posterior blood island, which is a small cluster of cells in the developing tail of zebrafish (95). In adult zebrafish, hematopoiesis occurs in the kidney with some hematopoiesis also occurring in the spleen (96).

An analysis of hematopoietic tissue of adult zebrafish has revealed the presence of neutrophils along with basophils/eosinophils and macrophages, additional members of the myelolid family (39, 97). Beyond two days post fertilization, the development of neutrophils

occurs within the caudal hematopoietic tissue (98, 99). Like mammalian neutrophils, zebrafish neutrophils can be identified by the production of myeloperoxidase (MPO), an enzyme involved in respiratory burst. The protein sequence of zebrafish MPO is 76.5% similar to human MPO, with 49.6% identical amino acids between the homologues. The expression of MPO in zebrafish was analyzed by *in situ* hybridization and *mpo* mRNA is expressed 18 hours post fertilization and localized anteriorly spreading along the yolk sac. At three days post fertilization, *mpo* is expressed in the anterior yolk, the posterior ICM and posterior blood islands and at four days post fertilization, *mpo* is expressed throughout the embryo. The adult kidney also contains *mpo* positive cells (95). Studies have shown that neutrophils are recruited in response to tissue wounding in larval stage zebrafish embryos (96, 100) and thus a tail wounding model has been developed for the study of *in vivo* neutrophil migration. The model is quite simple; three day post fertilization embryos are embedded in low melting point agarose containing Tricane (an aquatic species sedative) and the tails of the embryos are wounded with a 25-guage needle. After incubation, the embryos are fixed and stained with a leukocyte peroxidase kit, which enables the visualization of MPO and the number of neutrophils that have migrated in proximity to the wound are quantified (101).

There are limitations to the above approach in that live imaging of neutrophil migration cannot be obtained. Live imaging of neutrophil migration is advantageous because it allows for the analysis of single larvae over time as well as the ability to determine multiple parameters such as total distance, velocity and direction of migration. Thus, two transgenic

zebrafish lines have been developed in which expression of green fluorescent protein (GFP) is driven by the MPO promoter. One transgenic line, produced by Huttenlocher and collegues, was generated by screening a P1 artificial chromosome clone (PAC) library for the zebrafish *mpo* cDNA sequence and cloning the 8kb 5' untranslated region of *mpo* upstream of GFP and a SV40 polyadenylation sequence. Wild type embryos were injected in the single cell stage and raised to maturity and mated with wild type embryos to produce the F1 generation, which was screened for GFP expression (102). The second transgenic line, produced Renshaw and colleagues, was generated by a bacterial artificial chromosome (BAC) containing 130kb of the 5' untranslated sequence of *mpo* that has enhanced green fluorescent protein (EGFP) and a SV40 polyadenylation site inserted at the *mpo* ATG start codon. The BAC was linearized and injected into single cell embryos and stable transgenic lines were generated (100). One of the key differences between the Huttenlocher and Renshaw lines is that the Huttenlocher line has two distinct populations of cells (GFP^{bright} and GFP^{dim}) whereas the Renshaw line solely expresses GFP^{bright} cells. Using the transgenic fish produced by Huttenlocher, investigators have shown that the GFP^{dim} cells constitute a population of monocyte/macrophages, based on the morphology and the fact that monocytes/macrophages are capable of expressing low levels of MPO activity (96, 100, 103).

Both of these transgenic lines, termed MPO:GFP, have been used to study *in vivo* neutrophil migration. Studies by Huttenlocher have shown that neutrophils undergo a retrograde chemotaxis event in which neutrophils migrate towards the wound and then back

into circulation (96), which is paradigm that is not generally accepted by neutrophil biologists. Results reported by Renshaw and colleagues, however, contradict these findings and demonstrate that after neutrophils migrate into the wound, they die via apoptosis (100, 104), a mechanism for the resolution of neutrophilic inflammation that is widely accepted. Further studies with transgenic MPO:GFP zebrafish lines have elucidated the importance for both PI3K and Rac in mediating neutrophil motility *in vivo* (105). Further the MPO:GFP model has also been used to demonstrate *in vivo* evidence of exacerbated neutrophil migration in a model for chronic inflammation (106), a link between muscle degradation and infiltration of neutrophils (107) and *in vivo* evidence of macrophage uptake of apoptotic neutrophils during the resolution of inflammation (104). Ultimately, the use of MPO:GFP transgenic zebrafish provide a useful model for the study of *in vivo* neutrophil migration and function.

5. Comparison of neutrophil and fibroblast migration

Neutrophils are terminally differentiated cells and survive upwards of 24 hours in circulating blood and are therefore cannot be maintained *in vitro* (18). The HL-60 cell line, which can differentiate into a neutrophil-like cell upon DMSO treatment (108, 109), has been used in various studies as a model for neutrophil migration (110). However, performing genetic studies in differentiated HL-60 cells has been difficult until recent advances allowing for HL-60 transfection by nucleofection (110). Fibroblasts, a mesenchymal cell, are also

capable of chemotaxis and thus are another cell type that can be used to study mechanisms of neutrophil migration. Fibroblasts are easily transfected and both primary fibroblasts as well as commercially available fibroblast cells lines (NIH-3T3 cells) have been utilized to study mechanisms of cell migration. Various techniques have been used to study fibroblast migration including scratch or wounding assays, Boyden chambers, or microfluidics devices (111).

Like neutrophils, fibroblasts are essential to the process of inflammation and in particular are involved in the process of repairing inflamed tissue. Fibroblasts migrate into wounds or inflamed tissue, synthesize extracellular matrix proteins, proliferate and remodel the tissue in a mechanism resulting in wound closure and/or tissue repair (112-115). However, also like neutrophils, fibroblasts can contribute to chronic inflammation and have been linked to the pathogenesis of various diseases, including COPD and cancer (116-120).

Both neutrophils and fibroblasts have been classified as "professional" migratory cells. This classification is given because these cells both migrate via chemotaxis and with an asymmetrical morphology. This morphology is characterized by polarization and spreading of the front of the cell towards a chemoattractant source with adhesive contacts forming between the cell and the extracellular matrix. Subsequent contraction of the cell rear results in the propulsion of the cell in the direction of migration (30, 32, 121). Various signaling molecules, including PIP_3, PI3K, Rac and Cdc42, asymmetrically localize to the pseudopod of migrating neutrophils and fibroblasts, while Rho GTPases and PTEN localize to the trailing edge uropod (31, 32, 121).

Although the morphology of migrating neutrophils and fibroblasts and the signaling molecules involved in their regulation are similar, there are many differences between these two cell types. The obvious difference is that neutrophils respond to a variety of chemoattractants (IL-8, fMLP, C5a, and LTB_4), which signal through GPCRs, whereas fibroblasts primarily respond to platelet derived growth factor (PDGF), which signals through a receptor tyrosine kinase (RTKs) (41, 122). PDGF is produced by macrophages and platelets and the PDGF family consists of four members (PDGF-A, PDGF-B, PDGF-C and PDGF-D) which form dimers of disulfide-linked polypeptide chains. A homodimer of PDGF-B subunits (PDGF-BB) is the key regulator of fibroblast migration *in vivo* and *in vitro* and the receptor for PDGF-BB consists of two PDGF receptor (PDGFR)-β chains, which dimerize upon PDGF-BB ligation. Dimerization of the PDGFRβ results in autophosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptors. The phospho-tyrosines on the cytoplasmic tail serve as docking sites for various signaling molecules and adaptor proteins. PI3K interacts with the phospho-tyrosine residues of the RTK via Src homology 2 (SH2) domains, resulting in its activation and subsequent activation of Rac and Cdc42 and actin polymerization. PLCγ also interacts with the cytoplasmic domain of PDGFR, resulting in its activation via phosphorylation. The adaptor proteins, Grb2 and Shc, bind to PDGFR via SH2 domains and lead to activation of Ras and the MAPK pathways. Further, PDGFRβ can interact with Na^+/H^+ exchanger regulatory factors (NHERFs) that are capable of activating PTEN and linking PDGFRβ to the cortical actin cytoskeleton through its association with focal adhesion kinase (FAK) (122-124).

One signaling molecule that differs between neutrophil and fibroblast migration is PI3K. Both neutrophils and fibroblasts utilize class I PI3Ks because this class of PI3K is responsible for the generation of PIP_3, which is essential for mediating chemotaxis. However, fibroblasts utilize class 1α PI3Ks for migration whereas neutrophils utilize class 1 β and γ PI3Ks (45-47, 125). An additional difference is that neutrophils migrate at fast speeds (~ 20 μm/min) and require a shallow gradient of chemoattractant to migrate whereas fibroblasts migrate at slower speeds (~ 1 μm/min) and require a steep gradient of chemoattractant. This is due to stronger adhesive contacts between fibroblasts and the extracellular matrix that are mediated by integrins (126). Neutrophils express the β_2-integrins LFA-1 and Mac-1, which allow adhesion to plasma proteins, immune complexes and other cell types (endothelial and epithelial cells) (1, 15, 127, 128) as well as $\alpha_6\beta_1$ integrin, which interacts with laminins (27). Fibroblasts, however, do not express β_2-integrins and instead, primarily express β_1-integrins, including, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_{11}\beta_1$. These integrins recognize extracellular matrix proteins, specifically fibronectin and collagen (129).

Neutrophils and fibroblasts adopt a similar morphology and utilize similar intracellular signaling pathways during migration, thus making fibroblasts a useful model for studying neutrophil migration. However, one must keep in mind the various differences between the two cell types and how these differences may alter the migration of their respective cells.

B. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS)

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a 32 kDa acidic, unfolded, rod shaped protein initially identified as a substrate for protein kinase C (PKC) (130-134). First identified in 1982 as an 80 kDa protein given its size on a SDS-PAGE (135), MARCKS is an ubiquitously expressed protein with expression observed in neural and CNS tissue (131, 136), connective tissue (137), skeletal muscle (138), renal tissue (139), leukocytes (132, 140-142), and in the gastrointestinal, respiratory and reproductive tracts (143-145). Characterization of *MARCKS* mRNA and protein expression has led to the discovery that numerous vertebrate species, both mammalians and non-mammalian, express MARCKS, including; human (146, 147), mouse (132, 137), rat (148, 149), bovine (150), chicken (151), and *Xenopus laevis* (152)[16]. The *MARCKS* gene (previously termed *Macs*) encodes *MARCKS* mRNA with a single intron splice site towards the 5' end (149) and the *MARCKS* gene is upregulated by stimulation with LPS, TNF-α and β-amyloid peptides (153-156). There are subtle differences in the size and amino acid sequence of MARCKS protein expressed by different species; however, the conserved domains of the protein remain constant (131, 149, 151, 157). MARCKS contains three conserved domains; a myristoylated amino-terminal domain, a MH2 domain, and the phospho-site domain (PSD), which serves as the phosphorylation site for PKC (130, 158). The MH2 domain contains the intron-splice site and has homology to the internalization domain of the mannose-6 receptor, but has no known function (130).

In resting cells, MARCKS is anchored to cellular membranes via its myristoylation motif and electrostatic interactions between the basic amino acids of the PSD and the acidic phospholipid bilayer, which is enriched in phosphatidylserine and PIP_2 (159, 160). MARCKS is myristoylated post-translationally by the addition of the myristoyl moiety (C_{14} saturated fatty acid) to the amino-terminal glycine residue. The myristoyl moiety of MARCKS inserts itself hydrophobically into the phospholipid bilayer of the plasma membrane (130, 160-166). The basic residues within the PSD of MARCKS electrostatically interact with acidic phospholipids, further anchoring MARCKS to cellular membranes (164-166). Both myristoylation and electrostatic interactions tether MARCKS to cellular membranes in an independent, yet additive manner with the electrostatic interactions having a higher affinity for cellular membranes than the myristoyl moiety. However, neither myristoylation nor electrostatic interactions within the PSD alone are enough to sustain MARCKS binding to cellular membranes (162, 163, 167, 168). Phosphorylation of MARCKS results in the incorporation of negative charges into the PSD, thus weakening the electrostatic interactions between MARCKS and the phospholipid bilayer. This weakening of the electrostatic interactions causes complete dissociation of MARCKS from cellular membranes resulting in cytosolic localization of MARCKS (162, 163, 165, 166), which has been termed the “myristoyl-electrostatic switch” mechanism (167).

1. Regulation of MARCKS function by phosphorylation

MARCKS was originally described as a substrate for PKC and phosphorylation of MARCKS by PKC occurs via both direct and indirect mechanisms. PKC and PKC-related kinase 1 (PRK1) directly phosphorylate rat MARCKS on three serine residues within the PSD: Ser^{152}, Ser^{156} and Ser^{163} (169-171). PKC activation occurs with signaling through GPCRs or RTKs, which results in the cleavage of PIP_2 by PLC into inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG). DAG remains in the plasma membrane whereas IP_3 opens calcium channels in the endoplasmic reticulum and thus increases intracellular calcium concentrations. PKC is activated by cytosolic to membrane translocation, where it interacts with DAG and Ca^{2+}. There are various PKC isozymes, including conventional PKCs (α, βI, βII and γ), which require both DAG and Ca^{2+}; novel PKCs (δ, ε, θ, η), which require DAG only; and atypical PKCs (ζ, ι, λ), which do not require DAG or Ca^{2+} (172). Both novel PKCs (PKC-ε, PKC-δ and PKC-θ) and classical PKCs (PKC-α and PKC-βII) have been shown to phosphorylate MARCKS (138, 144, 173, 174). MARCKS phosphorylation occurs on cellular membranes by activated membrane-bound PKC, thus resulting in MARCKS localizing to the cytosol (175). Specifically, PKC phosphorylation of rat MARCKS at Ser^{152} is involved in the regulation of MARCKS binding to phosphatidylserine on the plasma membrane (176). Further, pharmacological inhibition of Rho-associated kinase (ROCK) signaling has shown that ROCK phosphoyrlates human MARCKS on Ser^{159} (Ser^{152} in mice) which is an example of MARCKS indirect

phosphorylation by PKC, as ROCK activates PKC (177-182). Activation of protein kinase A (PKA), a serine/threonine kinase activated by adenylyl cyclase and cyclic AMP (cAMP), in parotid acinar cells results in activation of PKCδ and subsequent amylase release (183). Previous research has shown that PKC is activated by PKA in other cells (184), suggesting that MARCKS phosphorylation and function may be indirectly regulated by the cAMP depenendent/PKA pathway.

MARCKS phosphorylation can also occur independently of PKC activation. Activation of protein kinase G (PKG), a protein kinase activated by cyclic GMP (cGMP) (185), results in the phosphorylation of MARCKS in NIH-3T3 fibroblasts. However, activation of the PKG pathway in these cells results in decreased total MARCKS protein levels, indicating that the PKG pathway is involved in the degradation of MARCKS (186). Further, the p42 Mitogen Activated Protein Kinase (MAPK) phosphorylates mouse MARCKS on Ser^{113}, a serine residue located outside of the PSD (187). The p42 MAPK is a proline-directed kinase that is capable of phosphorylating serine or threonine residues that are flanked by proline in the +1 position (188, 189). MARCKS is phosphorylated on multiple serine residues by proline-directed kinases, including cdc2 kinase and tau protein kinase II at a unique serine phosphorylation site within the carboxy-terminal domain (Ser^{299}) of bovine MARCKS (190-192). The exact role of proline-directed kinase phosphorylation of MARCKS is yet to be determined; however phosphorylation of chicken MARCKS at Ser^{25} results in the ability of MARCKS to bind and stabilize F-actin filaments in retinal neuroblasts (193). Phosphorylation of chicken MARCKS on Ser^{25} appears to be unique to cells of the

nervous system (194, 195), suggesting that phosphorylation of MARCKS outside the PSD, presumably by proline-directed kinases, may be involved in the regulation of MARCKS function and this mechanism may vary depending on the cell type.

Upon phosphorylation of MARCKS in the PSD, MARCKS dissociates from the plasma membrane resulting in localization to the cytosol (158, 167, 168, 196) or other cellular membranes, such as the lysosome (197). Additionally, cytosolic MARCKS can also be phosphorylated by PKC isozymes that are activated in the cytosol and do not require translocation to the membrane for activation (175). Cytosolic phosphorylated MARCKS is dephosphorylated by protein phosphatases, specifically by protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) or calcineurin (191, 198-201). PP1 and PP2A are activated by a variety of pathways, including p38 MAPK (202), and in respiratory epithelia, PP2A activation is mediated by PKG and inhibition of PKG results in MARCKS remaining in a phosphorylated state (143). Once dephosphorylated, MARCKS can be bound by calcium-bound calmodulin, cross linked by filamentous actin (F-actin), or re-associate with cellular membranes.

2. Regulation of MARCKS function by calmodulin

Besides PKC, calmodulin (CaM) is also activated by GPCRs and RTKs as well as increased intracellular Ca^{2+} concentrations and studies have revealed that MARCKS is also a major substrate for calcium-bound CaM (151). Calcium-bound CaM binds MARCKS

localized on cell membranes, causing the MARCKS/CaM complex to dissociate and localize to the cytosol (164). Typically, CaM substrates are α-helical or become α-helical upon CaM binding. However, the PSD of MARCKS remains non-helical, in a flexible confirmation, upon binding by calcium-bound CaM (203, 204). Interestingly, studies have shown that CaM binds with higher affinity to recombinant PSD than to full length MARCKS. The same is also true for unmyristoylated MARCKS, in that CaM binds to unmyristoylated MARCKS with higher affinity than to wild type MARCKS (205).

Like PKC, calcium-bound CaM inhibits the ability of MARCKS to crosslink F-actin in the cytosol (206). However, when the cellular Ca^{2+} concentrations decrease, CaM releases MARCKS, which is able to bind F-actin and/or return to cellular membranes (130, 207). Both PKC and CaM bind the PSD of MARCKS in a mutually exclusive manner, in that phosphorylation of MARCKS by PKC inhibits CaM binding to MARCKS and MARCKS is unable to be phosphorylated by PKC when bound by CaM (130, 151, 208-210). This suggests that MARCKS may be involved in cross-talk between the PKC and CaM signaling pathways with two potential scenarios for MARCKS mediated cross-talk. First, if PKC is activated first, MARCKS will become phosphorylated, leaving CaM the ability to activate its other substrates, such as MLCK and CaM kinase II. However, if CaM is activated first and forms a complex with MARCKS, PKC will phosphorylate other known substrates, such as calpain (134, 211). The second scenario is that MARCKS is involved in PKC mediated CaM localization in cells. Depolarization of smooth muscle cells results in CaM and MARCKS colocalization to the cytosol. However, activation of PKC results in an initial translocation

of CaM followed by subsequent translocation of MARCKS at a later time point (212).

3. MARCKS is an actin binding protein involved in reorganization of the actin cytoskeleton

Many investigators have reported that MARCKS is capable of binding to actin. However, the exact mechanism by which MARCKS binds to actin is still unclear. A generally accepted hypothesis is that the PSD of MARCKS contains two actin binding motifs on the N-terminal and C-terminal halves of the PSD that are responsible for F-actin cross linking. Phosphorylation of MARCKS results in a conformational change within the PSD, resulting in the blockade of one of the two actin binding motifs. Dephosphorylation of MARCKS results in a conformational change that reveals both actin binding motifs, allowing for F-actin cross-linking (206, 213, 214). The ability of MARCKS to cross-link F-actin is enhanced by both myristoylation and cleavage of MARCKS (205) and the integrity of the MARCKS associated actin filaments correlates with sustained phosphorylation of MARCKS at Ser^{25} in retinal neuroblasts (193). However, a conflicting report suggests that MARCKS contains a single actin binding site within the PSD and actin cross-linking is the result of actin dimerization or a second actin binding site that is located within the MH-2 domain (130, 133). Further, the PSD of MARCKS is capable of bundling F-actin filaments in that the PSD binds to negatively charged residues of F-actin, thus eliminating the electrostatic repulsions between F-actin filaments (215). Also, the PSD domain of MARCKS is capable

of polymerizing globular actin (G-actin) into F-actin, and this ability is dependent on the N-terminal pentalysine sequence present in the PSD. The ability of MARCKS to polymerize G-actin is inhibited by PKC phosphorylation or calmodulin binding (216).

It is unclear if MARCKS plays a role in the rearrangement of F-actin. One thought is that MARCKS is involved in the movement of F-actin around cells and that MARCKS assists in the association of F-actin to other cytoskeletal proteins. Myoblast alpha-5 integrin mediated focal adhesions requires MARCKS function (173) and MARCKS and F-actin are colocalized in blebs, membrane ruffles and the lamellae of fibroblasts (217). Further studies have shown colocalization of MARCKS with membrane associated cytoskeletal proteins such as α_3-integrins and tetraspanins, which are proteins that form membrane complexes with integrins and participate in integrin-mediated cellular migration (218, 219). Further, in resting macrophages, MARCKS has a punctuate distribution, resulting in localization of MARCKS to filapodia. In these filopodia, MARCKS was colocalized with the cytoskeletal proteins vinculin and talin (220). However, none of these studies demonstrate colocalization of F-actin with MARCKS and the cytoskeletal proteins that MARCKS associates with.

An additional hypothesis for the mechanism by which MARCKS regulates the actin cytoskeleton is by sequestration of PIP_2. PIP_2 is continuously present in the plasma membrane of cells and recent evidence suggests that PIP_2 is involved in signaling pathways regulating actin dynamics. Various actin binding proteins, such as WASP and ERM, bind to and are activated by PIP_2. It is thought that increased concentrations of free PIP_2 is a signal for anchoring the actin cytoskeleton to cell membranes and decreased PIP_2 concentrations is

the signal for the release of the actin cytoskeleton from membranes (216, 221, 222). The PSD of MARCKS binds to the plasma membrane phospholipids phosphatidylserine and PIP_2 via electrostatic interactions (159, 164, 166, 223), and MARCKS and PIP_2 have also been shown to colocalize to lipid rafts, phagosomal membranes, focal adhesion sites and membrane ruffles (197, 224, 225). The unphosphorylated PSD of MARCKS binds to PIP_2 within the plasma membrane and binding of MARCKS to PIP_2 inhibits PLC mediated hydrolysis of PIP_2 (226). MARCKS clusters PIP_2 molecules on the plasma membrane and PKC and CaM mediated displacement of MARCKS from the plasma membrane releases the sequestration of PIP_2, allowing for PLC induced hydrolysis of PIP_2 (216, 224, 226). Studies have shown that MARCKS increases the number, length and motility of dendrites and neurites in a manner that is dependent on PIP_2 (227). Therefore, it appears that one of the mechanisms by which MARCKS regulates actin dynamics is through the sequestration of PIP_2.

4. MARCKS is the substrate for various proteases

The lysosomal cysteine protease cathepsin B cleaves MARCKS resulting in a p40 fragment, corresponding to a carboxyl-terminal fragment that is associated with the mitochondrial/lysosomal fraction of fibroblasts (228). Stefin B is a ubiquitously expressed inhibitor of lysosomal cathepsin B and mice deficient in stefin B have an increased rate of MARCKS cleavage in the kidney and liver (229). The mechanism for cathepsin B cleavage

of MARCKS is unknown, however, it is hypothesized that cathepsin B is involved in the targeting of MARCKS to the lysosome for degradation (228). PKG mediated phosphorylation of MARCKS results in the degradation of MARCKS (186) and therefore, PKG and cathepsin B may act cooperatively to decrease the amount of MARCKS in cells.

Calpain is a calcium dependent non-lyososomal cysteine protease that has also been shown to cleave MARCKS. In myoblasts, inhibition of calpain is associated with an accumulation of MARCKS at the plasma membrane and subsequent inhibition of myoblast migration (230, 231). Further research reveals that in migrating myoblasts, a 55 kDa (on SDS-PAGE) MARCKS fragment is generated. When calpain is inhibited by calpastatin, a calpain inhibitor, there is a significant decrease in the appearance of the 55 kDa MARCKS fragment that is associated with decreased myoblast migration (232). Inhibition of calpain results in increased PKC mediated phosphorylation of MARCKS upon phorbol ester or N-methyl-D-aspartic acid stimulation of rat hippocampal cells (233). However, calpain proteolysis of MARCKS depends on PKCα mediated phosphorylation of MARCKS in myoblasts (232).

The exact location of calpain cleavage of MARCKS is unknown. However, a cleavage site within the amino-terminus of MARCKS has been identified between Lys^6 and Thr^7. Cleavage at this site is myristoylation dependent and cleavage between Lys^6 and Thr^7 results in decreased binding of MARCKS to membranes (234). Given that calpain inhibition in myoblasts results in the accumulation of MARCKS on the plasma membrane (230), it is hypothesized that calpain cleaves MARCKS at this amino-terminal site. It is thought that the

tertiary structure of a protein, not the primary amino acid sequence, directs calpain cleavage. The most common calpain cleavage sites have a small hydrophobic amino acid at the P2 position and a large hydrophobic amino acid at the P1 position (235). In MARCKS Thr^{7} is in the P1 position and Lys^{6} is in the P2 position and both of these are polar hydrophilic amino acids. The MARCKS cleavage site between Lys^{6} and Thr^{7} does not appear to follow the predicted amino acid cleavage site for calpain. However, given that the exact cleavage site of calpain is yet to be determined, it is still plausible that calpain cleaves MARCKS on the amino-terminal site and further analysis is required.

5. A role for MARCKS in developmental biology

Attempts to generate a homozygous *Marcks* deficient mouse strain have led to the discovery that MARCKS is essential during development. *Marcks* deletion in mice is embryonic lethal and mouse pups that survive birth die within hours (236). Severe abnormalities are observed in *Marcks* deficient pups, including runting, exencephaly, and omphalocele, and the brain of homozygous *Marcks* deficient pups is decreased in size compared to wild type and heterozygous littermates. Further analysis reveals separation between the cerebral hemispheres of the brain and disruption of the corpus callosum, hippocampal commisure and anterior commisure in homozygous *Marcks* deficient mouse pups (236). This data supports a role for MARCKS in neural tube closure. MARCKS and F-actin are apically colocalized in cells during closure of the neural tube in chick embryos,

specifically during the bending process. After the neural tube is closed, MARCKS disappears from apical regions and is localized to the periphery whereas F-actin remains apically localized (237).

A human *MARCKS* transgene is capable of rescuing homozygous Marcks deficient mouse pups. However, a human nonmyristoylated MARCKS (*MARCKS A_2/G_2*) transgene complemented some, but not all abnormalities observed in homozygous *Marcks* deficient mice. Specifically, 25% of MARCKS-A_2/G_2 mice survived the perinatal period and those that survived had a decreased body size. Interestingly, the neural and anatomical defects that were observed in the homozygous Marcks deficient mouse pups were corrected by expression of the *MARCKS-A_2/G_2* transgene (238). However, recent evidence indicates that in the developing cerebral cortex, myristoylation of MARCKS is required for the proliferation, placement and differentiation of radial glia (239), questioning the results of the human *MARCKS-A_2/G_2* transgene study.

Unlike myristoylation, phosphorylation of MARCKS in the PSD is required for postnatal survival. A human *MARCKS* transgene with the phosphorylatable serines in the PSD replaced by arganines (*Asn-Marcks*) did not complement the postnatal mortality observed in homozygous *Marcks* deficient mice (240). Evidence suggests that prior to neural tube closure, both Marcks and PKC-α colocalize to the plasma membrane of cells that give rise to the neural tube (the surface ectoderm, neuroepithelial cells and underlying mesenchyme) and that Marcks is phosphorylated in embryonic day 8.5 (E8.5), when neurulation occurs (241). However, the neural defects observed in the homozygous Marcks

deficient pups were not observed in the Asn-Marcks complemented pups (240). This is further supported by a study in which a PSD null MARCKS transgene complemented the cerebral cortex abnormalities observed in Marcks deficient mice (239). Therefore, a role for MARCKS in neural tube closure has been established, although it appears that it does not involve PKC mediated phosphorylation of MARCKS in the PSD.

Low but sustained phosphorylation of MARCKS occurs in neural and non-neural embryonic tissue before the onset of neural differentiation. However, phosphorylation of MARCKS increases drastically during early differentiation of the neural retina and in the spinal cord (242). These studies were performed using a monoclonal antibody (mAb) specific for MARCKS that was generated by immunizing mice with chick retinal tissue. This antibody recognizes a phosphorylated form of MARCKS that is present in the neural tissue (136, 242) and additional research revealed that this mAb recognizes chicken MARCKS that is phosphorylated on Ser^{25}. Research has also revealed that MARCKS phosphorylated at Ser^{25} is involved in embryonic development of retinal tissue, but that this unique phosphorylation of MARCKS is lost after retinal histogenesis is complete (243). Interestingly, abnormal retinal layering in homozygous Marcks deficient mouse pups is also observed. Specifically, the nuclei-free layer (transient fiber layer of Chievitz (244)) is completely absent in the retinas of Marcks deficient mouse pups (236). Therefore, MARCKS is required for the histogenesis of retinal tissue and it appears that it may involve phosphorylation of MARCKS on chicken Ser^{25}.

The role of MARCKS in neural development has been well established, in that MARCKS is required for neural tube closure and retinal histogenesis during later embryonic stages (194, 236). Marcks deficiencies in mice results in embryonic lethality (236), suggesting that MARCKS is also involved in early embryonic events. Interestingly, MARCKS deficient *Xenopus laevis* exhibit defects in gastrulation that are associated with impaired convergent extension (245); a process by which cells converge medio-laterally and extends anterior-posteriorly, resulting in elongation of the body axis (246, 247). The noncanonical Wnt signaling pathway functions upstream of MARCKS mediated convergent extension movements in *Xenopus laevis* (245). This data confirms that MARCKS is involved in earlier embryonic events and may explain the embryonic lethality and defects observed in Marcks deficient mice (236).

The noncanonical Wnt pathway regulates cell polarization during convergent extension and organogenesis by signaling through frizzled receptors, which are 7-transmembrane GPCRs (248, 249). Signaling through Wnt ligands/frizzled receptors result in the activation of intracellular signaling molecules leading to cellular polarization. PKC, calmodulin and RhoGTPases are all activated by the noncanonical Wnt Signaling pathway (247, 250) and are involved in mediating MARCKS function.

6. MARCKS involvement in cell migration

Given MARCKS mediated regulation of the actin cytoskeleton, it is not surprising that a role for MARCKS in cell migration has been established. As previously described, MARCKS is involved in the morphogenetic movements of embryogenesis, specifically during gastrulation and neurulation (236, 245). However, MARCKS is also involved in the migration of other cells, including fibroblasts (217), myoblasts (198), human embryonic kidney cells (251), human hepatic stellate cells (252), vascular smooth muscle cells (253), neuroblastoma and cholangiocarcinoma cells (254, 255), neutrophils (142) and mesenchymal stem cells (256).

One of the first steps during cell migration is adherence of cells to the extracellular matrix and a role for MARCKS in the regulation of cell adhesion and migration has been established (142, 173, 217, 219, 257). Expression of a mutated MARCKS, which the myristoyl moiety is replaced by a palmitoyl moiety, thus interfering with MARCKS myristoyl-electrostatic switch mechanisms, results in abrogation of fibroblast spreading on a fibronectin substrate. The palmitoylated MARCKS construct interfered with early stages of cell spreading, which was indicated by a rounded morphology with multiple membrane blebs. Further, a decreased number of membrane ruffles and lamellae at the leading edge of the cell was observed in fibroblasts expressing palmitoylated MARCKS. This study ultimately revealed that the defect was due to the inability of the cell to adhere to a

fibronectin substrate (217). Further supporting a role for MARCKS in cell adhesion, neutrophils pretreated with a peptide inhibiting MARCKS function have inhibited β2-integrin mediated adhesion upon stimulation with fMLP, PMA and immune complexes (142). Glioblastoma multiforme cells expressing a constitutively active variant of the epidermal growth factor receptor (EGFR) undergo decreased adhesion, spreading and invasion when transfected with siRNA against MARCKS (257).

The adherence, spreading and migration of myoblasts is dependent on MARCKS function. MARCKS deficient α_5 integrin myoblasts are unable to spread on a fibronectin substrate and MARCKS is localized to focal adhesion sites in α_5 integrin expressing myoblasts during attachment and spreading (173). Myoblasts expressing either wild type or unmyristoylated MARCKS were capable of spreading on fibronectin whereas myoblasts expressing MARCKS PSD null mutants were incapable of spreading on fibronectin, indicating that phosphorylation and bi-lateral translocation of MARCKS are required for cell migration. Further examination reveals that MARCKS is localized to the membrane during the initial attachment of myoblasts whereas MARCKS is phosphorylated and localized to the cytosol of adherent myoblasts. MARCKS subsequently returns to the membranes once myoblasts are fully spread (198). MARCKS has also been associated with the migration of melanoma cells. Besides classic focal adhesions, melanoma cells contain more extensive, highly dynamic, irregularly shaped adhesions that tend to occur along the lamellepodia and are involved in motility. These highly dynamic adhesions are regulated by both PKC and MARCKS in that disassembly of the adhesions occurs upon PKC mediated phosphorylation

of MARCKS. Over expression of MARCKS results in the inability of the cells to respond to PKC and cells remained spread with MARCKS localized along the plasma membrane and cell edges. This differed from control cells, which retracted rapidly upon PKC stimulation and indicates that nonphosphorylated MARCKS promotes adhesive contacts in melanoma cells (219). Ultimately, these studies have revealed a role for PKC mediated bi-lateral translocation of MARCKS in the regulation of cell migration.

Platelet derived growth factor-BB (PDGF-BB) is a mitogen and chemoattractant for hepatic stellate cells, fibroblasts and smooth muscle cells (252, 258, 259). PDGF-BB has been shown to stimulate the membrane to cytosolic translocation of MARCKS (171) as well as subsequent phosphorylation of MARCKS (260, 261) in Swiss 3T3 fibroblasts. MARCKS is downstream of PDGF-BB signaling during the migration of hepatic stellate cells and MARCKS is phoshporylated by PKCε upon PDGF-BB stimulation. MARCKS associates with the PDGF-BB receptor but not focal adhesion kinase in non-stimulated and PDGF-BB stimulated hepatic stellate cells, with greater association observed in non-stimulated cells. Over expression of MARCKS results in decreased PDGF-BB mediated chemotaxis in hepatic stellate cells whereas a siRNA against MARCKS results in a slight increase in PDGF-BB mediated chemotaxis (252). This data supports the notion that bi-lateral translocation of MARCKS is required for the regulation of cell migration. This data further suggests that MARCKS is involved in stabilizing the actin cytoskeletal structure of migratory cells, but that phosphorylation of MARCKS must occur to allow for reorganization of the actin cytoskeleton and promotion of cell migration.

Contradictory to this is that MARCKS is overexpressed in cholangiocarcinoma cells (262) and this over expression correlates to increased metastasis and decreased host survival. Over expression of endogenous MARCKS results in increased cell attachment whereas transfection of cholangiocacinoma cells with a siRNA against MARCKS significantly reduced cell attachment. Further, TPA stimulated cholangiocarcinoma cells expressing a MARCKS siRNA had a significantly decreased ability to migrate in a Boyden chamber experiment compared to control transfected cells. Pretreatment of control or MARCKS siRNA transfected cholangiocarcinoma cells with a PKC inhibitor and TPA resulted in further inhibition of cell migration compared to pretreatment with TPA alone. This ultimately indicates that MARCKS is required for the migration and metastasis of cholangiocarcinoma cells in a manner that is dependent on PKC phosphorylation (255).

Calpain, a calcium activated protease, has been shown to be involved in the regulation of cell migration. Neutrophil chemotaxis towards IL-8, fMLP and C5a are regulated by calpain activity and calpain 2 is localized to the leading edge of polarized neutrophils during chemotaxis and its activity is required for regulating pseudopod formation in the direction of the chemoattractant source (263, 264). Calpain is also involved in the regulation of integrin function in that LFA-1 mediated adhesion is strengthened by activation of calpain in T lymphocytes through a mechanism involving altering intracellular calcium concentrations (265). Further, overexpression of μ-calpain results in increased cleavage of proteins localized to focal adhesions and increases the number of stress fibers and focal adhesions in bovine aortic endothelial cells, indicating that calpain is involved in focal

adhesion formation (266). In myoblasts, a rounded morphology and disorganized stress fibers are observed in cells lacking calpain activity, resulting in decreased myoblast attachment and spreading (267). As previously described, MARCKS is a substrate for calpain and cleavage of MARCKS by calpain has been shown to be involved in the regulation of cell migration (232). Decreased expression of μ- and m-calpain or overexpression of the calpain inhibitor, calpapastatin, in myoblasts results in approximately a 50% increase in MARCKS protein expression. This increased expression of MARCKS correlates to decreased myoblast migration, which was confirmed by overexpressing MARCKS in myoblasts, also resulting in decreased myoblast migration (230, 268).

7. Regulation of mucin secretion by MARCKS and a role for the myristoylated amino-terminus in regulating MARCKS function

In the respiratory epithelium, secretion of mucin is associated with the pathophysiology of various respiratory diseases, including asthma and cystic fibrosis. Mucin secretion is dependent on both PKC and PKG activity and previous research by Adler and colleagues has demonstrated that MARCKS is involved in the regulation of mucin secretion in the respiratory epithelium (269, 270). The proposed mechanism by which MARCKS regulates mucin secretion is via PKC phosphorylation and subsequent translocation of MARCKS from the plasma membrane to the cytoplasm. PKG has been shown to activate PP2A, which subsequently dephosphorylates MARCKS resulting in targeting of MARCKS to the membranes of mucin granules (143). Further, two chaperone

proteins, cysteine string protein (CSP) and heat shock protein 70 (hsp70) are involved in MARCKS mediated regulation of mucin secretion. MARCKS is associated in a trimeric complex with CSP and hsp70 on mucin granules in normal human bronchial epithelial (NHBE) cells (271). Specifically, MARCKS interacts with Hsp70, which also interacts with CSP, thus making Hsp70 the bridging factor between MARCKS and CSP (272). Knockdown of any of these three proteins by siRNA results in decreased mucin secretion (271), indicating that MARCKS interacts with these chaperone proteins in the regulation of mucin secretion. Further, MARCKS and CSP interact with unconventional myosin isoforms, specifically myosin V. Binding of MARCKS or CSP to myosin V was enhanced by stimulating NHBE cells with PMA, which is a stimulator of mucin secretion (272). Ultimately, this work has elucidated a mechanism for MARCKS in the regulation of mucin secretion that involves MARCKS interaction with chaperone proteins and myosin.

Adler and colleagues further demonstrated a role for the myristoylated amino-terminus is regulating mucin secretion within the airway epithelium. Utilizing a myristoylated peptide consisting of the first 24 amino acids of MARCKS (MANS), they have demonstrated that this peptide significantly decreases mucin secretion in both *in vitro* and *in vivo* studies (143, 273). During *in vitro* studies, mucin secretion stimulated via activation of the PKC and PKG pathways were significantly inhibited when NHBE cells were preincubated with the MANS peptide. However, pretreatment with a myristoylated random scrambled control peptide (RNS) did not result in decreased mucin secretion upon activation of PKC or PKG (143). During *in vivo* mouse models of asthma utilizing methylcholine

induced mucus hypersecretion, intratracheal or intranasal administration of the MANS peptide results in significantly decreased mucin hypersecretion and airway obstruction. As in the *in vitro* experiments, the RNS missense control peptide did not have an effect on mucin secretion in these mice (273, 274). Further studies reveals that the MANS peptide inhibits MARCKS association with the membrane of mucin granules (274).

Further research has revealed a role for the myristoylated amino-terminus of MARCKS in the regulation of leukocyte degranulation. Pretreatment of neutrophils, eonsinophil-like cells, monocyte-like cells and NK cells with the MANS peptide results in decreased myeloperoxidase, eosinophil peroxidase, lysozyme and granzyme C release, respectively. However, pretreatment with the RNS control peptide did not result in impaired leukocyte degranulation (141). Additional research has also elucidated a role for the myristoylated amino-terminus in the regulation of cell migration. Pretreatment of human neutrophils with the MANS peptide results in decreased migration towards the chemoattractants fMLP, IL-8 and LTB_4. The decreased migration of neutrophils can be attributed to decreased adhesion, as fMLP and immune complex mediated adhesion was reduced in MANS pretreated neutrophils (142). Further, primary bone marrow derived mesenchymal stem cells undergo decreased directed chemotaxis when pretreated with the MANS peptide (256). In both of these studies, the RNS peptide did not alter neutrophil (142) or bone marrow derived mesenchymal stem (256) cell migration.

8. **MARCKS-related protein expression and function**

There is an additional member of the MARCKS family of proteins, termed MARCKS-related protein (MRP: also termed MARCKS-like protein, MacMARCKS or F52). MRP is a 20 kDa acidic rod shaped protein with a single intron that is expressed in the central nervous system (CNS), reproductive tract, and leukocytes (130, 133, 134). MRP expression has been observed in humans (134), mice (275), rabbits (275), and *Xenopus laevis* (276) and MARCKS and MRP share the three conserved domains (the myristoylated amino-terminus, MH-2 domain and the PSD) with subtle differences. Within the PSD, MRP has a proline substitution where the second phosphorylatable serine is located in MARCKS (133). The myristoylated amino-terminus is the most different between MARCKS and MRP. They share a conserved myristoylation motif, with myristoylation occurring on the amino-terminal glycine residue with approximately 50% amino acid homology (130, 133, 134).

Like MARCKS, MRP is upregulated by LPS and β-amyloid proteins and is a protein substrate of both PKC and calmodulin (130, 133, 155, 210). Further, MRP is capable of binding to F-actin and PIP_2 and thus regulates the actin cytoskeleton (223, 275). However, one difference between MARCKS and MRP is in their interaction with F-actin. As previously discussed, F-actin binds to MARCKS at two binding sites within the PSD (213) whereas MRP has a series of six positively charged amino acids within the amino-terminus that appear to be crucial for its interaction with F-actin (277). Further, MARCKS and MRP are targeted to different intracellular localizations. For example, MARCKS is localized to

phagosomes whereas MRP is localized to phagocytic cups (130). Interestingly, MARCKS is associated with phagocytosis in macrophages but MRP does not appear to be involved (278), suggesting non-redundant roles for these proteins. Further supporting a non-redundant role for MARCKS and MRP is that homozygous deletion of either gene results in severe developmental defects and perinatal death (236, 279, 280).

As stated earlier, MARCKS is involved in the regulation of cell migration (142, 256). However, the role of MRP in cell migration is not completely understood. Downregulation of MRP is associated with both decreased macrophage chemotaxis (281) and increased migration of tumor cell lines (282). Both MARCKS and MRP are involved in the regulation of cell proliferation of endothelial cells and retinal cells, respectively (283, 284). Given that MARCKS is uniformly expressed and MRP is expressed in the CNS, reproductive tract and leukocytes, this data suggests that MARCKS and MRP play similar yet non-redundant roles in the respective cell types that express these molecules. Therefore, given differences in expression and function, it is thought that MARCKS and MRP act cooperatively to integrate signaling through PKC and calmodulin to regulate the actin cytoskeleton (130, 133, 134).

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CHAPTER 2

REGULATION OF FIBROBLAST MIGRATION BY MARCKS

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Abstract

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitously expressed actin binding protein involved in many cellular processes, including cell migration. Previously, we demonstrated that MARCKS regulates neutrophil adhesion and migration and that MARCKS functions in these processes via a mechanism that involves the amino-terminus. In this study, we hypothesized that MARCKS protein is involved in the regulation of fibroblast migration. Our approach was to use a myristoylated peptide identical to the first 24 amino acids of MARCKS (called the MANS peptide) that inhibits MARCKS function in NIH-3T3 fibroblasts to test our hypothesis. Treatment with the MANS peptide inhibited NIH-3T3 cell migration in a scratch wounding assay and in a transmembrane migration assay in response to platelet derived growth factor-BB (PDGF-BB). In contrast, treatment with a control peptide consisting of the same amino acids as MANS in a scrambled sequence (RNS peptide) did not affect migration in either assay. PDGF-BB signaling in fibroblasts results in the phosphorylation of MARCKS in both adherent and non-adherent cells and MANS peptide treatment did not alter MARCKS phosphorylation. Expression of EGFP fusion proteins consisting of the MANS peptide or unmyristolyated version of the MANS (UMANS) peptide in NIH-3T3 cells revealed that MANS is targeted to the membrane whereas UMANS is predominantly localized to the cytosol. However, expression of either MANS or UMANS fusion proteins significantly decreased migration of NIH-3T3 cells in scratch wounding and PDGF-BB transmembrane migration assays, indicating that

myristoylation of the MANS peptide is not required for the ability of MANS to inhibit cell migration. Our conclusion is that MARCKS is essential for NIH-3T3 fibroblast migration and that the amino-terminus regulates this function. In addition, our data suggest the novel concept that the peptide sequence of the amino-terminal 24 amino acid region corresponding to the MANS peptide has a role in regulating MARCKS function in migration independent from the role of the myristic acid in mediating membrane binding.

Introduction

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitously expressed protein kinase C (PKC) substrate that has been shown to regulate actin dynamics. MARCKS is tethered to cell membranes via amino-terminal myristoylation and electrostatic interactions between the basic effector domain of MARCKS and the acidic phospholipids of the plasma membrane (1, 2). Phosphorylation of MARCKS by PKC results in the complete repulsion of MARCKS from the plasma membrane into the cytosol (3). This translocation of MARCKS has been associated with the reorganization of the actin cytoskeleton (4, 5) and thus MARCKS function has been associated with various cellular processes, including endo- (6), exo- (7), and phagocytosis (8, 9), and cell migration (10, 11).

Recently, a unique reagent called MANS, which is a myristoylated peptide corresponding to the first 24 amino acids of MARCKS, was developed by Adler and colleagues (12). Utilization of the MANS peptide has demonstrated a role for MARCKS, specifically its myristoylated amino terminus, in the regulation of *in vitro* (12) and *in vivo* (13, 14)(15) mucin secretion as well as leukocyte degranulation (14). Thus, we utilized the MANS peptide to determine the role of MARCKS, specifically the amino-terminus, in the regulation of neutrophil migration. Our studies revealed that MANS peptide treatment results in decreased neutrophil migration and β_2-integrin dependent adhesion, whereas the myristoylated scrambled control peptide (RNS) did not have an effect (16).

The results from our neutrophil study (16) prompted us to investigate what aspect(s) of the MANS peptide and/or the amino terminus of MARCKS is involved in the regulation of cell migration. The first aspect of the MANS peptide that we are interested in is amino-terminal myristoylation because previous studies have shown that myristoylation of MARCKS is not required for cell spreading and adhesion (17, 18). Therefore, to answer this question, we proposed a genetic structure function analysis in which we transfect cells with mutated MANS constructs. Given that neutrophils are terminally differentiated cells and do not survive in culture, we chose a fibroblast cell line, NIH-3T3 cells, to perform our studies. Fibroblasts are advantageous for these studies because they are a migratory cell line that is easily transfected with various techniques available to study migration including, scratch wounding assays and Boyden chamber transmembrane chemotaxis assays (19, 20). Fibroblasts, including NIH-3T3 cells, express MARCKS (21-23) and studies have shown a role for MARCKS in regulating fibroblast migration. Epidermal growth factor (EGF) induces myosin contraction and motility of 3T3 fibroblasts in a mechanism dependent on MARCKS phosphorylation by PKCδ (24). Further, expression of palmoyltalated MARCKS reveals a role for MARCKS bilateral translocation in regulating membrane blebbing and lamellae formation and ultimately a role for MARCKS in mediating fibroblast adhesion (25). Thus, NIH-3T3 fibroblasts provide an excellent model for studying the regulation of cell migration by MARCKS.

An additional reason for utilizing the fibroblast model to study the role of the amino terminus of MARCKS in the regulation of cell migration is that fibroblasts solely express MARCKS (26, 27) whereas leukocytes express both MARCKS and MARCKS-like protein (MLP; also called MARCKS-related protein (MRP) or MacMARCKS) (28-30). MLP is a member of the MARCKS family that is expressed in leukcoytes, the central nervous system and the reproductive tract (27, 31, 32). Both MARCKS and MLP are myristoylated on the amino terminus; however, the amino acid composition of the amino terminal domain of MLP is subtly different to MARCKS (27) with approximately 50% homology. Studies reveal that MARCKS and MLP appear to play similar roles and both regulate cell migration. MARCKS is involved in mesenchymal stem cell (33), myoblast (34) and hepatic stellate cell (10) migration, and reduced expression of MLP in macrophages is associated with decreased chemotaxis (35). Therefore, our previous work utilizing the MANS peptide does not rule out the role of MLP in the regulation of neutrophil migration (16) and utilizing NIH-3T3 fibroblasts will help to determine the role of the MANS peptide in regulating MARCKS function independent of MLP expression and/or function.

Herein, we utilize the MANS peptide to determine the role of MARCKS in the regulation of fibroblast migration. Two established migration assays are utilized, a scratch wounding assay and PDGF-BB transmembrane chemotaxis assay. Further, a genetic structure function analysis with MANS and unmyristoylated MANS (UMANS) fusion proteins is performed to determine if amino-terminal myristoylation is required for MANS peptide regulated fibroblast migration. These studies will not only elucidate mechanisms by

which the amino-terminus of MARCKS regulates cell migration, but will provide further evidence for targeting MARCKS for the treatment of diseases that are caused by exacerbated cell migration (36).

Results

MANS peptide treatment inhibits fibroblast migration on fibronectin and collagen substrates

To determine if MANS peptide treatment results in decreased fibroblast migration, we performed a fibroblast scratch wounding assay in the presence of various concentrations of MANS peptide. Fibroblasts were seeded on 10 μg/mL fibronectin or collagen coated coverslips and grown to confluency. Scratch wounding assays were performed in the presence of 1, 50 or 100 μM MANS peptide or the control myristoylated random N-terminal sequence (RNS) peptide and incubated for 18 hours. As shown in Figure 1, MANS peptide treatment results in significantly decreased fibroblast migration on both fibronectin (Figure 1A and B) and collagen (Figure 1C) substrates in a dose dependent manner. However, scratch wounding assays performed in the presence of RNS control peptide did not significantly affect fibroblast migration, and migration was similar to non-treated fibroblasts (Figure 1B and C). As a positive control for the inhibition of migration, fibroblasts were incubated with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin (100 nM) (37) and wortmannin treatment results in significantly decreased fibroblast migration compared to

non-treated cells (Figure 1). Taken together, these results demonstrate that MANS peptide treatment of NIH-3T3 fibroblasts results in decreased migration on both fibronectin and collagen substrates.

PDGF-BB stimulation of fibroblasts results in the phosphorylation of MARCKS

Previous studies have shown that PDGF-BB results in membrane to cytosol translocation and phosphorylation of MARCKS in Swiss 3T3 fibroblasts (38-40). Therefore, we asked if MARCKS is phosphorylated upon PDGF-BB stimulation in adherent and non-adherent NIH 3T3 fibroblasts. We stimulated adherent and non-adherent cells with increasing concentrations of PDGF-BB (0.1-100 nM) for 1 minute, prepared lysates and immunoblotted for phospho-MARCKS and total MARCKS. As shown in figures 2A and C, PDGF-BB, at concentrations of 100, 10, 1 and 0.1 nM, results in phosphorylation of MARCKS in adherent cells. Similarly, PDGF-BB, at concentrations of 100, 10 and 1 nM, results in the phosphorylation of MARCKS in non-adherent cells (figures 2B and C). We next performed a kinetics study of MARCKS phoshporylation upon PDGF-BB stimulation in adherent and non-adherent cells. For both adherent and non-adherent cells, 10 nM PDGF results in phosphorylation of MARCKS at 1, 5, 10 and 20 minutes with greatest phosphorylation observed at 1 minute (figure 2 D-F). In non-adherent NIH-3T3 fibroblasts, higher background phosphorylation was observed (T=0 and vehicle control (VC) treated samples) compared to adherent cells (figure 2 D-F), suggesting differential activation of PKC

in adherent versus non-adherent NIH-3T3 fibroblasts. Ultimately, this data demonstrates that MARCKS is phosphorylated by PDGF-BB and that phosphorylation of MARCKS may be involved in the regulation of NIH-3T3 fibroblast migration given the stuble differences between adherent and non-adherent cells.

Previously, we demonstrated that MANS peptide pretreatment of neutrophils increases phosphorylation of MARCKS upon fMLF stimulation (E.J.S., unpublished data). To determine if MANS peptide treatment alters PDGF-BB mediated MARCKS phosphorylation, adherent NIH-3T3 fibroblasts were pretreated with 50 μM MANS, 50 μM RNS or PBS (VC) for 30 minutes and then stimulated with 10 nM PDGF-BB for 1 minute. As shown in figure 3, MANS pretreatmentment did not alter PDGF-BB mediated phosphorylation of MARCKS, as the level of phosphorylation was similar with PBS, MANS or RNS treatment.

MANS pretreatment results in the inhibition of PDGF-BB mediated fibroblast chemotaxis

PDGF-BB is a known chemoattractant for NIH-3T3 fibroblasts (41, 42) and we therefore asked if MANS peptide treatment alters PDGF-BB mediated chemotaxis of NIH-3T3 fibroblasts. For these studies we utilized a Boyden chamber approach with fibronectin coated transwells to assess transmembrane migration. Treatment with 50 μM MANS resulted in the inhibition of NIH-3T3 fibroblast migration towards 1 nM PDGF-BB (figure 4) compared to RNS peptide or vehicle (PBS) treatment. Like the scratch wounding assays,

MANS peptide inhibition of migration was comparable to treatment with 100 nM wortmannin (figure 4). Interestingly, unstimulated migration in control wells lacking PDGF-BB was not affected by MANS treatment. Taken together, MANS peptide treatment decreases PDGF-BB mediated NIH-3T3 transmembrane migration, further supporting an essential role for MARCKS in the regulation of NIH-3T3 fibroblast migration.

Myristoylation of MANS is not required for the inhibition of fibroblast migration

Our data suggest that the amino-terminus of MARCKS has a critical function in regulating MARCKS during migration. We next asked whether myristoylation is required for this function. Our approach was to modify the MANS peptide to create an unmyristoylated version. We used a genetic approach to introduce genes that encode these peptides into NIH-3T3 cells since eliminating the myristoylation of the MANS peptide was likely to affect cell permeability. We created fusion proteins by expressing genes encoding the MANS peptide sequence (MANS:EGFP) and a G2A mutant of MANS to eliminate the myristoylation signal sequence (UMANS:EGFP) fused to the amino-terminus of EGFP. We expressed these fusion proteins in NIH-3T3 cells using the expression vector pEGFP-N1 and expressed EGFP alone as a control. Western blot analysis using anti-EGFP antibody revealed equal expression of EGFP, MANS:EGFP and UMANS:EGFP 24 hours after transfection (figure 5A). Interestingly, we observed similar expression of MARCKS in EGFP, MANS:EGFP and UMANS:EGFP transfected cells while expression of MARCKS in transfected cells was

decreased compared to non-transfected cells (figure 5A). Subcellular fractionation revealed that MANS:EGFP is targeted to the membrane fraction compared to EGFP and UMANS:EGFP, which were predominantly localized to the cytosol (figure 5B). Further, fluorescence microscopy revealed that MANS:EGFP has a peri-nuclear distribution whereas EGFP and UMANS:EGFP has a uniform distribution throughout NIH-3T3 cells (figure 5C). These results demonstrate that MANS:EGFP is targeted to cell membranes, and that amino-terminal myristoylation is a required localization signal.

Scratch wounding assays revealed that expression of MANS:EGFP significantly decreased migration compared to non-transfected cells and cells expressing EGFP and the ability of MANS:EGFP expression to inhibit migration was similar to 50 μM MANS peptide treatment (figure 6). Interestingly, expression of UMANS:EGFP also significantly inhibited NIH-3T3 fibroblast migration, similar to 50 μM MANS peptide treated and expression of MANS:EGFP (figure 6). Expression of MANS:EGFP and UMANS:EGFP in NIH-3T3 cells also significantly inhibited migration in response to PDGF-BB stimulation in a transmembrane migration assay compared to untreated cells and cells expressing EGFP (figure 7). The ability of the MANS peptide sequence to inhibit migration in the absence of myristoylation suggests an essential role for the amino-terminal peptide sequence in regulating MARCKS function during migration.

Discussion

In the present study, we demonstrate a role for MARCKS in the regulation of NIH-3T3 fibroblast migration. This was performed by utilizing the MANS peptide, a myristoylated peptide corresponding to the first 24 amino acids of MARCKS that has been shown to inhibit MARCKS function (12). Treatment of NIH-3T3 cells with the MANS peptide results in decreased migration in both scratch wounding and PDGF-BB transmembrane chemotaxis assays. The data presented here supports previous research demonstrating a role for MARCKS in neutrophil adhesion and migration (16) as well as stem cell chemotaxis (33). Further, this data also suggests that aspects of the amino-terminus of MARCKS are required for regulating MARCKS function during cell migration, given inhibited cell migration in the presence of the MANS peptide.

In the present study, we utilize a genetic approach to express MANS and unmyristoylated MANS (UMANS) EGFP fusion proteins in fibroblasts and observe similar expression of EGFP, MANS:EGFP and UMANS:EGFP while we do not observe expression of EGFP in non-transfected cells, as expected. Further, in EGFP, MANS:EGFP and UMANS:EGFP expressing cells we observe similar expression of MARCKS with non-transfected cells expressing higher levels of MARCKS than transfected cells. Likewise, previous studies have revealed that transformed 3T3 fibroblasts express decreased MARCKS levels compared to non-transformed cells (43, 44) and together with our data, suggest that transformation of 3T3 cells results in decreased MARCKS expression. Previously, we have

demonstrated that both the MANS and RNS peptides are loaded into neutrophils, presumably via the amino-terminal myristoyl moiety. Further, subcellular fractionation studies demonstrate that the MANS peptide displaces MARCKS from cell membranes in untreated neutrophils, whereas the RNS peptide allows for MARCKS to remain localized to the membrane (16). In the present study, subcellular fractionation studies reveal that MANS:EGFP fusion proteins are preferentially targeted to the membrane fraction of NIH-3T3 fibroblasts whereas EGFP and UMANS:EGFP are localized to the cytosol. This data supports our previous data in neutrophils, and suggests that the MANS peptide localizes to cell membranes and competes with MARCKS for membrane binding sites within cells.

Our previous results also elucidated that β_2-interin mediated neutrophil adhesion is regulated by MARCKS function (16). In the present study, we demonstrate a role for MARCKS in regulating fibroblast migration on both fibronectin and collagen substrates. Fibroblasts express integrins that recognize fibronectin and collagen; including $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (45), which recognize collagen, as well as $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (46, 47), which recognize fibronectin. Studies have revealed that NIH-3T3 cells express both fibronectin and collagen integrins (46, 48) and together with our data, suggest that MANS peptide mediated inhibition of NIH-3T3 migration occurs independent of specific integrins expressed by the cell.

Myoblasts and HEK 293 embyonic kidney cells expressing unmyristoylated MARCKS are capable of spreading and adhering to extracellular matrix substrates, respectively (17, 18). Supporting this research, herein we demonstrate that the mechanism by which the MANS peptide inhibits MARCKS regulated fibroblast migration is independent

of myristoylation, as performed by a genetic structure function approach. Introduction of UMANS:EGFP fusion proteins into NIH-3T3 fibroblasts resulted in decreased migration in scratch wounding and PDGF-BB transmembrane chemotaxis assays comparable to MANS:EGFP transfected and MANS peptide treated cells. This is the first report demonstrating that myristoylation of the MANS peptide is not involved in regulating MARCKS function and further suggests that membrane localization of the MANS peptide (presumably via myristoylation) is not required for inhibition of cell migration. Our data further suggests that other aspect(s) of the MANS peptide and the amino-terminus of MARCKS are involved in regulating cell migration. One potential aspect of the amino-terminus of MARCKS that may be involved in regulating cell migration is cleavage between Lys^6 and Thr^7. Cleavage at this site is performed by an unidentified protease (49) although calpain has been named a potential candidate. Calpain is a calcium activated protease that is localized to the leading edge of polarized neutrophils and is involved in pseudopod formation and neutrophil chemotaxis (50, 51). Calpain is also involved in LFA-1 mediated T-lymphocyte adhesion as well as focal adhesion formation in bovine aortic endothelial cells (52, 53). Studies have revealed that calpain cleaves MARCKS resulting in a 55 kDa fragment in myoblasts (54). Inhibition of calpain activity results in decreased myoblast migration that is associated with an accumulation of membrane bound MARCKS (34, 54-56). Thus, it appears that generation of the six amino acid fragment that is generated by cleavage between Lys^6 and Thr^7 may be involved in regulating cell migration, with further experimentation necessary to confirm this hypothesis.

PDGF-BB, a known mitogen and chemoattractant for fibroblasts, signals through a receptor tyrosine kinase, PDGF-BB receptor (PDGFR-BB). Signaling through PDGFR-BB results in increased intracellular Ca^{2+} concentrations and subsequent PKC activation, both of which are involved in mediating MARCKS function (57, 58). Previous studies have shown that PDGF-BB results in the translocation of MARCKS from the membrane to cytosol in Swiss 3T3 cells and subsequent phosphorylation (38-40). Our results described here support these results, in that PDGF-BB stimulation results in the phosphorylation of MARCKS in NIH-3T3 fibroblasts, an event that causes membrane to cytosolic translocation (3, 59). Previous studies have revealed that MARCKS is phosphorylated by the conventional PKCα and the novel PKC-ε and –θ in NIH-3T3 fibroblasts (23). Interestingly, PDGF-BB results in the activation of PKC-α in various fibroblast lines (60, 61) and PDGF-BB mediated MARCKS phosphorylation is dependent on both PKC-α and –ε during the migration of human hepatic stellate cells (10).

An interesting finding that we present here is increased basal MARCKS phosphorylation in non-adherent NIH-3T3 fibroblasts compared to adherent cells. This was of interest to us given that cell migration occurs through a series of adherent and non-adherent events and suggests that PKC activation may be differentially regulated in adherent versus non-adherent fibroblasts. A similar mechanism has been reported regarding protein kinase A (PKA) signaling. Stimulation of PKA activity in neutrophils results in decreased immune complex and fMLP mediated adhesion while inhibition of PKA activity promotes fMLP mediated β2-integrin expression and mediates β2-integrin adhesion and migration of

neutrophils (62, 63). Given that increased PKA activity is associated with de-adhesion of neutrophils, it could be possible that PKC activity is also elevated in non-adherent NIH-3T3 fibroblasts. Again, this is supported by the increased basal MARCKS phosphorylation in non-adherent NIH-3T3 fibroblasts that we observed in the present study. Supporting our observations, phosphorylation of MARCKS is associated with the loss of adhesive contacts in cholangiocarcinoma cells, melanoma cells and growth cones (11, 64, 65) However, there is evidence that PKC activity promotes adhesion (66-68) and PKC mediated translocation of MARCKS is required for the initial stages of cell adhesion in myoblasts (69). Thus further research to determine if PKC is differentially regulated in adherent versus non-adherent cells and to elucidate the role that MARCKS may play in these processes is warranted.

An additional finding that we observed was that MANS peptide treatment did not alter PDGF-BB mediated phosphorylation of MARCKS. This was surprising because previous studies have shown that MANS peptide treatment of neutrophils increases the phosphorylation of MARCKS upon fMLF treatment (E.J.S., unpublished data). Our explanation for these results is that PDGF-BB signals through a receptor tyrosine kinase whereas fMLF signals through a seven-transmembrane G protein coupled receptor (GPCR) (57, 70). Thus, differential signaling through these receptors may explain why we do not observe altered MARCKS phosphorylation in MANS treated PDGF-BB stimulated fibroblasts while observing increased MARCKS phosphorylation in MANS treated neutrophils stimulated with fMLF.

In summary, we have demonstrated a role for MARCKS in regulating NIH-3T3 fibroblast migration. Specifically, we have demonstrated that MANS peptide treatment of NIH-3T3 fibroblasts inhibits migration and that myristoylation of MANS is not involved in regulating this inhibition. Given that the MANS peptide has been shown to inhibit neutrophil and stem cell migration (16, 33) as well as mucin secretion in models of respiratory inflammation (12, 13, 15), the results reported here provide further support for targeting MARCKS as a therapeutic approach for various respiratory and other inflammatory diseases.

Materials and Methods

Reagents and cell culture

MANS and RNS were synthesized as previously described (12) and resuspended in sterile PBS. Wortmannin was obtained from Sigma (St. Louis, MO) and a stock solution was made in DMSO (Sigma). PDGF-BB and fatty acid free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO) and were resuspended in sterile water or PBS, respectively. Type II rat tail collagen and fibronectin were also purchased from Sigma and were resuspended in 0.1% acetic acid v/v or sterile water, respectively.

NIH-3T3 fibroblasts (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) and 0.2% Penicillin (10,000 U/ml) Streptomycin (10,000 μg/ml) solution (Gemini Bio-Products).

Recombinant plasmids and transfections

Primers for MANS amino-terminal constructs were generated from Integrated DNA Technologies (Coralville, IA) and *EcoRI* and *BamHI* restriction sites were engineered into the 5' end of the leading and lagging strands, respectively, with a four nucleotide overhang. MANS and UMANS inserts were PCR amplified from pCDNA4/TO wt MARCKS plasmid, with glycine (GGT) to alanine (GCT) point mutation in the second position for UMANS. MANS amino-terminal constructs were cloned into the *EcoRI* and *BamHI* restriction sites of pEGFP-N1 (Clonetech, Mountain View, CA) and colonies were selected on LB agar containing 30 μg/mL kanamycin. Colonies were screened by colony PCR using pEGFP-N1 sequencing primers and positive colonies were sequenced (MWG, Huntsville, AL).

For transient transfections for cell migration analysis, NIH-3T3 fibroblasts were transfected by nucleofection using the Amaxa® Cell Line Nucleofector Kit® R (Lonza, Basel, Switzerland) following manufacturer's protocol with 10 μg of plasmid per reaction. Plasmid DNA was prepared using the EndoFree® Plasmid Maxi-Kit (Qiagen, Valencia, CA). Transfected cells were plated into 6 well tissue culture plates and for scratch assays two nucleofection reactions ($2x10^6$ cells total) were used per well, which containted fibronectin coated coverslips. Expression of EGFP fusion proteins was determined by fluorescent microscopy 18 hours after transfection and cells were used for cell migration studies within 24 hours. Expression of EGFP fusion proteins was determined by western blot for EGFP.

Scratch Wounding Assay

Fibroblast scratch assays were performed as described by Beurden, *et al*. (71). Briefly, sterile 22 mm coverslips (Fisher Scientific, Pittsburgh, PA) were coated with 10 μg/mL fibronectin or collagen in sterile 6-well tissue culture plates for two hours at room temperature. Coated coverslips were washed in sterile PBS and NIH-3T3 fibroblasts were seeded in complete media and cultured until confluent. For scratch assays with transfected cells, two nucleofection reactions per scratch were performed ($1x10^6$ cells/nucleofection or $2x10^6$ cells/scratch) with scratch assays starting 18-24 hours after transfection. Two parallel scratches in the monolayer were made using a standard sterile 200 μL pipette tip. The coverslip was washed once with sterile PBS and replaced with DMEM containing 2% FBS and antibiotics. In some experiments, media was supplemented with 50 μM MANS, 50 μM RNS, sterile PBS (VC) or 100 nM wortmannin. The T=0 coverslip was immediately removed from the plate and processed prior to incubating the remainder of the plate for 18 hours at 37 ˚C at 5% CO_2. Coverslips were processed by fixing in 10% neutral buffered formalin solution (Fisher) and stained with harris hematoxylin (Sigma) following manufacturer's instructions. Coverslips were mounted onto microscope slides and an ocular micrometer was used to measure the wound distance at ten locations along the scratch under a 4X objective. The ocular micrometer measurement was used to calculate distance in μm and the wound closure distance for each sample was determined by subtracting the distance for each sample from the initial T=0 wound distance with data represented as percent wound closure.Photographs of the scratches were obtained using a Nikon AZ100 microscope

(Nikon, Melville, NY) under bright field conditions.

Transmembrane Chemotaxis Assay

Fibroblast chemotaxis assays were performed as described by Kramer, *et al*. (72). Briefly, transwell inserts (8 μm pore size, 6.6 mm diameter; Corning, Corning, NY) were coated with 10 μg/mL fibronectin for two hours at room temperature. Transfected or non-transfected NIH-3T3 fibroblasts were gently rinsed in sterile PBS prior to trypsinizing and cells were resuspended in serum-free DMEM. Cells were washed once and resuspended in sterile serum-free DMEM at a concentration of 5 x 10^5 cells/ml. In some experiments, cells were pretreated in with 50 μM MANS, 50 μM RNS, sterile PBS (VC) or 100 nM wortmannin for 30 minutes at 37˚C. Chemotaxis buffer was serum free DMEM containing containing 1 mg/mL fatty acid free BSA and 1 nM PDGF-BB or VC (sterile water) were added. Chemattractant, VC or media alone was placed into a well of a 24-well plate and a fibronectin coated transwell was placed on top. NIH-3T3 cells (100 μL or 5x10^4 cells) were placed in the top chamber of each transwell and the plate was incubated for 4 hours at 37˚C and 5% CO_2. Cells on the upper part of the filter were dislodged with a sterile cotton swab and rinsed with sterile PBS. Filters were fixed in 10% neutral buffered formalin solution and stained with Harris Hematoxylin (Sigma) following manufacturer's guidelines prior to mounting on glass microscope slides. The number of cells on the bottom of the filter was counted in 10 high powered fields (40 x) of a light microscope.

Western blotting and PDGF stimulation

For EGFP expression, lysates were prepared from adherent transfected and non-transfected cells by scraping cells into RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50 mM sodium fluoride) containing protease inhibitors (1 mM phenylmethanesulphonylfluoride (PMSF) and 1:100 dilution of Sigma protease inhibitor cocktail). Lysates were incubated on ice with agitation for 20 minutes and lysates were cleared by centrifugation for 10 minutes at 9,000 x g at 4˚C and protein concentrations were determined by the BCA assay (Pierce, Rockford, IL). Samples were diluted in 5X Sample Buffer containing 2-ME and boiled for 5 minute prior to storing at -20˚C or analyzed by 12% SDS-PAGE. Equal protein concentrations were analyzed by SDS-PAGE and transferred to Immobilon-P PVDF transfer membrane (Millipore, Billerica, MA) and membranes were blocked with 5% non-fat dry milk in tris buffered saline with Tween-20 (TBS/T; 136 μM NaCl, 20 μM Tris-base (pH 8.0) and 0.1% Tween-20 v/v, pH 7.4) for one hour at room temperature with gentle agitation. Membranes were incubated with 1:500 dilution of anti-EGFP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% non-fat dry milk in TBS/T overnight at 4˚C with gentle agitation. Membranes were washed in TBS/T and incubated with 1:4000 dilution of goat anti-rabbit (H+L) horseradish peroxidase (HRP) secondary antibody in 5% non-fat dry milk in TBS/T for 1 hour with gentle agitation. Membranes were washed and immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Thermo

Scientific, Rockford, IL) following manufacturer's protocol and exposed to radiographic film.

For PDGF stimulation of non-adherent cells, confluent NIH-3T3 fibroblasts were trypsinized and isolated in serum free media containing 1 mg/mL fatty acid free BSA and stimulated with PDGF-BB in a cell suspension at the indicated concentration or time at 37˚C. Cells were immediately placed on ice and centrifuged at 3,500 x g for 10 minutes at 4˚C. Cells were washed once in cold sterile PBS and resuspended in cold RIPA buffer containing protease inhibitors and placed on ice with agitation for 20 minutes. Lysates were cleared, processed and analyzed by 10% SDS-PAGE.

For PDGF stimulation of adherent cells, 6 well plates were coated with 10 µg/mL fibronectin for 2 hours at room temperature and washed with PBS. Confluent NIH-3T3 fibroblasts were seeded and grown to 90% confluence. Cells were serum starved for 4 hours in serum free DMEM plus antibiotics and 1 mg/mL fatty acid free BSA. In some experiments, cells were pretreated with 50µM MANS, RNS or VC (PBS) for 30 mimutes at 37˚C. Cells were then stimulated with the indicated concentration of PDGF-BB for indicated time. Plates were then placed on ice and washed with ice cold sterile PBS and 500 µL of RIPA buffer containing protease inhibitors. Cells were scraped and placed into a microcentrifuge tube, vortexed and incubated on ice for 20 minutes with gentle agitation. Lysates were cleared, processed and analyzed by 10% SDS-PAGE as described above.

Western blots for phospho-MARCKS expression are as follows; membranes were blocked in 5% non-fat dry milk in TBS/T for one hour at room temperature and incubated

with 1:500 dilution of α-phospho-MARCKS (Ser 152/156) (Cell Signaling Technology, Danvers, MA) in antibody dilution buffer (5% BSA in TBS/T). Membranes were washed in TBS/T and incubated with 1:2000 goat anti-rabbit IgG (H+L) HRP (Santa Cruz Biotechnology) in 5% non-fat dry milk in TBS/T and washed again in TBS/T. Proteins were detected by enhanced chemiluminescence and exposed to radiographic film. Western blots for total MARCKS expression are as follows; membranes were blocked with BSA blocking buffer (150 μM NaCl, 10 μM Tris-HCl (pH 8.0), 1% BSA and 0.1% Tween-20) for one hour at room temperature with gentle agitation. Membranes were incubated in α-MARCKS (N-19) antibody (Santa Cruz Biotechnology) at a concentration of 1:750 in BSA blocking buffer overnight at 4˚C with gentle agitation. Membranes were washed at room temperature in TBS/T prior to incubation with 1:5000 donkey anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology) in BSA blocking buffer and membranes were washed again prior to protein detection by enhanced chemiluminescence and radiographic exposure.

Subcellular Fractionation

Subcellular fractionation was performed as previously described (16). Briefly, cells were transfected by nucleofection and plated in 6-well plates. Cells were washed with ice cold PBS and scraped into eppendorf tubes. Cells were pelleted at 1,000 rpm for 5 minutes at 4°C and resuspendend with ice cold lysis buffer (50 mM Tris, 150mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail). Cells were sonicated three times for 15 seconds each and the nuclei and cell debris was collected by centrifugation at 500 x g for 2

minutes. The supernatant was transferred to a new eppendorf tube and centrifuged at 20,000 x g for 45 minutes at 4° cells. The supernatnant was collected as the cytosol fraction and the remaining pellet was resuspended in lysis buffer containing 1% Triton X-100. This mixture was sonicated as above and centrifuged at 20,000 x g for 30 minutes and the supernatant was collected and saved as the membrane fraction. Protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA) and samples were diluted in 5x sample buffer and boiled for 5 minutes. Equal protein concentrations were loaded onto a 12% SDS-PAGE and western blots were performed for EGFP expression, as described above.

Epi-fluorescence microscopy

For epi-fluorescence microscopy, 10,000 cells were plated on fibronectin coated coverslips (10 μg/mL) and allowed to adhere for 3 hours in a 37°C incubator. Cells were washed twice with sterile PBS and fixed in 10% neutral buffered formalin solution and washed again with PBS twice. Coverslips were then mounted face down on glass coverslips and allowed to dry overnight in the dark. Immunofluorescence images were obtained using the Nikon AZ100 microscope.

Statistical analysis

Statistical analysis was performed by Sigma Stat (Systat Software, Inc, Chicago, IL) using a paired t-test, with $P<0.05$ considered statistically significant.

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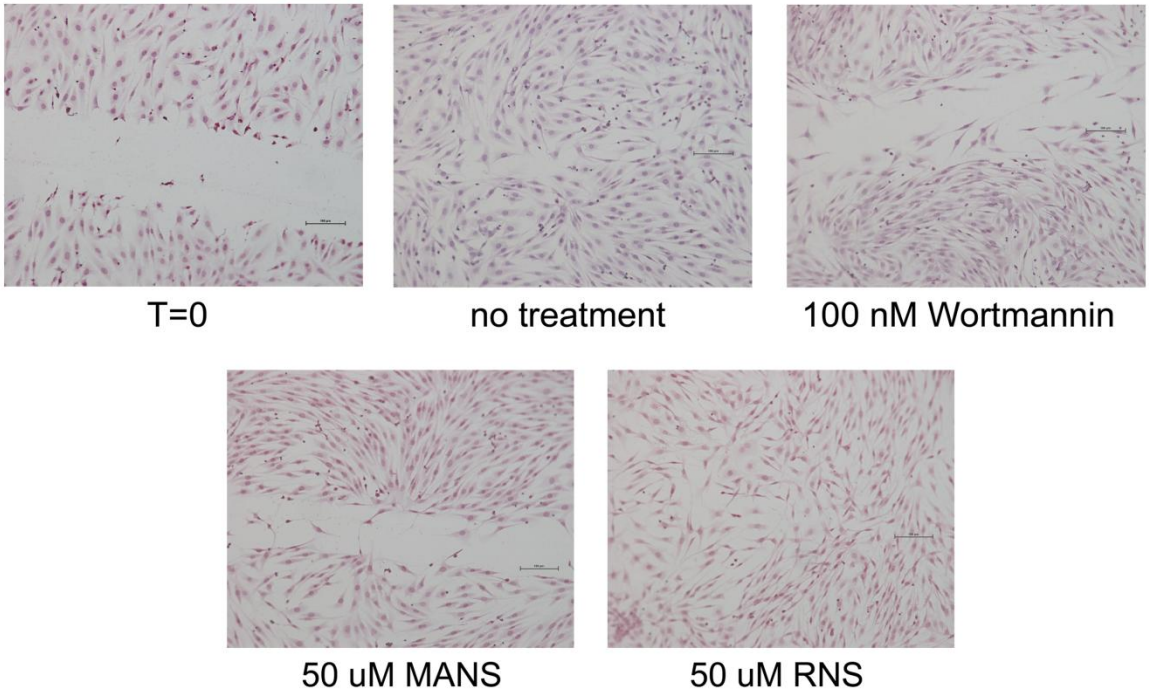
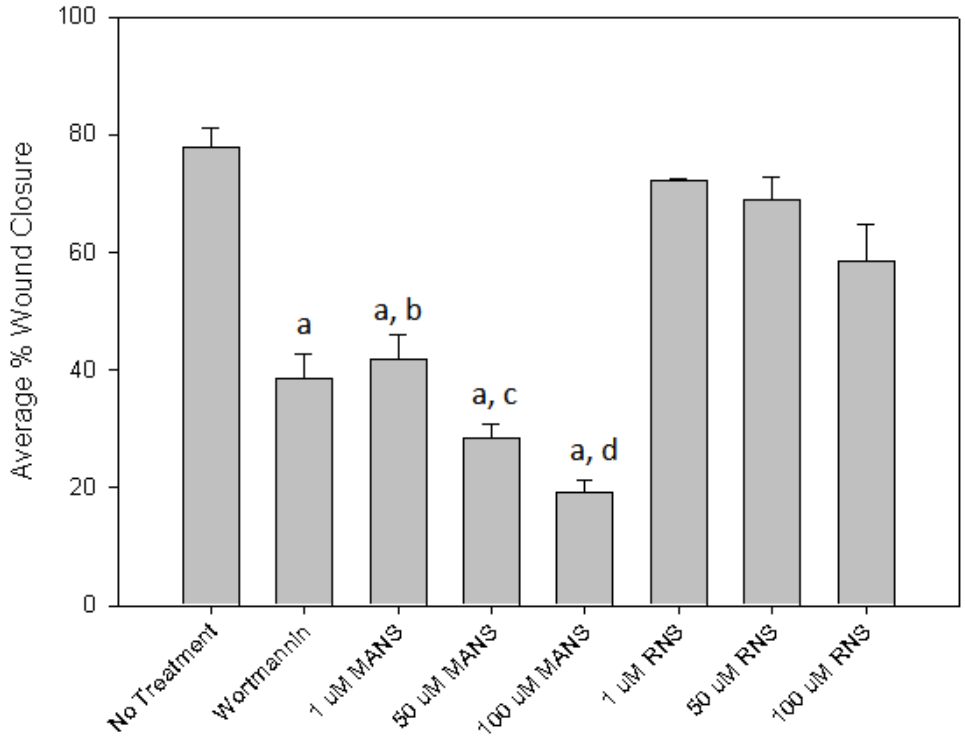
Figure 1: MANS peptide treatment results in the inhibition of NIH-3T3 fibroblast migration. NIH-3T3 fibroblasts were grown to confluency on fibronectin (A, B) or collagen (C) coated coverslips and scratches were made in the monolayer in the presence of increasing concentrations (1, 50 or 100 μM) of MANS or RNS, VC (PBS) or 100 nM wortmannin. After 18 hours, coverslips were fixed, stained and the width of the scratch was measured by an ocular micrometer. Photos are representative from one experiment on fibronectin coated coverslips (A). The average percent wound closure from four independent experiments are shown on fibronectin substrate (B) or collagen substrate (C). Statistcal analysis ($p<0.05$) was performed where "a" denotes a significantly decreased ability to migrate back into the wound relative to no treatment and "b", "c", and "d" denote a statistically significant ability to migrate back into the wound relative to 1 μM, 50 μM and 100 μM RNS, respectively.

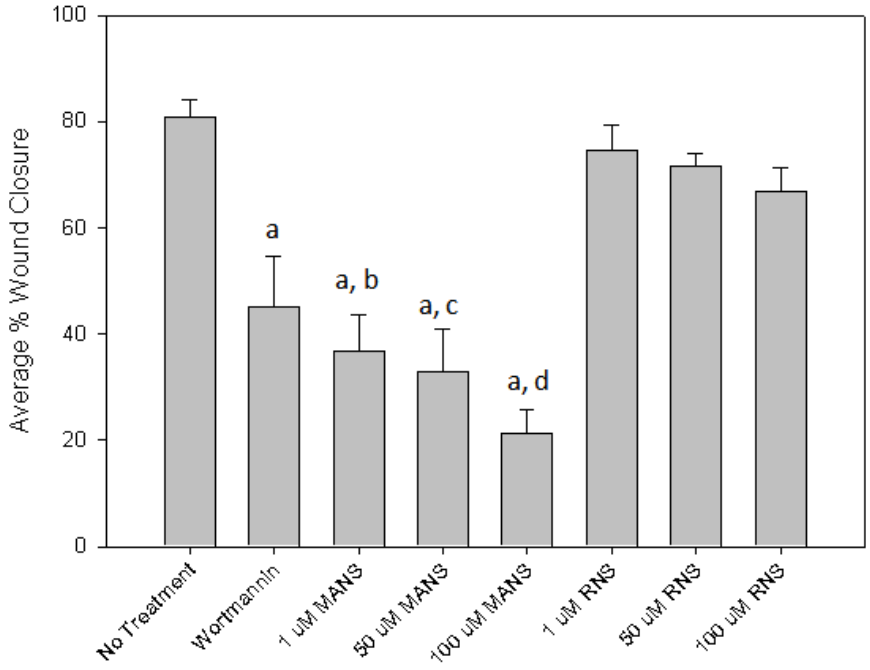
A.



B.


Average % Wound Closure
100
80
60
40
20
0
a
a, b
a, c
a, d
No Treatment
Wortmannin
1 uM MANS
50 uM MANS
100 uM MANS
1 uM RNS
50 uM RNS
100 uM RNS

C.


Average % Wound Closure
100
80
60
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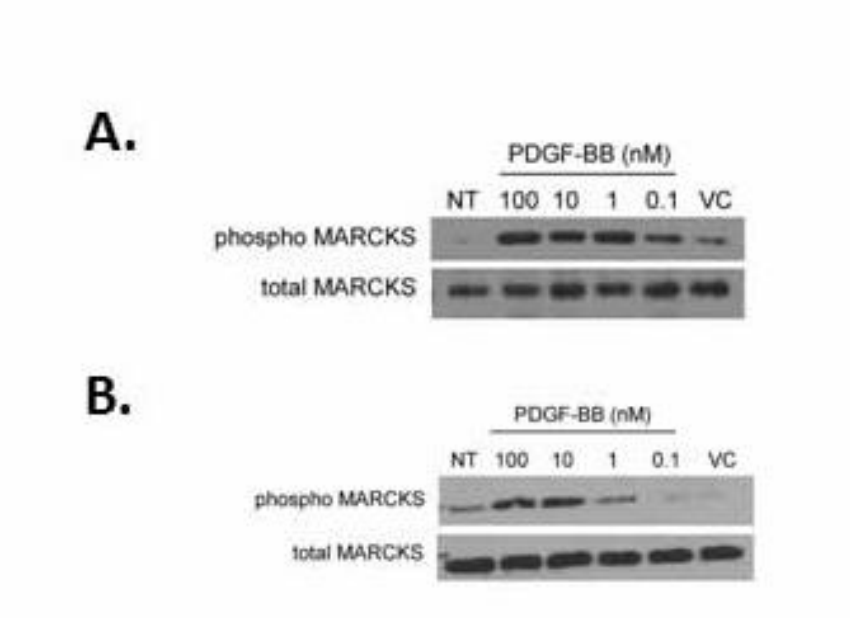
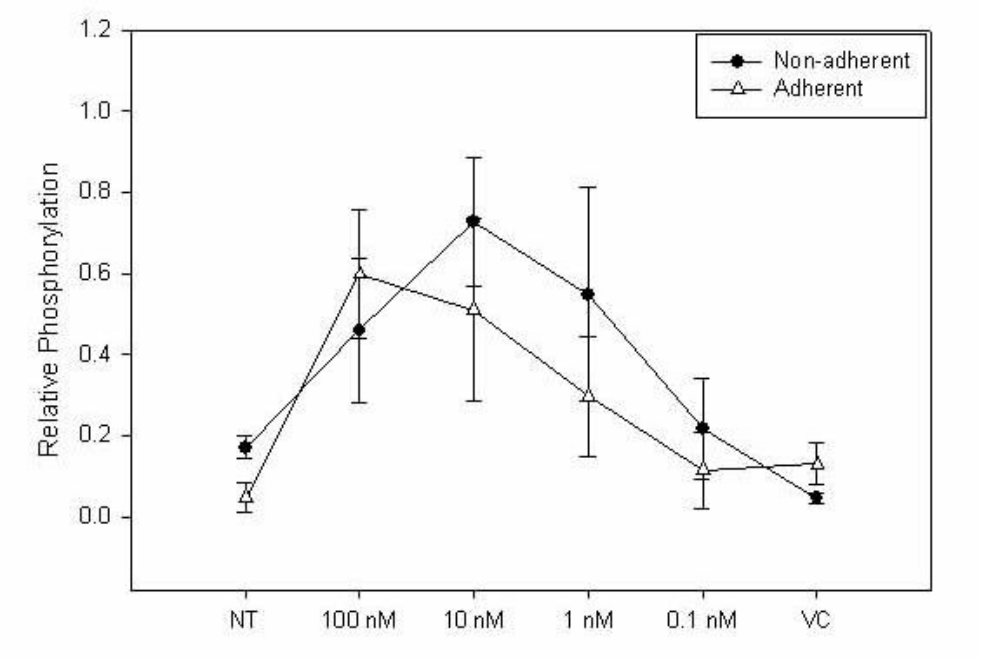
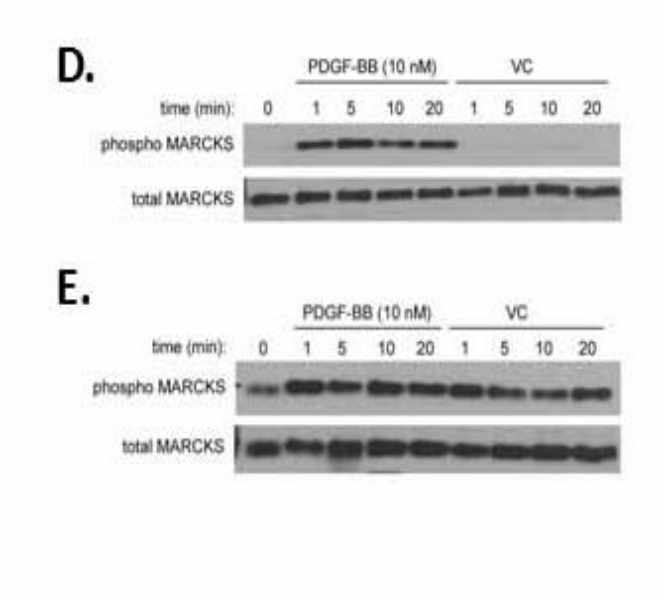
No Treatment
Wortmannin
1 uM MANS
50 uM MANS
100 uM MANS
1 uM RNS
50 uM RNS
100 uM RNS

<u>Figure 2:</u> PDGF-BB stimulation results in the phosphorylation of MARCKS in adherent and non-adherent NIH-3T3 fibroblasts. Adherent (A and D) or non-adherent (B and E) fibroblasts were stimulated with the indicated concentration of PDGF-BB for the indicated time. Cell lysates were prepared and analyzed by western blot analysis for both phospho-MARCKS and total MARCKS. Representative dose response (1 min stimulation) studies from three separate experiments in adherent (A) and non-adhernt (B) cells are depicted with densitometry analysis comparing adherent versus non-adherent % MARCKS phosphorylation (C). Representative kinetics analysis (10 nM PDGF-BB) in adherent (D) and non-adherent (E) cells from three (adherent) and four (non-adherent) experiments are depicted with densitometry analysis comparing % MARCKS phosphorylation in adherent versus non-adherent cells (F) with an * denoting a significant difference between PDGF-BB and VC treated samples in adherent cells ($p<0.05$), # denoting a difference between PDGF-BB and VC treated samples in non-adherent cells ($p>0.05$) and † denoting a significant difference between VC treated adherent and non-adherent cells ($p<0.05$)

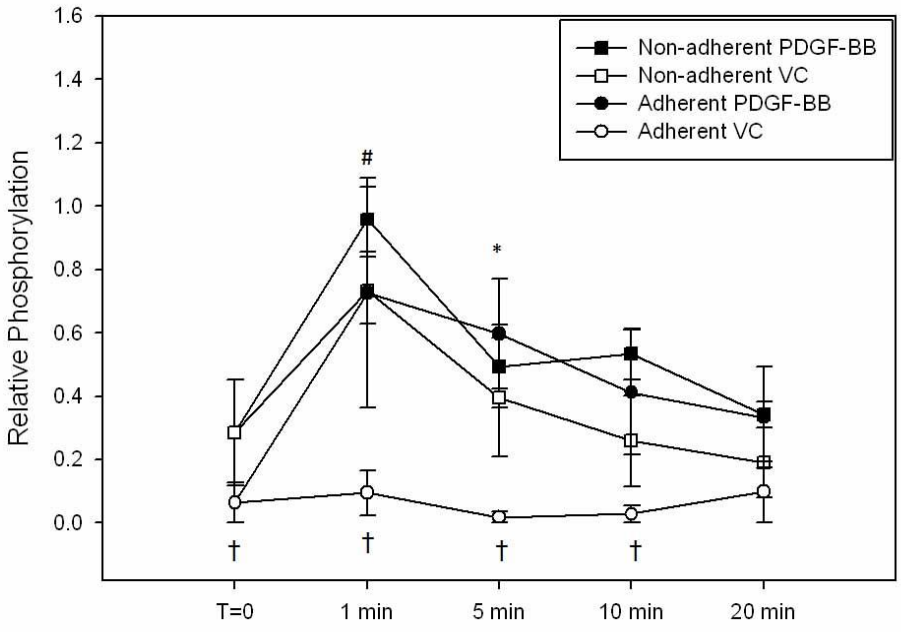

A.
PDGF-BB (nM)
NT 100 10 1 0.1 VC
phospho MARCKS
total MARCKS
B.
PDGF-BB (nM)
NT 100 10 1 0.1 VC
phospho MARCKS
total MARCKS

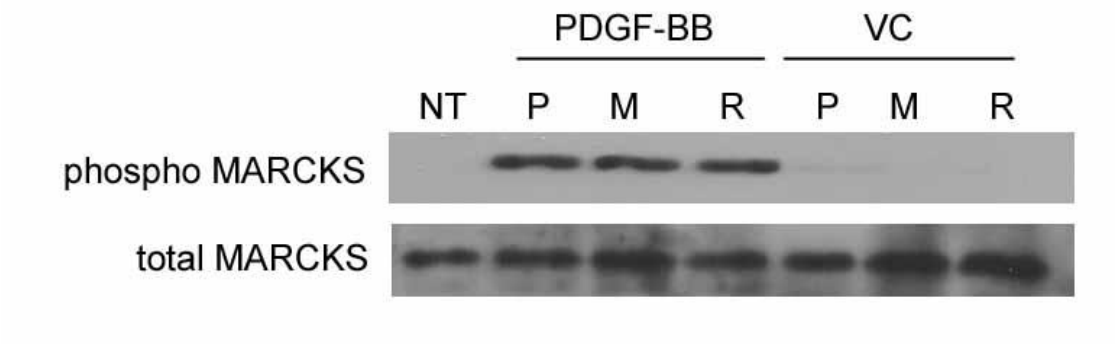
C.


Relative Phosphorylation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Non-adherent
Adherent
NT
100 nM
10 nM
1 nM
0.1 nM
VC


D.
PDGF-BB (10 nM)
VC
time (min):
0
1
5
10
20
1
5
10
20
phospho MARCKS
total MARCKS
E.
PDGF-BB (10 nM)
VC
time (min):
0
1
5
10
20
1
5
10
20
phospho MARCKS
total MARCKS

F.


Relative Phosphorylation
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Non-adherent PDGF-BB
Non-adherent VC
Adherent PDGF-BB
Adherent VC
#
*
†
†
†
†
T=0
1 min
5 min
10 min
20 min



<u>Figure 3:</u> MANS pretreatment does not alter PDGF-BB mediated MARCKS phosphorylation. Adherent fibroblasts were pretreated with 50 μM MANS, 50μM RNS or PBS (VC for MANS or RNS peptides) for 30 minutes prior to stimulation with 10 nM PDGF-BB for 1 minute. Cell lysates were prepared and westerm blot analysis for phospho and total MARCKS was performed. Data is representative of three separate experiments.

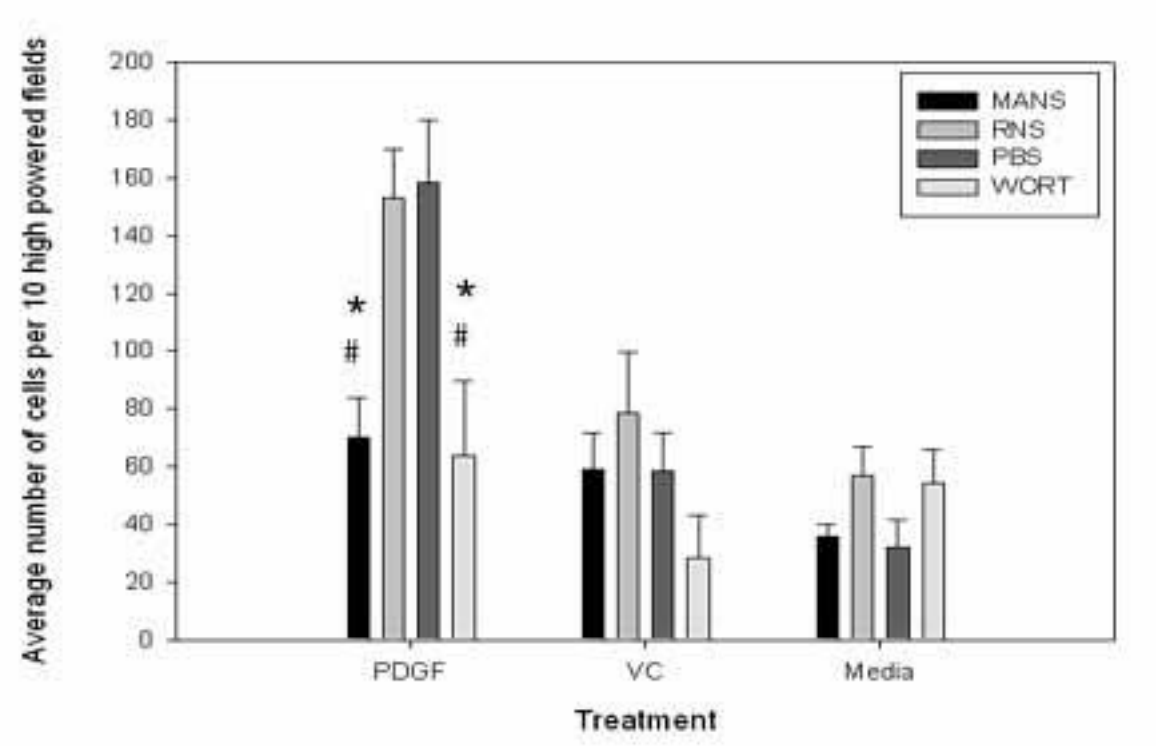
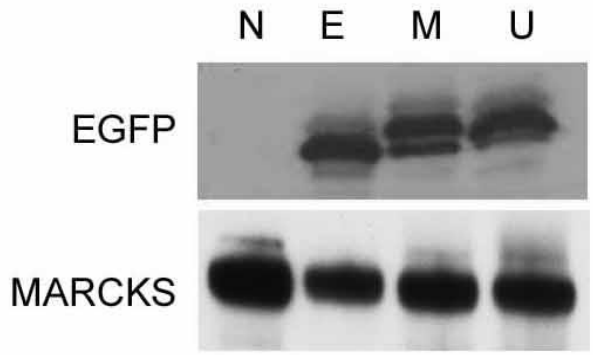


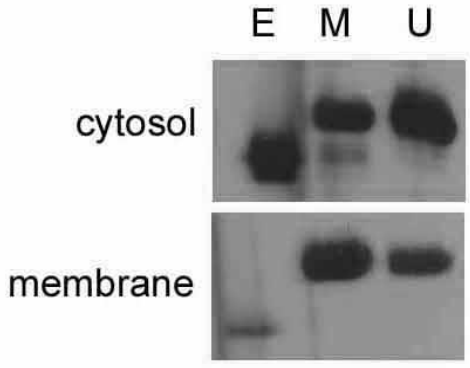
Figure 4: PDGF-BB mediated NIH-3T3 fibroblast chemotaxis is inhibited by MANS pretreatment. NIH-3T3 fibroblasts were pretreated with 50 μM MANS, 50 μM RNS, PBS (VC) or 100 nM wortmannin (WORT) for 30 minutes prior to adding the cells to fibronectin coated transwells with 1 nM PDGF, VC or media alone in the bottom chamber. Transwell chambers were incubated for 4 hours and the transwell inserts were fixed, stained and mounted on microscope slides and the number of cells on the bottom side of the filter was counted in 10 high powered fields. Data is represented as the average number of cells in 10 high powered fields from four individual experiments with # and * denoting a significant decrease in % wound closure relative to RNS and VC treatment treatment, respectively ($p<0.05$).

Figure 5: Expression and cellular localization of MANS:EGFP and UMANS:EGFP fusion proteins. Transfection of EGFP (E), MANS:EGFP (M) and UMANS:EGFP (U) results in similar expression of EGFP in NIH-3T3 fibroblasts, with no expression of EGFP in non-transfected (N) cells as determined by western blot analysis. MARCKS expression in transfected and non-transfected NIH-3T3 fibroblasts was also determined by western blot with similar expression of MARCKS observed in EGFP (E), MANS:EGFP (M) and UMANS:EGFP (U) transfected cells and decreased expression of MARCKS in transfected cells compared to non-transfected (N) cells (A). Subcellular fractionation (B) and immunofluorescence analysis (C) demonstrates that EGFP (E) and UMANS:EGFP (U) are preferentially localized to the cytosol while MANS:EGFP (U) is targeted to cell membranes. Figures are representative of three independent experiments.

A.

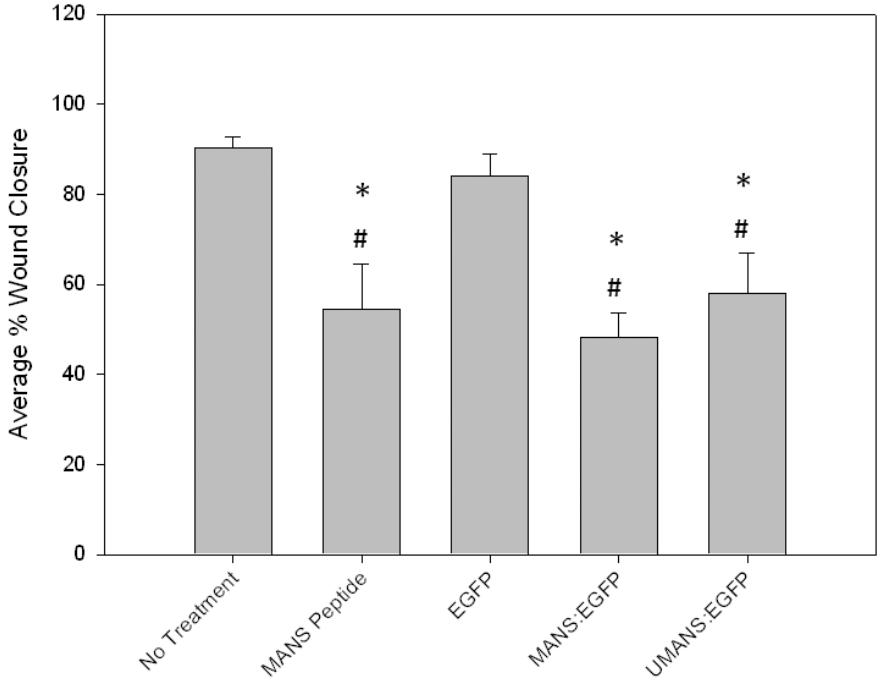


B.

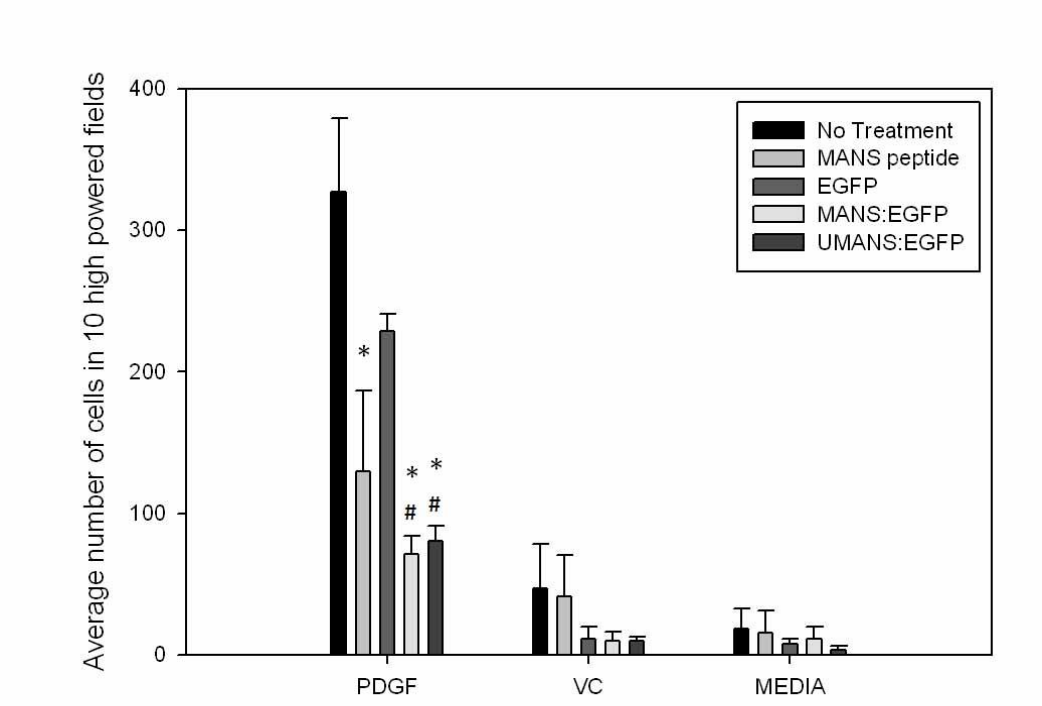


C.

EGFP MANS:EGFP UMANS:EGFP



<u>Figure 6:</u> Myristoylation of MANS is not required for fibroblast migration. NIH-3T3 fibroblasts transfected with EGFP, MANS:EGFP or UMANS:EGFP as well as non-transfected cells were plated on fibronectin coated coverslips and scratch assays were performed as described in Materials and Methods. MANS peptide (50 μM) treated cells as well as MANS:EGFP and UMANS:EGFP transfected cells had significantly reduced migration compared to non-treated and EGFP transfected cells, as denoted by an * and #, respectively ($p<0.05$). Data is representative of five independent experiments.



<u>Figure 7:</u> Myristoylation of MANS is not required for PDGF-BB mediated fibroblast migration. PDGF-BB chemotaxis assays were performed with NIH-3T3 fibroblasts transfected with EGFP, MANS:EGFP or UMANS:EGFP as described in Materials and Methods. MANS peptide (50 μM) treated cells as well as MANS:EGFP and UMANS:EGFP transfected cells have a significantly reduced migration compared to non-treated cells, as denoted by an * ($p>0.05$). Further, MANS:EGFP and UMANS:EGFP transfected cells had a significantly reduced migration towards PDGF-BB compared to EGFP transfected cells, as denonted by an # ($p<0.05$). Data is representative of five independent experiments.

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CHAPTER 3

TWO MYRISTOYLATED ALANINE-RICH C-KINASE SUBSTRATE (MARCKS) PARALOGS ARE REQUIRED FOR NORMAL DEVELOPMENT IN ZEBRAFISH

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Abstract

Myristoylated alanine-rich C-kinase substrate (MARCKS) is an actin binding protein substrate of protein kinase C (PKC) and critical for mouse and *Xenopus* development. Herein two MARCKS paralogs, *marcksa and marcksb*, are identified in zebrafish and the role of these genes in zebrafish development is evaluated. Morpholino-based knockdown of either MARCKS protein resulted in increased mortality and a range of gross phenotypic abnormalities. Phenotypic abnormalities were classified as mild, moderate or severe, which can be characterized by a slight curve of a full length tail, a severe curve or twist of a full length tail and a truncated tail, respectively. All three phenotypes displayed abnormal neural architecture. Histopathology of MARCKS deficient embryos revealed abnormalities in retinal layering, gill formation and skeletal muscle morphology. These results demonstrate that Marcksa and Marcksb are essential for normal zebrafish development and suggest that zebrafish are a suitable model to further study MARCKS function.

Introduction

Myristoylated Alanine-Rich C-kinase Substrate (MARCKS) is a ubiquitously expressed, acidic, rod shaped, actin binding protein. MARCKS is conserved among vertebrate species possessing three conserved domains; an amino-terminal myristoylation domain, a MH-2 domain and the phospho-site domain (PSD), which serves as the phosphorylation site for protein kinase C (PKC) (1-3). Phosphorylation of MARCKS by PKC results in dissociation of MARCKS from the plasma membrane to the cytosol (4, 5), where MARCKS is dephosphorylated and is subsequently able to bind filamentous actin (F-actin) (6, 7). F-actin bound MARCKS then returns to cell membranes (8), resulting in rearrangement of the actin cytoskeleton. Given its association with the actin cytoskeleton, it is not surprising that previous experiments have elicited a role for MARCKS in cell spreading (9, 10), phagocytosis (11, 12), and endo- and exo-cytosis (13-15).

One of the first characterized roles for MARCKS was in development. Blackshear and colleagues generated a homozygous Marcks deficient mouse strain by targeted disruption of the *Marcks* gene (also termed *Macs*), resulting in perinatal lethality and severe abnormalities. Abnormalities observed in *Marcks* deficient mice included decreased body size and severe muscular and neural abnormalities. The neural abnormalities observed included exencephaly, agenesis of the corpus callosum and defects in retinal layering (16), demonstrating a critical role for Marcks in neural development (17-19). *Marcks* mRNA expression is observed in embryonic mice as early as day 7.5 and expression is maximal by

day 8.5-9.5 (18), correlating with the neural abnormalities observed in Marcks deficient mice (16, 18). Interestingly, adult mice and rats express lower levels of *Marcks* mRNA than developing animals (18, 20), further supporting a functional role for MARCKS during embryonic development.

The cause of developmental defects in the absence of MARCKS can be attributed to the role of MARCKS in gastrulation. Blockade of MARCKS protein expression in *Xenopus laevis* embryogenesis resulted in defective morphogenetic movements during gastrulation, specifically during convergent extension (21). MARCKS function is also required during myogenesis (22, 23), further suggesting a role for MARCKS during gastrulation. Given that cell migration is one of the key processes during gastrulation (24); MARCKS may have a key role in the regulation of cell migration.

Previously, we and other laboratories have demonstrated an *in vitro* role for MARCKS in cell migration (25, 26). However, given that genetic disruption of Marcks in mice is embryonic lethal (16), it has yet to be determined if MARCKS is required for cell migration *in vivo*. With the recent descriptions of transgenic lines and *in vivo* cell migration assays (27, 28); we have chosen to explore the use of zebrafish larvae as a possible in *vivo* model to study the role of MARCKS in cell migration. We therefore sought to establish the phenotype of MARCKS deficient zebrafish embryos. To our knowledge, the role of MARCKS in zebrafish development has not been previously reported.

Herein, two zebrafish MARCKS genes, *marcksa* and *marcksb*, are identified and evaluated for their role in embryonic development. The *marcksa* and *marcksb* genes appear

to be paralogs derived from a progenitor MARCKS gene. Expression of *marcksa* and *marcksb* transcripts are detected throughout embryonic development and morpholino-based knock-down of either gene resulted in developmental defects reminiscent of those observed in MARCKS deficient mouse and *Xenopus* models. These results demonstrate that zebrafish is a viable model for the study of MARCKS in developmental biology as well as other biological processes, including cell migration.

Results and Discussion

Zebrafish express two MARCKS paralogs which are orthologous to mammalian MARCKS.

It has been predicted that, subsequent to its divergence from mammals, the bony fish lineage experienced a whole genome duplication event hundreds of millions of years ago. The remnants of this genome duplication event are detected by the presence of paralogs of approximately half of the genes in the zebrafish genome (29, 30). MARCKS appears to be one of such genes in zebrafish, as BLASTx searches using the human MARCKS protein as a query identified two zebrafish MARCKS cDNA sequences in GenBank. Both sequences were deposited as a result of a large scale cDNA sequencing effort (31). One sequence, which had been termed zebrafish *marcks* on both GenBank (NM_001015060) and the ZFIN database (ZDB-GENE-030131-1921), is located on chromosome 17. The other sequence, which has been termed *zgc: 109978* on the GenBank (NM_001024404) and ZFIN database (ZDB-GENE-050522-145), is located on chromosome 20. Based on our sequence comparisons and phylogenetic analyses (Fig. 1), we demonstrate that these genes are likely

paralogs and have renamed *zgc:109978* and *marcks* to be *marcksa* and *marcksb*, respectively. We PCR amplified, cloned and sequenced full-length cDNA sequences for both genes by rapid amplification of cDNA ends (RACE). We observed 11 nucleotide and seven amino acid differences between our *marcksa* cDNA sequence and its reference sequence (NM_001024404). We observed seven nucleotide and three amino acid differences between our *marcksb* cDNA sequence and its reference sequence (NM_001015060).

Zebrafish Marcksa and Marcksb are 40.6% identical. Marcksa is 48.8% identical to human MARCKS and 43.7% identical to mouse Marcks. Marcksb is 41.9% identical to human MARCKS and 42.1% identical to mouse Marcks. Protein sequence alignments reveal that the three conserved domains characteristic of MARCKS (2) (the myristoylated amino-terminus, the MH2 domain and PSD), are conserved in both zebrafish Marcks proteins (Fig. 1A). PKC phosphorylates MARCKS at Ser^{152}, Ser^{156} and Ser^{163} in rodents (32) and it appears that zebrafish Marcksa could be phosphorylated at equivalent serine residues. However, zebrafish Marcksb appears to be capable of serine phosphorylation at only two of these sites. Instead of having a serine at amino acid 119 (analogous to Ser^{156} in murine Marcks) zebrafish Marcksb encodes a proline at this position. Phosphorylation of MARCKS on Ser^{159} in humans (Ser^{152} in mice) occurs via the RhoA/ROCK pathways (33) and both zebrafish Marcksa and Marcksb encode serines at this position, presumably also capable of being phosphorylated. Phosphorylation of MARCKS occurs in chick neurons on Ser^{25} (34) and zebrafish Marcksa has a serine at that corresponding residue but zebrafish Marcksb does not. However, Marcksb does have a serine residue at position 26 that may be a candidate

serine residue capable of phosphorylation in a similar manner as Ser^{25} in other species. Taken together, these observations suggest that zebrafish Marcks proteins are homologous to MARCKS proteins expressed by other vertebrate species. The evidence also suggests that zebrafish Marcksa and Marcksb may be regulated by similar and perhaps different mechanisms, suggesting non-redundant roles for these proteins in zebrafish.

Phylogenetic analysis of vertebrate MARCKS and MARCKS-like protein (MLP) demonstrate that zebrafish Marcksa and Marcksb are more similar to MARCKS than to MLP (Fig. 1B). MLP is a member of the MARCKS family of proteins, sharing approximately 50% amino acid identity with MARCKS (35, 36). Further, Marcksa is more closely related to other vertebrate MARCKS proteins than Marcksb (Fig. 1B).

A comparison of genes flanking both zebrafish *marcksa* and *marcksb* demonstrate conserved synteny with the MARCKS locus on human chromosome 6q and mouse chromosome 10 (Fig. 1C). On zebrafish chromosome 20 *marcksa* is flanked by *zgc:162161*, *rev3l* and *zgc:172112* which are orthologous to *KIAA1919, REV3L* and *CNSKR3* on human chromosome 6q and *2010001E11Rik, Rev3l* and *Cnksr3* on mouse chromosome 10. On zebrafish chromosome 17 *marcksb* is flanked by *col10a1, LOC796410, manea* and *cx52.7* which are orthologous to *COL10A1, FUT9, MANEA* and *GJA10* on human chromosome 6q: of these genes, only *Col10a* is present on mouse chromosome 10. This data suggests that *marcksa* and *marcksb* are indeed paralogs.

Previously, Thisse and colleages demonstrated by *in situ* hybridization that *marcksa* and *marcksb* are expressed in developing zebrafish embryos, with uniform expression from

the single cell stage to 60 hours post fertilization (hpf). Both *marcksa* and *marcksb* were described in a non-spatially restricted pattern and high expression was evident in the brain and notochord (77). We validated these results by determining the mRNA expression of *marcksa* and *marcksb* during zebrafish development by reverse transcriptase PCR (RT-PCR). Maternal transcripts of *marcksa* and *marcksb* are detected in the fertilized egg (0 hpf) and somatic expression maintains consistent mRNA levels through 120 hpf (Fig. 2). We attempted to determine protein expression of zebrafish Marcks throughout development. However, given the lack of a commercially available zebrafish specific anti-Marcks antibody and differences in the amino acid sequences between mouse, human and zebrafish MARCKS proteins; we were unable to detect zebrafish Marcks protein expression by western blot (unpublished observation).

Knockdown of zebrafish Marcksa and Marcksb result in increased mortality and abnormal phenotypes

Given previous research reporting a role for MARCKS in mammalian and *Xenopus laevis* development, we hypothesized that both Marcksa and Marcksb play a role in the development of zebrafish. In order to test this hypothesis, we utilized anti-sense morpholinos (MOs) to evaluate Marcksa and Marcksb function in the zebrafish embryo. Two MOs were designed for both Marcksa and Marcksb; two translation blocking MOs for *marcksa* (one binding complementary to sequence including the AUG start codon and one binding complementary to sequence upstream of the AUG start codon) and a translation blocking and

splice blocking MO for *marcksb*. Single cell zebrafish embryos were injected with Marcksa-blocking or Marcksb-blocking MOs and phenotypes were characterized at 24, 48, 72 and 96 hpf. Disruption of Marcksa or Marcksb produced embryos with a similar range of phenotpyes that were classified into one of four phenotypic groups: normal, mild, moderate and severe (Fig. 3). The mild phenotype fish have a slight curve to their full length tail, whereas moderate phenotype fish have a full length tail with a severe curve or twist. Severe phenotype fish have a cropped or absent tail, with the tail not extending far beyond the yolk sac. Mild, moderate and severe phenotyped embryos all appear to have abnormal brain development, evidenced by the lack of normal eye and neural tissue architecture.

To further characterize the effect of MARCKS knock down on zebrafish development, we performed a MO dose response experiment and quantified the phenotypes of Marcksa and Marcksb MO injected embryos (Figs. 4 and 5, Supplemental Fig. 1 and 2, Supplemental Table 1). For Marcksa, we injected embryos with 0.5, 2 or 4 ng of Marcksa translation blocking MO that binds the AUG start site (MAT) (Fig. 4) or 2, 4 or 6 ng of Marcksa translation blocking MO that binds upstream of the AUG start site (MATU) (Supplemental Fig. 1). For Marcksb, we injected embryos with 2, 4 or 6 ng of Marcksb translation blocking MO (MBT) (Fig. 5) or Marcksb splice blocking MO (MBS) (Supplemental Fig. 2). The first obvious phenotype that we observed was an increased mortality in Marcksa and Marcksb MO injected embryos. There was significantly increased mortality as early as 24 hpf in the 6 ng MBT group compared to the 6 ng control MO and wild type groups as well as a significantly increased mortality in the 4 ng MAT and 2 and 4

ng MBT groups compared to the wild type groups (Figs. 4A and 5A, supplemental table 1). At 48 hpf, we observed a significant increase in mortality in the 4 ng MAT and 4 and 6 ng MBT groups compared to the control MO and wild type groups. We also observed a significant increase in mortality in the 2 ng MBT group compared to the wild type group (Figs. 4B and 5B, supplemental table 1). At 72 hpf, there was a significant increase in mortality in the 4 ng MAT and 4 and 6 ng MBT groups compared to control MO and wild type groups. We also observed a statistically significant increase in mortality in the 0.5 ng MAT and 2 ng MBT groups compared to the wild type group at 72 hpf (Figs. 4C and 5C, Supplemental Table 1). Finally, a significant increase in mortality was also observed at 96 hpf with the 4 ng MAT and 2, 4 and 6ng MBT groups having a higher mortality than control MO and wild type groups (Figs. 4D and 5D, Supplemental Table 1). At 96 hpf, we also observed a statistically significant increase in mortality in the 0.5 and 2 ng MAT groups compared to the wild type group (Fig. 4D, Supplemental Table 1). Increased mortality was also observed in the MATU and MBS injected embryos compared to the control MO and wild type embryos (Supplemental Figs. 1 and 2).

The increased mortality in Marcks deficient zebrafish is consistent with previous data in a mouse model. Marcks deletion in mice (*Marcks*$^{-/-}$) is embryonic lethal and mouse pups that survive birth die shortly thereafter (16). Interestingly, Marcks heterozygote mice (*Marcks*$^{+/-}$) appeared normal and were capable of reproduction (16) and homozygous non-myristoylated Marcks transgenic mice had an increased post natal survival rate compared to Marcks deficient mice, with 25% of the mouse pups surviving the perinatal period (37).

Since we did not observe 100% mortality in either Marcksa or Marcksb MO injected zebrafish embryos at any of the doses indicated, we therefore tested if co-injection of Marcksa and Marcksb MOs would result in 100% mortality. Two ng combinations of MAT, MBT or control MOs (4 ng total) were co-injected into 1-cell zebrafish embryos and mortality was determined at 24 hpf. Embryos co-injected with 2 ng MAT and 2 ng MBT had an increased mortality (97.1 ± 5.0%) compared to embryos co-injected with 2 ng MAT and 2 ng control MO (83.1±11.7%), 2 ng MBT and 2 ng control MO (58.22±20.1%) or non-injected, wild-type embryos (10.7±2.8%). The increase in mortality in the MAT and MBT co-injected embryos was statistically significant compared to MBT and control MO co-injected and wild type (non injected embryos), but was not significantly increased compared to MAT and control co-injected embryos (data not shown). Interestingly, when we injected 2 ng of MAT or MBT alone, we observed 40.8±7.6% and 55.0±32.8% mortality, respectively. Further, when we injected 4 ng MAT or MBT alone, we observed 80.8±26.9% and 86.5±7.4% mortality, respectively (Figs. 4 and 5). Given that the mortality in the MAT and MBT co-injected group was not 100% and that the mortality was similar to the mortality in the 4 ng MAT or MBT alone injected groups suggests that Marcksa and Marcksb may play an additive role in zebrafish development. However, we cannot determine if Marcksa and Marcksb play redundant or non-redundant roles because we are unable to determine protein expression levels and localization.

As previously stated, we observed four phenotypes in Marcksa and Marcksb MO injected embryos: normal, mild, moderate and severe (see Fig. 3). As for the mortality, we

also quantified the phenotypes at 24, 48, 72 and 96 hpf. For both Marcksa and Marcksb MOs, an increase in the number of mild, moderate and severe phenotypes were observed in comparison to control MO injected and wild type (non injected embryos) (Figs. 4 and 5, Supplemental Figs. 1 and 2). We further examined the phenotype by determining the number of normal versus abnormal phenotypes, with abnormal being the sum of the live embryos with mild, moderate or severe phenotypes. At 24 hpf the 2 and 4 ng MAT and 2, 4 and 6 ng MBT groups had a statistically significant increase in the number of abnormal phenotypes compared to control MO and wild type groups (Figs. 4A and 5A, Supplemental Table 1). Interestingly, the 0.5 ng MAT group had a decreased number of normal phenotypes compared to the wild type group, which was also statistically significant (Fig. 4A, Supplemental Table 1). At 48 hpf we observed a statistically significant increase in the number of abnormal phenotypes in the 2 and 4 ng MAT and 2, 4 and 6 ng MBT groups compared to respective control MO and wild type groups (Figs. 4B and 5B, Supplemental Table 1). At 72 hpf, we observed a significant increase in the number of abnormal phenotypes in the 2 ng MAT and 2 ng MBT groups compared to the 2 ng control MO and wild type groups and a significant decrease in the number of normal phenotypes in the 2 and 4 ng MAT and 2, 4 and 6 ng MBT groups compared to control MO and wild type groups (Figs. 4C and 5C, Supplemental Table 1). We observed the same trend at 96 hpf, with the 2 ng MAT and MBT groups having significant increases in the number of abnormal phenotypes compared to the 2 ng control MO and wild type groups and a significant decrease in the number of normal phenotypes in the 2 and 4 ng MAT and 2, 4 and 6 ng MBT groups

as compared to control MO and wild type groups (Fig. 4D and 5D, Supplemental Table 1). The number of normal phenotypes in the 0.5 ng MAT group was significantly decreased compared to the wild type group at 96 hpf (Fig. 4D, Supplemental Table 1). At both 72 and 96 hpf there was no difference in the number of abnormal phenotypes among embryos injected with higher doses of MAT (4 ng) and MBT (4 and 6 ng) MOs. However, we did observe an increased mortality in these populations (Figs. 4C, D and 5 C, D) as well as a decrease in the number of normal phenotypes. Likely, this accounts for the decreased number of abnormal phenotypes, in that embryos that were classified abnormal at 24 and 48 hpf died at the later time points. Again, an increased number of abnormal phenotypes was observed in MATU and MBS injected groups (Supplemental Figs. 1 and 2), indicating that the phenotypes observed in Marcksa and Marcksb deficient zebrafish are true phenotypes. Further, these findings support a role for Marcksa and Marcksb in zebrafish development.

The phenotypes that we observed in Marcksa and Marcksb deficient zebrafish occurred as early as 24 hpf, suggesting that MARCKS may play a role in the early development of zebrafish, potentially in gastrulation. In *Xenopus*, knockdown of MARCKS leads to defective gastrulation, which is the result of impaired involution of the mesoderm and failed blastopore closure. MARCKS deficiencies in *Xenopus* were also associated with impaired convergent extension (21). Convergent extension results in body axis elongation via polarized cell movements that converge medio-laterally and extends anterior-posteriorly (38, 39). Previous studies have shown the phenotype of convergent extension defective zebrafish embryos (40, 41) and the curved, twisted and truncated tails (mild, moderate and

severe phenotypes, respectively) observed in the current study (Fig. 3) suggest that MARCKS is also involved in zebrafish convergent extension.

Cell migration is crucial to the processes of gastrulation and convergent extension. During these processes, various molecules signal cells to migrate in a directed and organized manner and disruption of this directed cell migration leads to developmental abnormalities (42, 43). The Wnt pathway, which consists of canonical and noncanonical pathways, is involved in the regulation of morphogenetic movements during gastrulation (44). Wnt gradients help to direct the cell migration of mesendoderm cells (43) and signaling through the noncanonical Wnt pathway results in PKC activation (42), a protein kinase for which MARCKS is the substrate. Various PKC isoforms phosphorylate MARCKS, including PKCδ (45) which is also involved in convergent extension. Loss of PKCδ results in the inability of Dishevelled, a cytoplasmic phosphoprotein within the Wnt signaling pathway, to translocate upon Wnt pathway activation. These events result in decreased c-Jun N-terminal kinase (JNK) activation (46), which functions to regulate convergent extension (47). Further, PKC signaling through the Frizzeled-7 receptor, a G-protein coupled receptor (GPCR) also involved in Wnt signaling, controls cell sorting in the mesoderm and ultimately results in the dissociation of the anterior mesoderm from the ectoderm (48). Atypical PKCs also regulate gastrulation by regulating microtubule organization and balancing adherens junction symmetry resulting in the generation of planar cell planar polarity of epithelial cells (49). Interestingly, the noncanonical Wnt singaling pathway is involved in MARCKS mediated cortical actin dynamics during convergent extension (21). The noncanonical Wnt signaling

pathway is involved in generating planar cell polarity during convergent extension through the activation of Rho GTPases, which are also involved in cell polarization (39, 50-53). Noncanonical Wnt pathway mediated Rho GTPase activity is required for both *Xenopus* and zebrafish gastrulation (54-57) and a link between Rho GTPase and morphogenetic processes during neural tube closure has been made (58, 59). The Rho GTPase dependent kinase, Rho-Kinase, is involved in the phosphorylation of MARCKS in neural cells (60, 61). This suggests that during convergent extension, the noncanonical Wnt pathway may direct MARCKS function during embryonic development via both PKC and RhoGTPases.

Marcksa and Marcksb are required for neural development and retinal histogenesis in zebrafish

Our phenotypic characterization of Marcksa and Marcksb deficient zebrafish revealed that both proteins may be involved in neural development. With both MAT and MBT MOs, we observed neural abnormalities that were not present in the control injected or wild type, non-injected embryos (Fig. 3). Recently, Ekker and colleagues have shown that MOs can elicit off target effects, resulting in the activation of the p53 pathway and a "neural dead" phenotype (62). Therefore, to confirm that the neural phenotype that we observed in Marcksa and Marcksb deficient embryos was not due to off target effects of the morpholinos, we co-injected 2ng MAT and MBT with 4 ng p53 or control MOs and observed the phenotype at 24 hpf (Supplemental Fig. 3). When both MAT and MBT were co-injected with the control MO, the same neural phenotype (characterized by a lack of normal eye and

neural tissue architecture) was observed (Supplemental Fig. 3). When MAT or MBT were co-injected with the p53 MO, the neural phenotype was once again observed (Supplemental Fig. 3). These findings confirm that the neural phenotype observed in Marcksa and Marcksb deficient embryos are true phenotypes and not MO mediated artifacts. Further, it suggests that Marcks is involved in the neural development of zebrafish. Consistent with this result, MARCKS deficiencies in mice also result in neural abnormalities, including exencephaly resulting from a failure in neural tube closure (16, 18, 63). MARCKS deficiencies in non-exencephalic mice resulted in the failure of fusion of the cerebral hemispheres and agenesis of the corpus callosum and other forebrain commisures (16).

Histopathological analysis further revealed neural abnormalities in both Marcksa and Marcksb deficient zebrafish. MAT and MBT embryos were euthanized 72 hpf, fixed and routinely processed, and multiple sections stained with hematoxylin & eosin (H & E) were examined microscopically. The observed neural abnormality consists of irregular retinal layering in both Marcksa and Marcksb deficient embryos, whereas control injected and wild type embryos have normal retinas (Fig. 6). While the outermost layer, the retinal pigment epithelium, appears normal in Marcksa and Marcksb deficient zebrafish, the other layers essentially consist of a mass of poorly organized neural cell nuclei with no clear separation into normal retinal layers (Fig. 6B). In addition, the overall size of the eyes in these fish appears smaller. This data correlates with results that previously established MARCKS involvement in the histogenesis of the retina (16, 64). Specifically, it was found that the thick non-nuclear layer (layer of Chievitz) was absent in retinas of homozygous Marcks

deficient mice (16). Taken together, our data suggests that MARCKS plays a role in the neural development of zebrafish, specifically retinal histogenesis, a finding consistent with observations in other species. This data supports the use of zebrafish as a model to better understand the role of MARCKS in neural development.

Marcksa and Marcksb are required for gill formation and skeletal muscle cell morphology in developing zebrafish

Histopathology on 72 hpf Marcksa and Marcksb deficient zebrafish embryos also revealed abnormal gill formation. The gills in these specimens consist of jumbled layers of epithelial cells with no discernable separation into lamellar units that would normally be separated by a core of cartilage (Fig. 7 A and B). Abnormal gill formation could certainly contribute to the increased mortality in Marcksa and Marcksb deficient embryos. The role of MARCKS in respiratory physiology has been well studied as previous investigation has demonstrated that MARCKS is involved in mucin secretion in the respiratory epithelium (14, 65). However, to our knowledge, a role for MARCKS in respiratory development has yet to be demonstrated, making our observation of abnormal gill formation in MARCKS deficient zebrafish a novel finding. This finding also suggests the need for further research on the role of MARCKS in respiratory development of both mammalian and non-mammalian vertebrates.

Consistent with the curved or twisted tail phenotype, histopathology revealed that the skeletal muscle in the tails of Marcksa and Marcksb deficient zebrafish embryos contains

numerous curved/crescent-shaped fibers and plumper, more numerous nuclei as compared to control injected and wild type, non-injected embryos (Fig. 7 C and D). This abnormal morphology supports the argument that MARCKS may play a role during *in vivo* myogenesis. Interestingly, myocyte adhesion and spreading (9, 10) as well as migration (66) are associated with MARCKS function *in vitro*, as is fusion of embryonic myoblasts (23). Given these observations, zebrafish may be a suitable model for further studying the role of MARCKS in both respiratory and muscle development.

Conclusions

MARCKS has been identified as a protein involved in mammalian and *Xenopus* development and in the present study we demonstrate a role for MARCKS in zebrafish development. Zebrafish express two *MARCKS* genes, *marcksa* and *marcksb*; both of which are equally expressed throughout early development and have similar homology to *MARCKS* expressed by other vertebrate species. Knock down of Marcksa and Marcksb proteins in zebrafish results in increased mortality as well as several other developmental abnormalities. To our knowledge, we are the first to analyze the role of Marcks in zebrafish development. The role of MARCKS in the embryonic and neural development of other species has been previously examined (16, 21, 67, 68). Our results support this research and offers further evidence for the role of MARCKS in development biology.

Cell migration is critical to development biology, especially during the morphogenetic movements of gastrulation (42, 43, 69) and recent evidence has suggested a role for MARCKS in gastrulation (21). MARCKS function is also required for mesenchymal

and leukocyte cell migration. The growth hormone, platelet derived growth factor-BB (PDGF-BB) results in activation of PKCε, which in turn phosphorylates MARCKS leading to migration of hepatic stellate cells (26). MARCKS silencing is also associated with inhibition of vascular smooth muscle cell migration (70) and inhibition of calpain, a protease of which MARCKS is a substrate, leads to an accumulation of MARCKS and inhibition of myoblast migration (66, 71). We have demonstrated a role for the myristoylated amino-terminus of MARCKS in the regulation of neutrophil migration *in vitro* (25) and MARCKS has also been associated with the establishment of polarity of T lymphocytes (72). Taken together, these results indicate that MARCKS function is required for migration of diverse cell populations, which may explain the variety of developmental abnormalities observed in MARCKS deficient animals.

The experiments presented here describe a useful *in vivo* model to study MARCKS function. Zebrafish are a well established model for developmental biology and are becoming increasingly popular in other fields, including innate immunology (27, 28, 73). Given the results of these experiments, we now have a model to study MARCKS function *in vivo*, increasing our ability to better understand the mechanisms by which MARCKS acts.

Materials and Methods

Bioinformatics

Peptide sequences were aligned by Clustal W (74). For phylogenetic analysis, MARCKS and MARCKS-like protein sequences were aligned by Clustal W and neighbor joining trees were constructed from pairwise Poisson correlation distances with 2000 bootstrap replications with MEGA 4 software (75). Accession numbers for both the box shade alignment and phylogenetic tree are as follows: zebrafish Marcksa, GU563328; zebrafish Marcksb, GU563329; human MARCKS, NP_002347; mouse MARCKS, NP_032564; *Xenopus laevis* MARCKS, NP_001080075; chicken MARCKS, NP990811; salmon MARCKS, ACN11034; human MARCKS-like, NP_075385; mouse MARCKS-like, NP_034937; zebrafish Marcks-like, NP_998298; chicken MARCKS-like, NP_001074187.

For synteny analysis, genes flanking *marcksa* and *marcksb* were identified using the Entrez Gene database on the National Center for Biotechnology Information (NCBI) website. Homologous genes in human and mice were determined using the Homologene database on the NCBI website. The chromosomal location of the homologous genes in humans and mice were then determined by the Entrez Gene database.

Zebrafish maintenance and husbandry

Experiments were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC). Wild type, EKK adult zebrafish (EKKwill Waterlife Resources, Ruskin, FL) were maintained in a recirculating aquarium facility

(Aquatic Habitats, Apopka, FL) at 28˚ and were fed a commercial grade zebrafish diet. Zebrafish were mated and embryos were collected in egg water (0.005% Methylene blue and 60 µg/mL aquarium salt mixture).

mRNA isolation and RT-PCR

Twenty EKK embryos were collected at the indicated time and euthanized with 0.17% Tricaine methanesulfonate (Finquel MS-222, Argent Chemical Laboratories, Redmond, WA) and transferred to 1 mL RNA Later (Qiagen, Valencia, CA) and stored at -80˚C. Samples were thawed and transferred into RLT buffer (Qiagen) with β-mercaptoethanol added and homogenized with a pellet pestle (Kimble Kontes, Vineland, NJ) and passed through QIAshredder™ columns (Qiagen). Qiagen's RNeasy Protect mini kit was used to isolate mRNA following manufacturer's suggestions with an on column DNase digestion step (RNase-free DNase Set, Qiagen). Complementary DNA (cDNA) synthesis was performed as previously described (76).

The following primer (Integrated DNA Technologies, Coralville, IA) sequences and annealing temperatures were used for RT-PCR amplification of zebrafish *marcksa*, *marcksb* and *β-actin* (NM_131031): *marcksa* (annealing temperature 57.5˚C) forward primer 5' CAC AAA AAC AGC TGG AAA AG, reverse primer 5' ATC GCT TCT GTG TTT CCA TC; *marcksb* (annealing temperature 65.8˚C) forward primer 5'TCC AAA AAC GGA GCA AAA GAC GAG, reverse primer 5' TTC GCT GGA AGC TTC GGG CTT; *β-actin* (annealing temperature 57.5˚C) forward primer 5' GGA GAA GAT CTG GCA TCA CAC CTT CTA C, reverse primer 5' TGG TCT CGT GGA TAC CGC AAG ATT CCA T. The

optimal annealing temperature was determined for each primer pair by the temperature gradient aspect of the MyIQ thermocycler (Bio-Rad, Hercules, CA). Amplitaq Gold PCR master mix (Applied Biosystems, Foster City, CA) was used for both annealing temperature gradient PCR and RT-PCR and manufacturer's instructions were followed for a 40µL total PCR reaction. The thermal cycling conditions for RT-PCR are as follows: 94˚C for 10 minutes, 35 cycles of [94˚C for 15 sec, the appropriate annealing temperature for 60 sec, 72˚C for 2 min], and 72˚C for 10 min.

Morpholino injections and phenotypic observations

The following MOs were purchased from GeneTools (Philomath, OR): Marcksa translation blocking (MAT) 5' CTG TTT TTG TGA ATT GCG CTC CCA T; Marcksa upstream translation blocking (MATU) 5' AGC TCA ACA GAT CCC AAT ACA GAA C; Marcksb translation blocking (MBT) 5' TTT TGG AGA TTT GTG CTC CCA TGC T; Marcksb splice blocking (MBS) 5' ATA ATT AAA GTT ATT ACC TGC CCG T. Standard stocks of control and p53 MOs were purchased from GeneTools. MOs were suspended to100 mM with sterile water and these stock solutions were stored at room temperature. Prior to loading MOs into calibrated needles, MOs were diluted in 0.5% phenol red. Injected embryos were transferred to a petri dish containing egg water and incubated at 28˚C.

Embryos were dechorionated at 24 hpf using very fine forceps and phenotyped. For photomicroscopy, embryos were anesthetized in 0.017% Tricaine methanesulfonate and images were captured with a Nikon AZ100 microscope (Melville, NY) under bright field light.

Histopathology

Embryos were euthanized at 72 hpf with a 0.17% tricaine solution. Whole (dechorionated) embryos were then fixed in 10% neutral buffered formalin solution, routinely processed, embedded in paraffin, sectioned at 5µm, stained with hematoxylin and eosin (H & E) and examined via light microscopy.

Statistical Analysis

Statistical analysis was performed using Sigma Stat Software and a one-way ANOVA test. When a significant interaction was detected, a pairwise multiple comparison procedure (Fisher LSD method) was used to identify the source of the interaction. Significance was set at $P < 0.05$.

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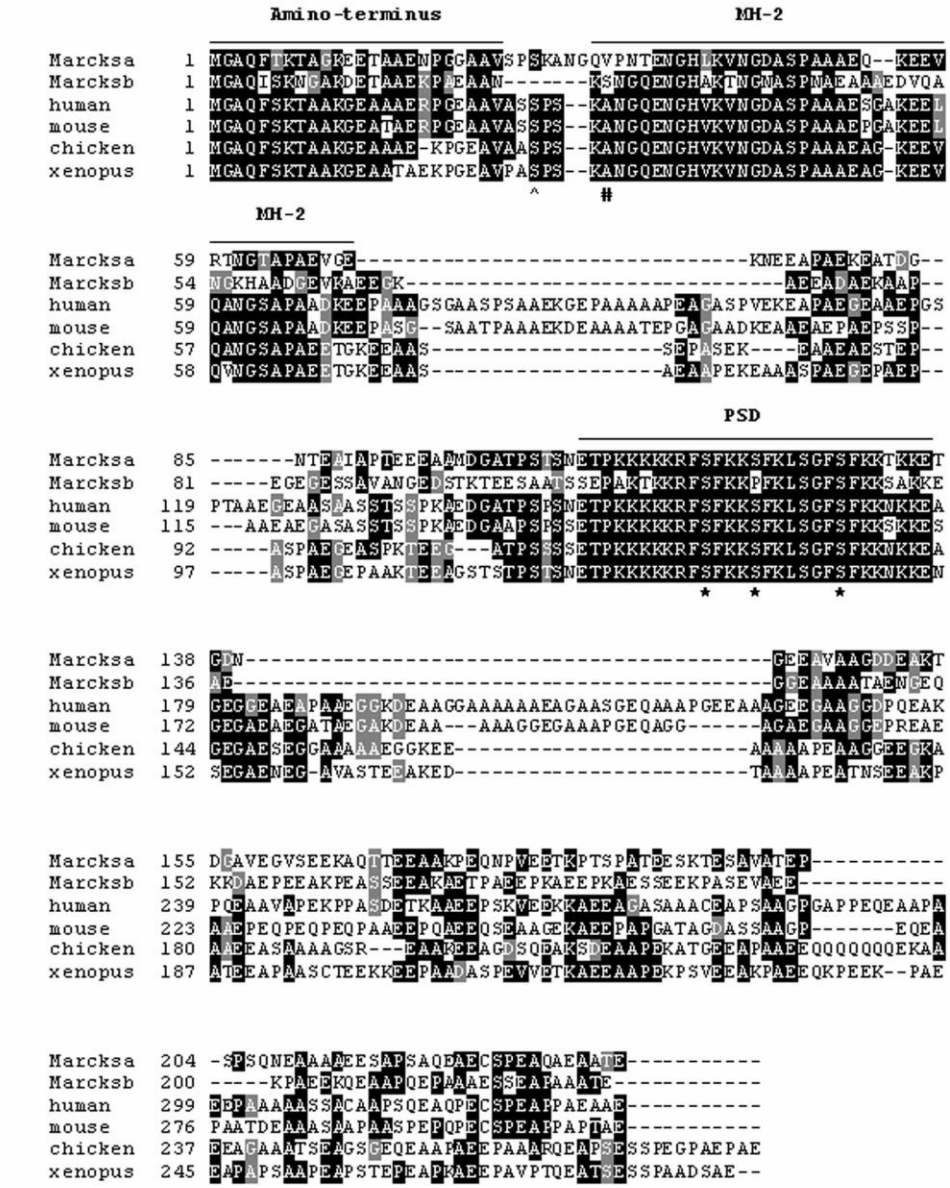
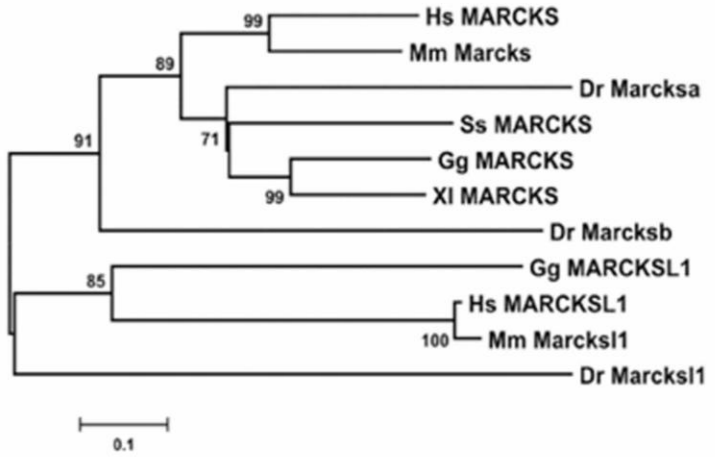
Sequence from this article have been deposited with the GenBank and ZFIN databases under accession numbers GU563328 and ZDB-GENE-050522-145 (*marcksa*), and GU563329 and ZDB-GENE-030131-1921 (*marcksb*).

Figure 1: Zebrafish Marcksa and Marcksb are similar to other vertebrate MARCKS sequences. (A) Zebrafish Marcksa and Marcksb protein sequences are aligned with MARCKS sequences from other vertebrate species. Black shading indicates identical amino acid alignment; gray shading indicates functionally similar amino acids. The three conserved domains of MARCKS are indicated: the myristoylated amino-terminus, the MH-2 domain and the phospho-site domain (PSD). The three serine residues phosphorylated by PKC are identified by an asterisk (*) and Ser^{25}, which is phosphorylated in neural tissue, is denoted by a carrot (^). The pound sign (#) denotes a candidate serine in Marcksb that may also be phosphorylated given its proximity to Ser^{25} in MARCKS expressed by other species. (B) Phylogenetic comparison of MARCKS and MARCKS-like protein sequences from vertebrate species. Branch lengths are measured in terms of amino acid substitutions with the scale indicated below the tree. (C) Zebrafish *marcksa* and *marcksb* loci both share conserved synteny with the MARCKS locus in human and mice. Genes flanking *marcksa* on chromosome 20 and *marcksb* on chromosome 17 were determined and the chromosomal location of orthologous genes in humans and mice were determined. Gray shaded areas indicate conserved synteny.

A.

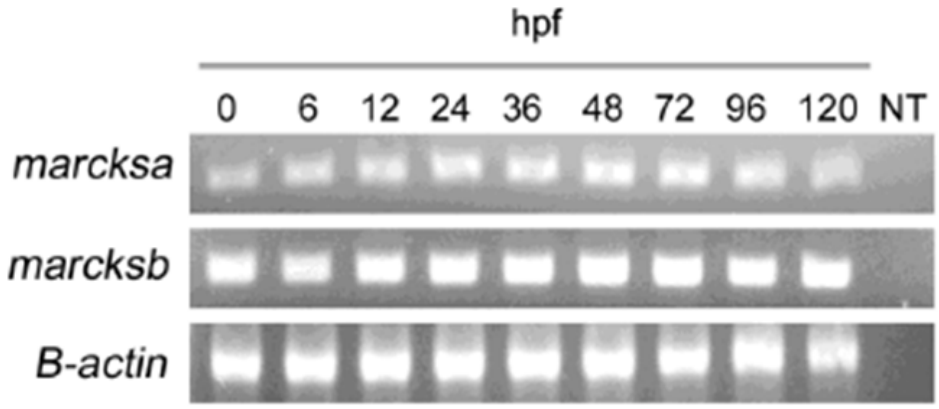

Amino-terminus
MH-2
MH-2
PSD
Marcksa
Marcksb
human
mouse
chicken
xenopus

B

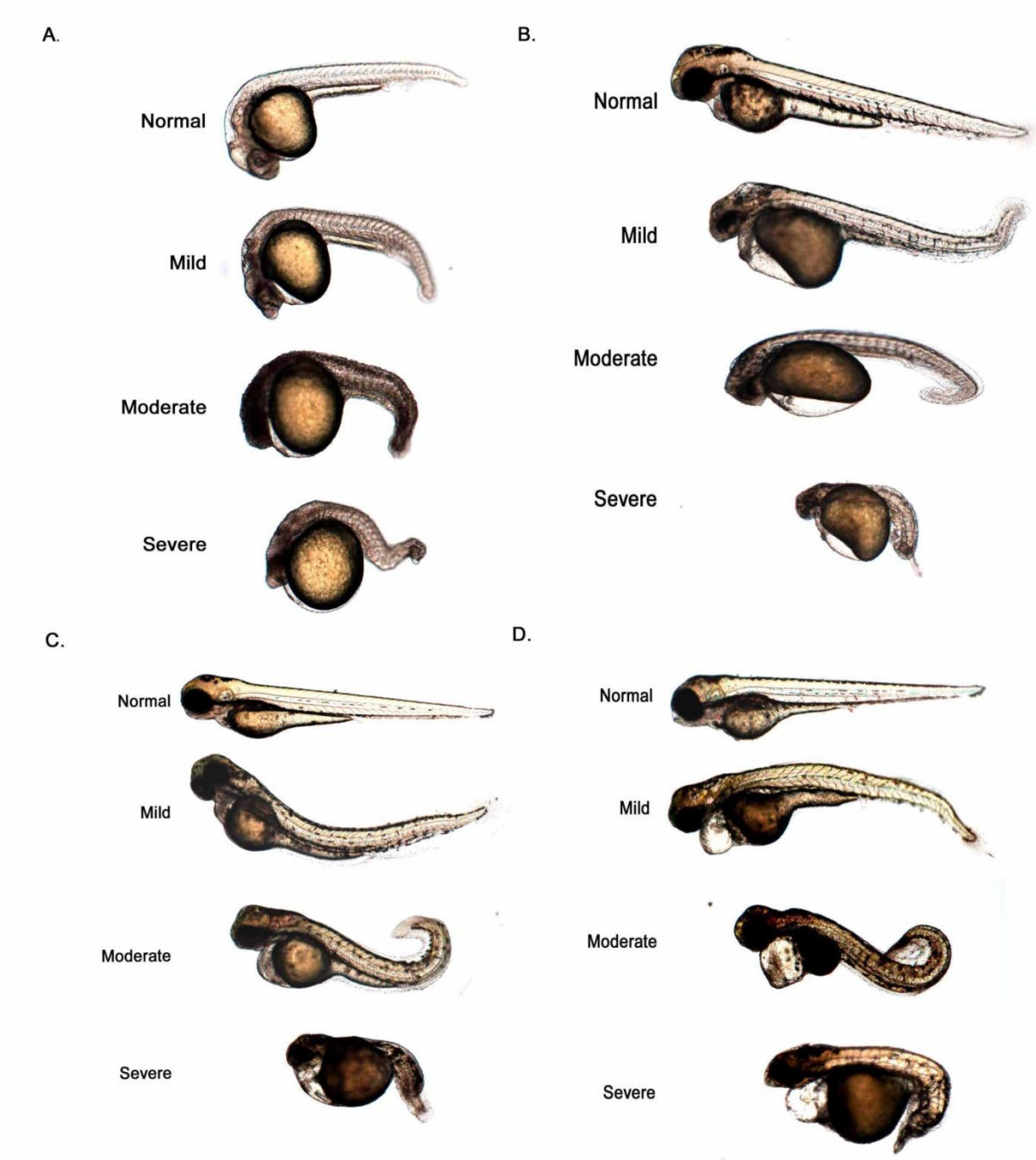


C.

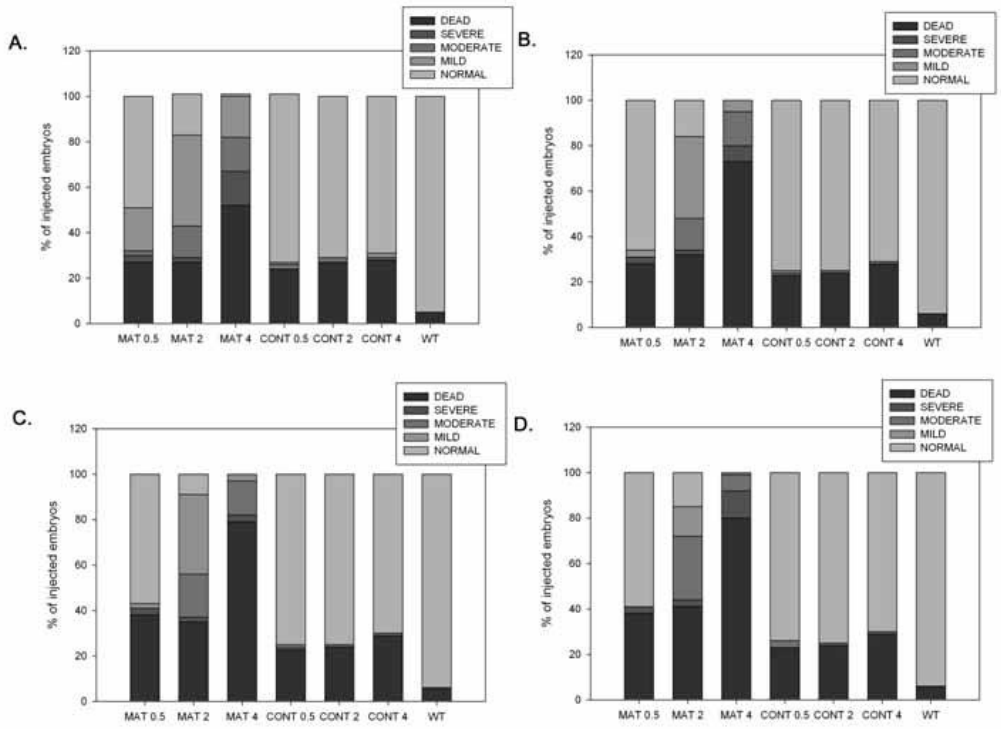
Zebrafish chromosome 20	Human		Mouse	
prpf39	*PRPF39*	14q21	*Prpf39*	12 C1
fau	*FAU*	11q13	*Fau*	19 A
zgc:77665	*ARF6*	14q21	*Arf6*	12 C2
zgc:153989	*C2orf56*	2p22	*2410091C18Rik*	17 E3
zgc:112104	*CEBPZ*	2p22	*Cebpz*	17 E3
marcksa (zgc:109978)	***MARCKS***	**6q22**	***Marcks***	**10 B1**
zgc:162161	*KIAA1919*	6q22	*2010001E11Rik*	10 B1
rev3l	*REV3L*	6q21	*Rev3l*	10 B1
zgc:171813	*ARV1*	1q42	*Arv1*	8 E2
LOC568246	*TTC13*	1q42	*Ttc13*	8E2
LOC568355	*CLEC11A*	19q13	*Clec11a*	7 B3-B5
Zgc:172112	*CNKSR3*	6q25	*Cnksr3*	10 A1
Zebrafish chromosome 17	**Human**		**Mouse**	
jkamp	*JKAMP*	14q23	*Jkamp*	12 C3
LOC792018	*VCAN*	5q14	*Vcan*	13 C3
xrcc4	*XRCC4*	5q13-q14	*Xrcc4*	13 C3
tmem167a	*TMEM167A*	5q14	*Tmem167*	13 C3
atg10	*ATG10*	5q14	*Atg10*	13 C3
marcksb (marcks)	***MARCKS***	**6q22**	***Marcks***	**10 B1**
col10a1	*COL10A1*	6q21-q22	*Col10a*	10
zgc:92375	*FHL2*	2q12-q14	*Fhl2*	1 B
LOC796410	*FUT9*	6q16	*Fut9*	4 A3
manea	*MANEA*	6q16	*Manea*	4 A3
cx52.7	*GJA10*	6q15-q16	*Gja10*	4 A5



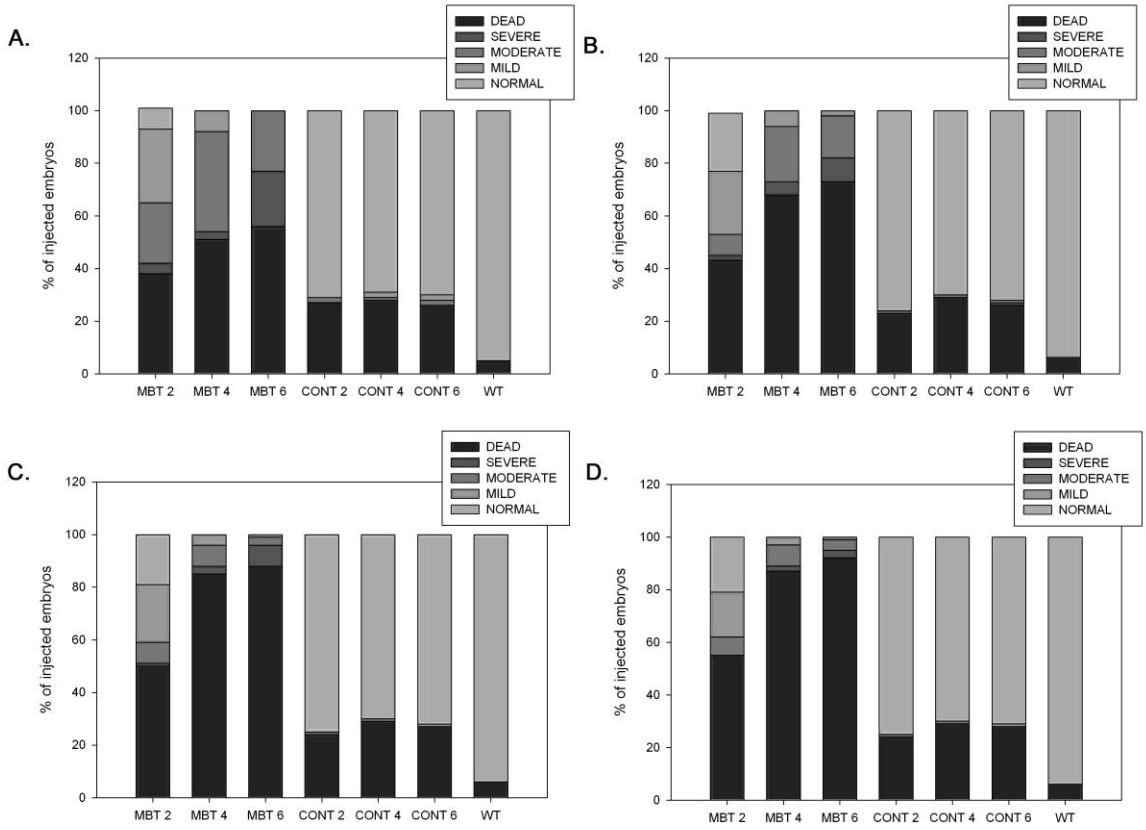
<u>Figure 2:</u> Zebrafish *marcksa* and *marcksb* are expressed throughout early development. Reverse transcriptase PCR was performed to detect zebrafish *marcksa, marcksb* and *β-actin* transcripts from embryo derived cDNAs. Age of embryos is indicated above each lane in hours post fertilization (hpf). Data is representative of 3 separate experiments and NT indicates no template control.



<u>Figure 3:</u> Phenotypic characterization of Marcksa and Marcksb deficient zebrafish morphants. Representative examples of normal, mild, moderate and severe phenotypes are depicted at 24 hpf (A), 48 hpf (B), 72 hpf (C) and 96 hpf (D).



<u>Figure 4:</u> Phenotypic quantification of zebrafish Marcksa deficient morphants. Embryos were injected at the single cell stage with 0.5, 2 or 4 ng of Marcksa translation blocking MO (MAT), control MO (CONT) or embryos were not injected (WT). Phenotypic characterization was then quantified at 24 hpf (A), 48 hpf (B), 72 hpf (C) and 96 hpf (D) and phenotypes were classified into five phenotypes: normal, mild, moderate, severe and dead. Data is represented as percentage of injected embryos, with pooled data from three separate experiments with a combined total of 120 embryos injected in the MAT groups, 110 embryos in the CONT groups and 120 embryos in the wild type (non-injected) group.



<u>Figure 5:</u> Phenotypic quantification of zebrafish Marcksb deficient morphants. Embryos were injected at the single cell stage with 2, 4 or 6 ng of Marcksb translation blocking MO (MBT), control MO (CONT) or embryos were not injected (WT). Phenotypic characterization was then quantified at 24 hpf (A), 48 hpf (B), 72 hpf (C) and 96 hpf (D) and phenotypes were classified into five phenotypes: normal, mild, moderate, severe and dead. Data is represented as percentage of injected embryos, with pooled data from three separate experiments with a combined total of 120 embryos injected in the MAT groups, 110 embryos in the CONT groups and 120 embryos in the wild type (non-injected) group.

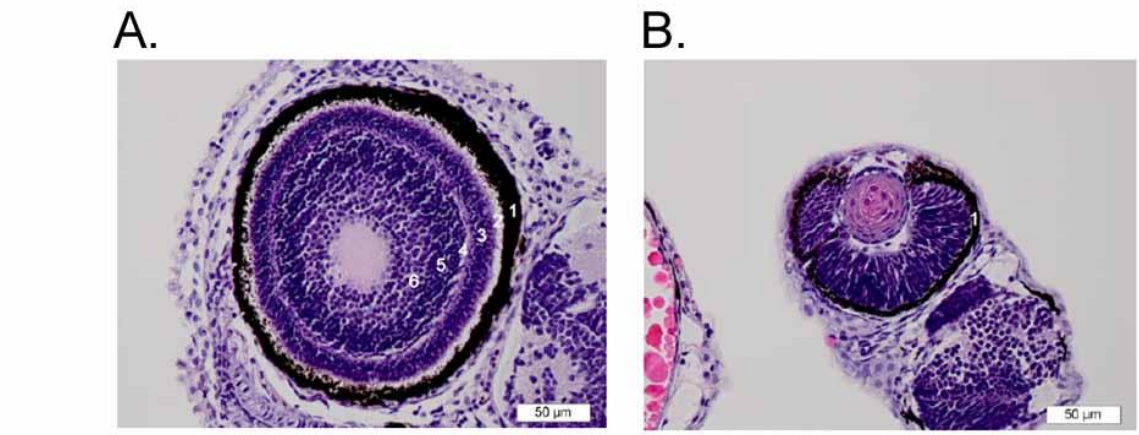


Figure 6: MARCKS is involved in retinal histogenesis of zebrafish. (A) Normal eye from 72 hpf wild type zebrafish embryo. Here, the layers of the retina are relatively well defined. Starting from the outermost layer and working toward the center of the eye: 1) the dark brown/black retinal pigment epithelium; 2) layer of rods and cones; 3) outer nuclear layer (dark blue/purple); 4) outer plexiform layer; 5) inner nuclear layer; 6) inner plexiform layer. The ganglion cell and nerve fiber layers are less distinct in this section. The plexiform layers consist mainly of synapses of the various sensory neural cells. (B) Eye from 72 hpf MAT injected zebrafish embryo (moderate phenotype). The retina consists of a mass of poorly organized neural cell nuclei with no clear separation into normal retinal layers. Only the retinal pigment epithelium forms a distinct layer (1). This morphology is consistent in both the MAT and MBT injected fish, and is most severe in the severe phenotype fish (data not shown).

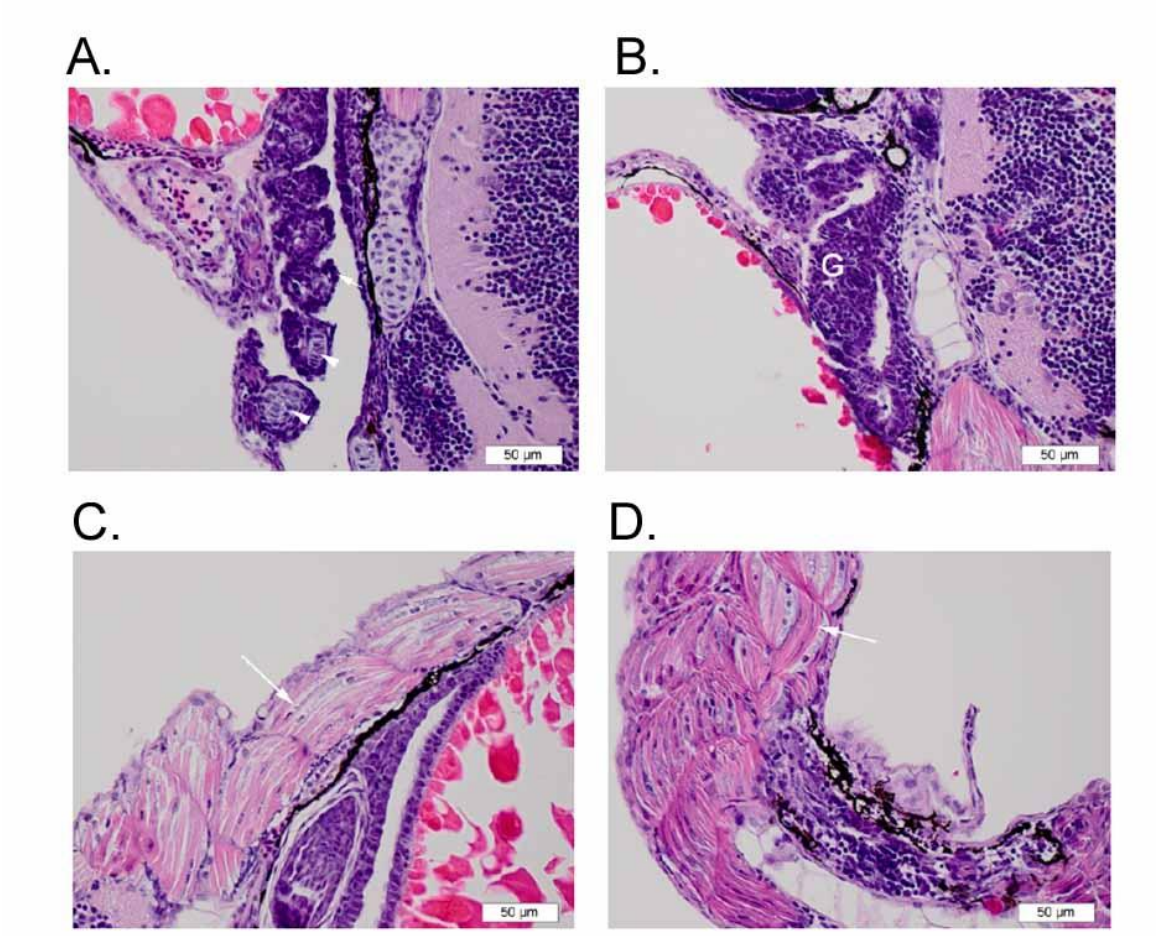
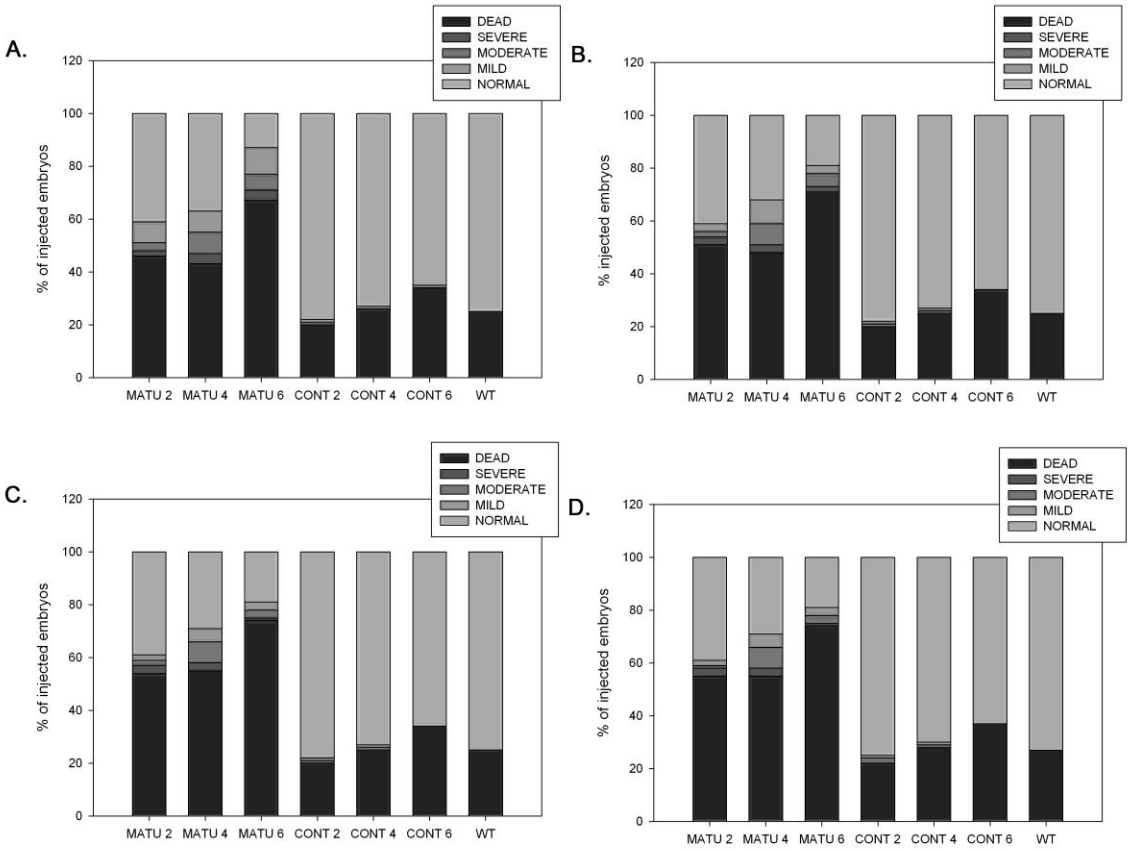
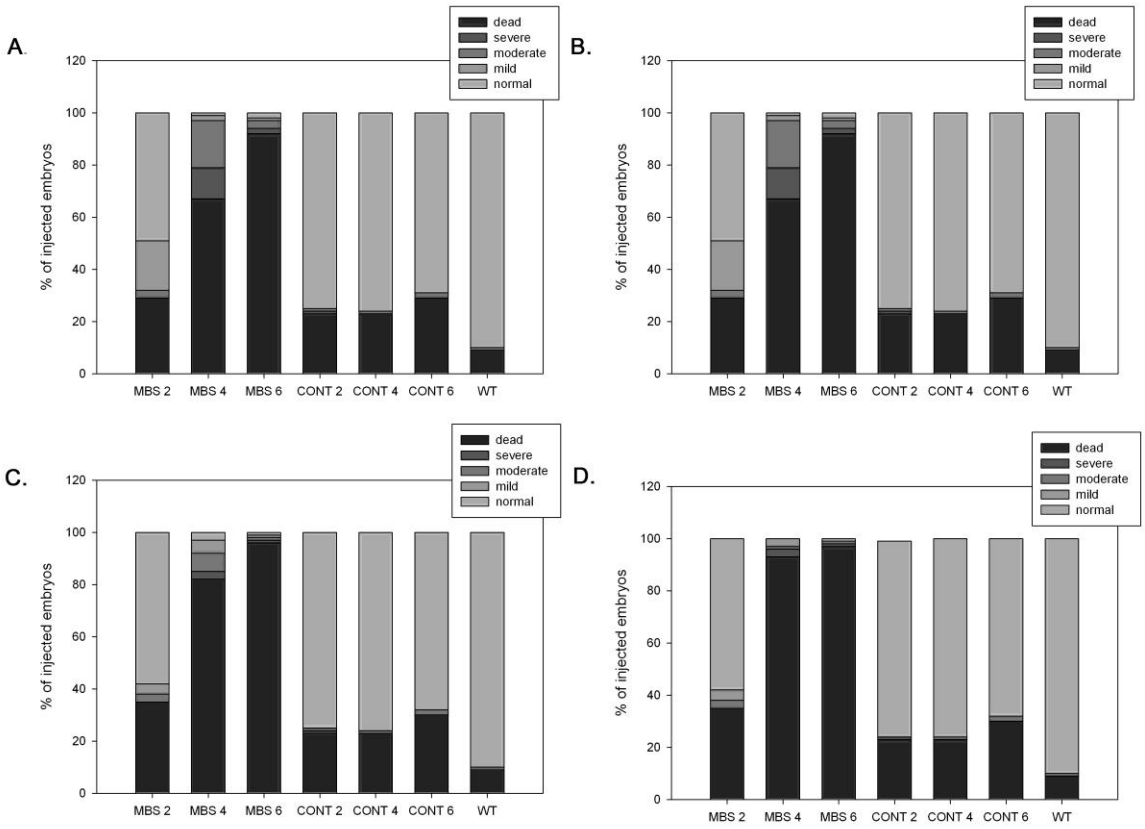


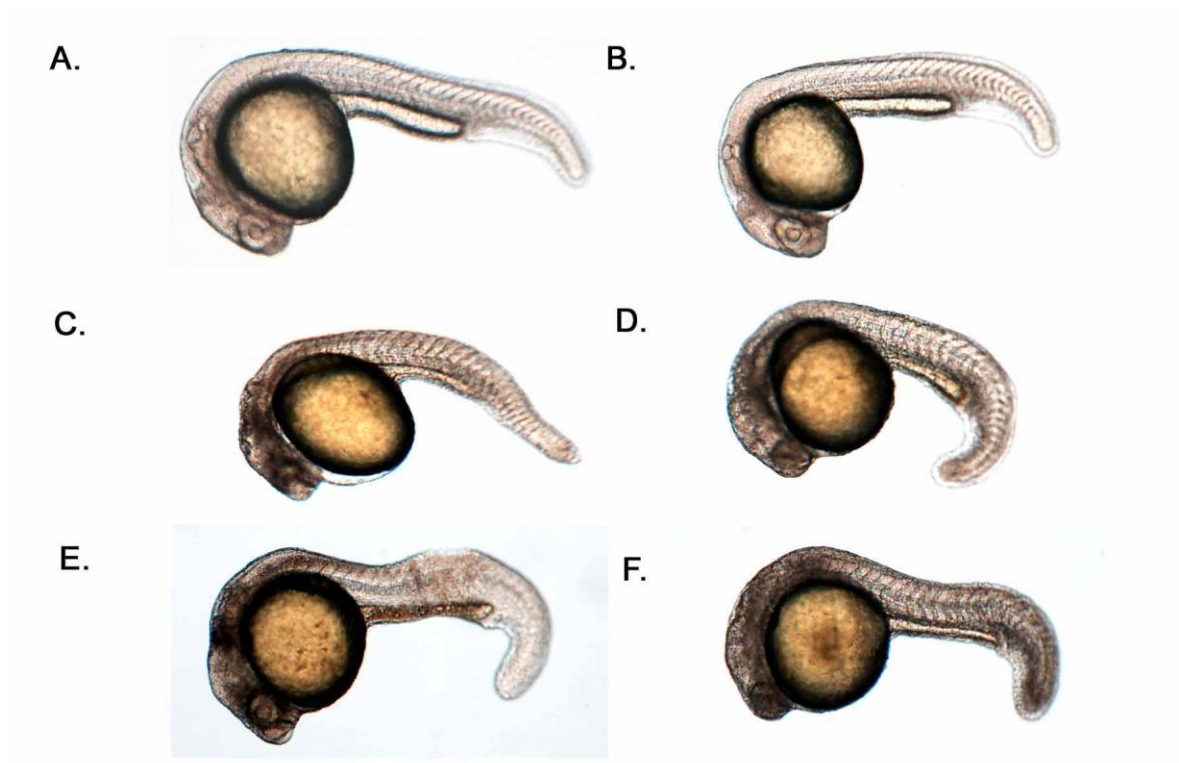
Figure 7: MARCKS deficiencies in zebrafish result in abnormal gill development and muscle cell morphology. (A) Normal gill tissue from 72 hpf wild type zebrafish embryo. The primary lamellae (filaments) of the developing gill are shown in cross section, with normal supporting chondrocytes (arrow heads) at the center and a covering of squamous epithelial cells (arrow). (B) Gill tissue (G) from 72 hpf MAT injected zebrafish embryo (moderate phenotype). In this section, the gill is comprised of a mass of numerous, poorly organized epithelial cells with no clear separation into primary lamellae and no discernable central supporting chrondrocytes. This morphology is consistent in both MAT and MBT injected fish, and is most severe in the severe phenotype fish (data not shown). (C) Skeletal muscle fibers (arrow) from the tail of 72 hpf control MO injected zebrafish are relatively straight, with thin, regularly spaced nuclei. (D) Skeletal muscle from the tail of 72 hpf MAT injected zebrafish (moderate phenotype) contains numerous curved/crescent-shaped fibers (arrow) and plumper, more numerous nuclei as compared to control and wild type fish. This morphology is consistent in both the MAT and MBT fish, and is most severe in the severe phenotype fish (data not shown).



<u>Supplemental Figure 1:</u> Phenotypic quantification of Marcksa upstream translation blocking (MATU) MO injected zebrafish embryos. Embryos were injected in the single cell stage with 2, 4 or 6 ng of MATU, control MO (CONT) or embryos were not injected (WT). Phenotypic characterization was then quantified at 24 hpf (A), 48 hpf (B), 72 hpf (C) and 96 hpf (D) and phenotypes were classified into 5 phenotypes: normal, mild, moderate, severe and dead. Data is represented as % of injected embryos, with pooled data from three separate experiments with a combined total of 110 embryos injected in the MAT groups, 98 embryos in the CONT groups and 107 embryos in the wild type (non-injected) group.



<u>Supplemental figure 2:</u> Phenotypic quantification of Marcksb splice blocking (MBS) MO injected zebrafish embryos. Embryos were injected in the single cell stage with 2, 4 or 6 ng of MBS, control MO (CONT)or embryos were not injected (WT). Phenotypic characterization was then quantified at 24 hpf (A), 48 hpf (B), 72 hpf (C) and 96 hpf (D) and phenotypes were classified into 5 phenotypes: normal, mild, moderate, severe and dead. Data is represented as % of injected embryos, with pooled data from three separate experiments with a combined total of 120 embryos injected in the MAT groups, 110 embryos in the CONT groups and 120 embryos in the wild type (non-injected) group.



<u>Supplemental figure 3</u>: Marcksa and Marcksb are involved in normal zebrafish neural development. Embryos were co-injected in the single cell stage with MAT or MBT MOs along with p53 or control MOs. Embryos were analyzed at 24 hpf for the presence of the neural dead phenotype. Representative embryos are shown; wild type, non-injected (A), 4 ng p53 MO + 2 ng control MO (B), 2 ng MBT + 4 ng p53 (C), 2 ng MAT + 4 ng p53 (D), 2 ng MBT + 4 ng control MO (E), 2 ng MAT + 4 ng control MO (F).

Supplemental Table 1: Phenotypic characterization of Marcksa and Marcksb MO injected zebrafish morphants. MBT, MAT, control and wild type non-injected zebrafish embryos were into one of three groups; dead, normal and abnormal, which is the sum of the mild, moderate and severe phenotypes at the indicated time point. Data is shown for all four timepoints examined and data is represented as average ± standard deviation. Statistical significance is set at ($p<0.05$) with * denoting a significant increase relative to wild type, † denoting a significant increase relative to control MO injected, ^{n} denoting a significant decrease relative to wild type and ‡denoting a significant decrease relative to control MO injected.

		24 hpf			48 hpf		
MO	**ng**	**Dead**	**Normal**	**Abnormal**	**Dead**	**Normal**	**abnormal**
MBT	0.5	10.67±3.51	19.67±9.45^{n}	9.67±6.35	11.00±3.00	26.67±5.03	2.33±2.10
	2	10.67±2.08	7.00±7.55^{n}‡	22.33±8.08*†	12.67±2.53	6.67±6.11^{n}‡	20.67±8.62*†
	4	20.67±9.02*	0.33±0.58^{n}‡	19.00±8.54*†	29.00±11.53*†	0.00±0.00^{n}‡	11.00±11.53*†
MAT	2	15.33±9.87*	3.00±5.20^{n}‡	18.67±1.15*†	17.33±9.30*	8.67±8.08^{n}‡	13.33±4.73*†
	4	20.33±11.24*	0.00±0.00^{n}‡	19.67±11.24*†	27.00±7.00*†	0.00±0.00^{n}‡	13.00±7.00*†
	6	22.33±9.61*†	0.00±0.00^{n}‡	17.67±9.61*†	29.00±5.30*†	0.00±0.00^{n}‡	11.00±5.30*†
CONT	0.5	8.67±1.53	27.67±7.51	0.00±1.00	8.33±3.06	27.33±7.51	0.00±1.00
	2	10.00±7.00	27.33±8.34	0.67±1.15	8.67±8.15	27.67±8.33	0.33±0.58
	4	10.33±6.66	25.33±10.41	1.00±1.00	10.67±7.10	26.00±10.15	0.33±0.58
	6	9.67±4.73	25.67±7.37	1.33±1.16	9.67±4.73	26.33±6.66	0.67±1.15
WT	-	1.67±2.89	35.00±5.00	0.00±0.00	2.33±2.52	34.33±4.04	0.00±0.00

Supplemental Table 1 Continued

		72 hpf			96 hpf		
MO	**ng**	**Dead**	**Normal**	**Abnormal**	**Dead**	**Normal**	**abnormal**
MBT	0.5	15.33±8.08*	22.67±9.02	2.00±2.00	15.33±8.08*	23.33±8.08	1.33±2.31
	2	14.00±4.58	3.67±4.73[n]‡	22.33±9.29*†	16.33±3.06*	6.00±5.20[n]‡	17.67±5.51*†
	4	31.67±11.15*†	0.00±0.00[n]‡	8.33±11.15	32.33±10.79*†	0.00±0.00[n]‡	7.67±10.79*
MAT	2	20.00±12.12*	8.00±7.21[n]‡	12.00±6.08*†	22.00±13.12*†	8.67±6.81[n]‡	9.33±7.51*†
	4	34.00±3.61*†	0.00±0.00[n]‡	6.00±3.60	34.67±2.90*†	0.00±0.00[n]‡	5.33±2.89
	6	35.33±2.08*†	0.00±0.00[n]‡	4.67±2.08	37.00±1.73*†	0.00±0.00[n]‡	3.00±1.73
CONT	0.5	8.33±3.06	27.33±7.51	0.00±1.00	8.33±3.06	27.33±7.51	0.00±1.00
	2	8.67±8.15	27.67±8.33	1.00±1.73	8.67±8.15	27.67±8.33	0.33±0.58
	4	10.67±7.10	25.67±10.26	0.33±0.58	10.67±7.10	25.67±10.26	0.33±0.58
	6	10.00±5.29	26.33±6.66	0.33±0.58	10.33±5.86	26.00±6.93	0.33±0.58
WT	-	2.33±2.52	34.33±4.04	0.00±0.00	2.33±2.52	34.33±4.04	0.00±0.00

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CHAPTER 4

THE ROLE OF MARCKS DURING *IN VIVO* NEUTROPHIL MIGRATION IN ZEBRAFISH

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Abstract

Neutrophilic inflammation is involved in the pathophysiology of various inflammatory diseases and inhibiting the migration of neutrophils has been the focus of many therapeutic stratagies. Previously, we have identified the protein kinase C (PKC) substrate and actin binding protein myristoylated alanine-rich C-kinase substrate (MARCKS) as a potential target for the treatment of neutrophilic inflammation, as inhibition of MARCKS function results in decreased neutrophil migration and adhesion *in vitro*. In the present study we hypothesize that MARCKS is involved in the regulation of neutrophil migration *in vivo* and utilized the transparent zebrafish model to test our hypothesis. Previous research has revealed that zebrafish express two MARCKS genes, *marcksa* and *marcksb*, and herein we utilize a morpholino approach to knockdown Marcksa or Marcksb in transgenic MPO:GFP zebrafish, in which the neutrophils express GFP. Our results show that both Marcksa and Marcksb are involved in the development of neutrophils as Marcksa or Marcksb deficient embryos have decreased neutrophil numbers relative to control animals. Further, employment of an established tail wounding assay to study neutrophil migration reveal that Marcksb appears to be involved in the regulation of neutrophil migration *in vivo*, as the number of neutrophils within the vicinity of a tail wound in Marcksb deficient embryos were reduced relative to control animals. However, neutrophils from Marcksb deficient zebrafish were capable of migrating the same total distance and velocity with a slight decrease in the persistence of migration. Thus, these results demonstrate a role for MARCKS during *in vivo*

neutrophil migration and indicate that further investigation of the role of MARCKS during *in vivo* neutrophil migration is warranted.

Introduction

Neutrophils are essential components of the immune response. They are the first cell type to respond to a bacterial invasion by efficiently migrating to the source of inflammation and killing bacteria by phagocytosis and release of enzymes and reactive oxygen species. However, during various inflammatory disorders, such as Crohn's disease and chronic obstructive pulmonary disorder (COPD), exacerbated neutrophilic inflammation has been reported (1-3). Thus, neutrophil migration has been the focus of many therapeutic stratagies for the treatment of inflammatory disorders.

One potential target for treatment of neutrophilic inflammation is myristoylated alanine-rich C-kinase substrate (MARCKS). MARCKS is a ubiquitously expressed actin binding substrate of protein kinase C (PKC) and calmodulin. PKC mediated phosphorylation or calmodulin binding of MARCKS results in the dislocation of MARCKS from the plasma membrane to the cytosol and this translocation event is associated with the regulation of actin dynamics (4-8). Thus, MARCKS function has been shown to regulate endo-, exo-, and phagocytosis, as well as cell migration (9-13).

Previously, we utilized a myristoylated peptide corresponding to the first 24 amino acids of MARCKS (MANS peptide) to demonstrate a role for MARCKS during *in vitro* neutrophil function. MANS peptide treatment displaces MARCKS from cell membranes of unstimulated neutrophils and results in decreased neutrophil migration and β_2-integrin dependent adhesion (14) as well as myeloperoxidase release (15). Further, *in vitro* and *in*

vivo mucin secretion (16-18) as well as mesenchymal stem cell chemotaxis (19) have been inhibited upon MANS peptide treatment. These findings ultimately demonstrate that MARCKS is an excellent candidate for a therapeutic target for the treatment of various inflammatory diseases involving exacerbated neutrophil inflammation.

Since our previous studies demonstrating a role for MARCKS during neutrophil migration were *in vitro* studies, our objective is to confirm our results in an *in vivo* model. Various neutrophil motility studies have been performed in zebrafish (20, 21) and recently two transgenic zebrafish lines have been generated in which green fluorescent protein (GFP) expression is driven by the zebrafish myeoloperoxidase (MPO) promoter (22, 23). One transgenic line, *Tg(zMPO:GFP)*^{uw}, developed by Huttenlocher and colleages, express GFP in both neutrophils (GFP^{hi}) and macrophages (GFP^{dim}) (22, 24). The other transgenic line, *Tg(mpx:eGFP)*^{i114}, which is a neutrophil specific transgenic line, was developed by Renshaw and colleages (23, 25). Both of the *MPO:GFP* zebrafish lines have been utilized to study apoptosis (23, 26) and retrograde chemotaxis (22) during the resolution of neutrophilic inflammation as well as phosphoinositide 3-kinase (PI3K) and Rac signaling in the regulation of neutrophil protrusion and polarity (27). Further, *MPO:GFP* zebrafish have been utilized to study neutrophil migration during various disease models. Crossing *Tg(MPO:GFP)*^{uw} fish with *spint1l*^{hi227}*/spng1l*$^{+}$ (mutated hepatocyte growth factor activator inhibitor 1 (*hai1*)) fish, which is a transgenic model for chronic inflammatory disorders and results in the accumulation of neutrophils in fins, demonstrate that neutrophils undergo random motility with alternating stationary and motile periods during chronic inflammation (28). Thus,

researchers are beginning to employ transgenic *MPO:GFP* lines to understand the molecular events of neutrophilic inflammation in various diseases, including respiratory diseases (25, 29).

Zebrafish express two MARCKS paralogs, Marcksa and Marcksb, and we have previously characterized the phenotype of developing zebrafish deficient in either Marcksa or Marcksb by a morpholino approach. MARCKS deficient zebrafish have a range of abnormalities; including an array of anatomical abnormalities with atypical muscle cell morphology as well as abnormal retinal layering and gill formation (Laura Ott, PhD thesis, Chapter 3). Interestingly, the abnormalities observed in MARCKS deficient zebrafish correlate with MARCKS deficiencies observed in other vertebrate species. MARCKS deficiencies in *Xenopus laevis* are associated with abnormal convergent extension (30) and homozygous MARCKS deficiencies in mice are embryonic lethal (31). Deficiencies in retinal layering have been reported in MARCKS deficient mice and chick embryos (31, 32) as well as an array of neural abnormalities (31, 33, 34).

Thus, taking advantage of the model we have generated to study MARCKS function in zebrafish we hypothesized that Marcksa and/or Marcksb deficient zebrafish will have reduced neutrophil migration (22, 35). For our studies, we chose to utilize and established tail wounding model in the neutrophil specific *Tg(mpx:eGFP)*^{i114} zebrafish line (23), which will be referred to herein as *MPO:GFP*. The results of these studies will not only provide *in vivo* evidence of MARCKS regulation of neutrophil migration, but will also identify

MARCKS as a potential therapeutic target for the treatment of exacerbated neutrophil migration during chronic inflammatory disorders.

Results

Our previous studies revealed that MARCKS deficiencies in zebrafish result in an array of phenotypic abnormalities, including neural and anatomical abnormalities. To further characterize the phenotype of MARCKS deficient zebrafish, we sought to determine if MARCKS deficiencies affect the total number of neutrophils in 3 day post fertilization (dpf) embryos. Therefore, we determined the number of GFP+ cells in *MPO:GFP* embryos that were injected with *marcksa* or *marcksb* translation blocking morpholinos (MAT and MBT, respectively) by flow cytometry. First, wefound that embryos injected with 2 ng MAT or MBT results in a significantly reduced number of neutrophils compared to 2 ng control injected and non-injected embryos (Figure 1A). Since 2 ng of MAT and MBT resulted in significantly reduced neutrophil numbers, we asked if decreasing the amount of morpholino (MO) injected has the same effect. Like 2 ng MO, injection of 1 ng MAT or MBT results in significantly reduced number of neutrophils compared to 1 ng control injected and non-injected embryos (Figure 1B). Interestingly, when 0.5 ng MBT was injected, we observed a significantly reduced number of neutrophils compared to 0.5 ng control injected and non-injected embryos (Figure 1C). However, we did not observe a difference between 0.5 ng MAT, 0.5 ng control injected or non-injected embryos (Figure 1C). Thus, MARCKS

deficiencies in zebrafish result in a decreased number of neutrophils in zebrafish embryos, although very low concentrations of MAT had no effect.

Next, we performed a zebrafish tail wounding assay to determine if MARCKS deficiencies in zebrafish result in altered neutrophil migration. We wounded the tails of 3 dpf *MPO:GFP* embryos that were either not injected or injected with 0.5, 1 and 2 ng of MAT, MBT or control MOs. We then counted the number of cells within 100 μm of the tail wound initially after wounding and 4 hours later. As shown in figure 2, an increase in the number of neutrophils that migrated within 100 μm of the tail wound was observed for all treatment groups. However, the number of cells that migrate within the vicinity of the wound at 4 hours post wounding was significantly decreased in all three doses of MBT injected embryos relative to both non-injected and respective control injected embryos (Figure 2B). Further, the number of cells that migrated into the vicinity of the wound in the 2 ng MAT injected embryos were statistically decreased relative to 2 ng control injected embryos, although there was not a statistical difference between 2 ng MAT injected and non-injected embryos (Figure 2B). Interestingly, migration observed after treatment with the lower concentrations of MAT (1 and 0.5 ng) were not statistically different from non-injected or 1 and 0.5 ng control injected embryos (Figure 2B). Thus, this data suggests that MARCKS deficiencies in zebrafish do result in decreased neutrophil migration *in vivo*, especially in Marcksb deficient embryos.

Given that we observed decreased numbers of total neutrophils in MAT and MBT injected embryos (Figure 1), we wanted to confirm that the observations made in our 4 hour

tail wounding experiment (Figure 2) were not an artifact of decreased neutrophil numbers in these embryos. Therefore, we performed single cell tracking on 3 dpf *MPO:GFP* embryos that were either non-injected or injected with 2 ng MAT, MBT or control MOs. From the single cell tracking data, we quantified three parameters of cell migration: total distance (μm), velocity (μm/sec) and persistence, which we defined as the ratio of the distance of the cell from the origin at the last frame captured over total distance traveled by the cell. This persistence measurement ultimately determines the directionality of the cell; cells migrating away from its original origin have a higher persistence than cells remaining in the vicinity of its original orgin. We did not observe a difference in total distance or velocity between any of the groups (Figures 3 A and B), signifying that cells from MARCKS deficient embryos are capable of migrating. However, we did observe decreased persistence in MBT injected embryos, although this decrease was not statistically significant (MAT versus non-injected and control injected embryos results in p-values of 0.080 and 0.134, respectively) (Figure 3C). We did not observe a difference in persistence between MAT injected, control injected or non-injected embryos (Figure 3C). Thus, MARCKS deficient embryos are capable of migrating, although Marcksb appears to potentially play a role in mediating neutrophil directionality during migration. Further experimentation is warranted to confirm this result.

Discussion

Neutrophil function is pivotal to the process of inflammation, and in cases of chronic inflammation targeting neutrophil function is a potential therapeutic option. Previously we have demonstrated an *in vitro* role for MARCKS in regulating neutrophil function (14, 15) and herein we present preliminary evidence of MARCKS function in the regulation of neutrophilic inflammation *in vivo*. Zebrafish deficient in Marcksa or Marcksb have decreased neutrophils relative to control animals (Figure 1) and Marcksa or Marcksb deficient embryos have decreased neutrophil migration in a 4 hour tail wounding assay (Figure 2). However, individual cell tracking experiments reveal that in Marcksa or Marcksb deficient animals, neutrophils migrate with the same total distance, velocity and persistence as control injected or non-injected animals, although there was a trend for decreased persistence in Marcksb deficient embryos (Figure 3).

We utilized a morpholino approach to determine the role of Marcksa and Marcksb in the regulation of neutrophil migration *in vivo*. Although morpholinos are a well accepted method to study protein function, there are caveats to their use. The first is that morpholino knock down of gene expression occurs throughout the animal. Traditionally, morpholinos have been utilized by developmental biologists to determine what role a particular protein plays in mediating the normal development of an organism (36). However, for our studies, knockdown of Marcksa and Marcksb throughout the animal does not rule out that other tissues affected by MARCKS knockdown are involved in the observed results. For instance,

we observed decreased neutrophil numbers in Marcksa and Marcksb embryos compared to control animals (Figure 1) with two potential explanations for these results. The first and most likely explanation is that MARCKS deficient zebrafish are phenotypically abnormal and thus the various tissues involved in neutrophil development are either damaged and/or developmentally delayed. Previously, we have characterized the phenotype of Marcksa and Marcksb deficient zebrafish embryos and found various anatomical abnormalities, including curved or twisted tails and abnormal muscle cell development. During zebrafish development MPO-expressing cells are detected as early as 18 hours post fertilization (hpf) in the posterior intermediate cell mass (ICM) and within 20 hpf MPO expressing cells are present in the anterior yolk sac. At 72 hpf, MPO-expressing cells are observed in the posterior ICM, anterior yolk sac and in posterior blood islands and by 96 hpf, MPO-expressing cells are distributed throughout the animal (37). Further evidence has indicated that development of neutrophils in zebrafish beyond 2 dpf occurs within the caudal hematopoietic tissue (38). Since many of the areas that are involved in neutrophil development occur in the tail of developing zebrafish, it could be hypothesized that these tissues are incapable of supporting neutrophil development in MARCKS deficient zebrafish. Although we used normal to mild embryos for our studies herein, we cannot rule out that the slightest abnormalities in these animals that are not observed in our phenotypic analysis affect neutrophil development.

An additional possible explanation for decreased number of neutrophils in Marcksa and Marcksb deficient embryos is that the neutrophils are incapable of migrating to the

various locations involved in neutrophil development. We have previously demonstrated a role for MARCKS in regulating neutrophil migration *in vitro* (14) and a role for MARCKS in the regulation of migration in other cell types have also been reported (9, 10, 19). However, our migration analysis in zebrafish embryos does not support this hypothesis, as we did not observe statistical differences in total distance, velocity or persistence in Marcksa or Marcksb deficient embryos. This observation brings up caveat two of morpholino usage; the dosage of morpholino used in experiments. We chose the maximum MO dose of 2 ng for this study based on our phenotypic analysis showing that injection of 2 ng MO resulted in the majority of normal to mild phenotype animals that survive to 3 dpf in comparison to higher MO doses (Laura Ott, PhD thesis, Chapter 3). We chose to utilize the lowest dose of MO possible that consistently results in predominantly mild or normal phenotypes to reduce any effects that MARCKS knockdown has on the entire animal, while still having an effect on neutrophil migration. However, the 2 ng dose of MO that we utilized for these studies are relatively low with many other studies utilizing higher concentrations of MO (5-6ng) and thus the knockdown rate in our experiments is likely to be small (36, 39). Utilizing a higher dose of morpholino for our studies may have resulted in greater inhibition of neutrophil migration in Marcksa and Marcksb deficient animals. Figures 2 and 3 provide evidence for this, in that we did not observe a difference in neutrophil migration between Marcksa deficient and non-injected or control embryos and thus to observe inhibited neutrophil migration in Marcksa deficient zebrafish may require a higher dosage of morpholino. Higher doses of morpholino, however, would also result in more severe phenotypes in our animals, potentially leading to

an even further reduction in neutrophil numbers. Higher doses of morpholino would also raise further concerns of off-target effects (36, 39) or if the inhibition of neutrophil migration was due to effects on the neutrophil itself or to abnormalities in the surrounding tissue. However, a few recent technologic advances resulting in cell specific gene disruption have been reported, including: generation of caged compounds (40-42), generation of stable cell specific short hairpin RNA (shRNA) transgenic zebrafish lines (46) and generation of transient transgenic zebrafish embryos by utilizing the Tol2 transposon system (47).

Caged compounds are morpholinos, DNA or RNA generated with ultraviolet (UV) sensitive caging groups attached. There have been multiple types of caged morpholinos designed, with the technology behind them being that they remain inactive in the zebrafish embryo until the embryos are exposed to ultraviolet (UV) light, resulting in activation of the morpholino (40-42). For our purposes, one approach would be to generate caged morpholinos against Marcksa or Marcksb and inject them in the single cell stage in the absence of light. Once embryos have developed to 24-48 hpf, we would uncage the morpholino by exposure to UV light and perform neutrophil migration experiments at 72 hpf. Theoretically, this would allow the embryos to develop normally and would eliminate any of the concerns that developmentally abnormal phenotypes may play on neutrophil migration or function. However, uncaging the morpholino would result in MARCKS knockdown throughout the entire embryo and again question if the effects observed are specific to neutrophils and not due to MARCKS knockdown in surrounding tissue.

An additional method besides morpholinos to knock down Marcksa or Marcksb in zebrafish is to use a micro RNA (miRNA) approach. Although the use of antisense RNA in zebrafish has been reported to result in off target effects (43), recent advances demonstrate that utilizing naturally occuring miRNA in zebrafish is a feasible option. miRNAs act as posttranslational repressors by binding to the 3' untranslated region (UTR) of target mRNA and usually result in gene silencing by the generation of RNA duplexes and activation of the RNA-induced silencing complex (RISC) (44, 45). Recently, Dong, *et al* described a technique in which a shRNA designed against the 3' UTR of a gene is inserted into the naturally occurring zebrafish miRNA-30e precursor. The resulting mir-shRNA is then embedded into an intron of a β-actin genomic fragment that is in frame with a fluorescent reporter and placed under a ubiquitous or tissue specific promoter. Microinjection of this construct results in the generation of both transient and stable transgenic zebrafish lines with knockdown of both exogenous and endogenous genes. Interestingly, insertion of multiple shRNA into the miRNA results in further knockdown of the gene of interest, indicating that different levels of knockdown can be achieved by this method (46). This system could be utilized for our purposes by designing shRNAs against both Marcksa and Marcksb and inserting them into the miRNA-30e precursor, generating mir-shRNA against *marcksa* and *marcksb*. Inserting this mir-shRNA under the direction of the MPO promoter would result in MARCKS knockdown specific to neutrophils and having an in frame fluorescent reporter (such as GFP) would allow for visualization of neutrophils with the shRNA construct. Generation of stable transgenic lines with these constructs and subsequent tail wounding

assays as performed herein would be an alternative approach for determining if MARCKS is required for *in vivo* neutrophil migration. This is ideal because knockdown of MARCKS would occur solely in neutrophils, thus eliminating any concern that knockdown of MARCKS in surrounding tissues would be involved in the outcome of the neutrophil migration experiment. Further, this method would eliminate any of the developmental abnormalities observed by global MARCKS knockdown by a MO and would give more insight into the role of MARCKS in neutrophil development. Specifically, it would assist in determining if the decreased number of neutrophils in MAT and MBT injected embryos was because of abnormal or damaged tissue or because of the inability of neutrophils to migrate to these tissues.

Previously, we have utilized the MANS peptide, which is a synthetic myristoylated peptide corresponding to the first 24 amino acids of MARCKS, to demonstrate a role for MARCKS during *in vitro* neutrophil migration (14). The MANS peptide has also been utilized to demonstrate a role for MARCKS during both *in vitro* and *in vivo* mucin secretion (15-18) and leukocyte degranulation (15) and thus it is hypothesized that the MANS peptide could be used as a therapeutic treatment for respiratory and other inflammatory disorders. However, to confirm that MANS peptide does interfere with neutrophil migration *in vivo*, further studies are required and zebrafish are an excellent model for these *in vivo* studies. The amino terminus of Marcksa, Marcksb and human MARCKS (used to generate the MANS peptide) are relatively homologous with only a few differences in the amino acid sequence. Thus, performing the zebrafish tail wounding assay described herein in the

presence of MANS peptide would demonstrate *in vivo* evidence that MANS peptide treatment results in decreased neutrophil migration. However, to do these experiments high volumes and/or concentrations of the MANS peptide would be required and if the outcomes of the experiments were not as expected, one concern that could be raised is that the peptide did not penetrate the chorion or embryos themselves and reach the neutrophils. Also, theoretically if the MANS peptide did enter the fish, again concerns would be raised if the MANS peptide was solely inhibiting neutrophils and not surrounding tissue. Therefore, an additional approach to determine if MANS peptide treatment results in decreased neutrophil migration *in vivo* would be to utilize the Tol2 transposon-mediated transgenesis system. The Tol2 transposon is a transposable element that was identified in medaka fish and studies have demonstrated that utilization of this element can result in the chromosomal integration of genes in zebrafish (47). Briefly, construction of a vector with the gene of interest with an in frame fluorescent tag (such as GFP) downstream of a cell specific promoter would be generated with flanking Tol2 sites. Co-injection of this vector with transposase mRNA that has been *in vitro* transcribed results in the integration of excised Tol2 fragment into the genome of the injected zebrafish. Expression of the gene of interest will be transiently expressed in a mosaic pattern and can be determined by GFP visualization (48-50). Thus cloning the MANS peptide sequence downstream of the MPO promoter and utilizing the Tol2 transposon system in zebrafish would produce transiently expressed MANS in zebrafish. This system is ideal because the MANS peptide will be expressed exclusively in neutrophils due to the utilization of the MPO promoter, and it is possible to determine which

cells are expressing MANS by visualization of GFP. Thus, tail wounding experiments on GFP+/MANS expressing cells would be an alternative approach to determine if MANS peptide treatment results in decreased neutrophil migration *in vivo*.

Taken together, the data presented herein provide preliminary evidence of a role for MARCKS during *in vivo* neutrophil migration. However, further experimentation is warranted with alternative approaches to alter MARCKS expression described above. The ultimate goal is to confirm that targeting MARCKS is a potential therapeutic option to combat neutrophilic inflammation, which we have previously presented *in vitro* evidence for.

Materials and Methods

Zebrafish maintenance and husbandry

Experiments were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC). Wild type, EKK (EKKwill Waterlife Resources, Ruskin, FL) and *MPO:GFP* (23) (a kind gift from Stephen Renshaw, The University of Sheffield) adult zebrafish were maintained in a recirculating aquarium facility (Aquatic Habitats, Apopka, FL) at 28°C and were fed a commercial grade zebrafish diet. Zebrafish were mated and embryos were collected in egg water (0.005% Methylene blue and 60 μg/mL aquarium salt mixture in reverse osmosis/deionized (RO/DI) water).

Morpholino Injections

The following MOs were purchased from GeneTools (Philomath, OR): standard control MO; Marcksa translation blocking (MAT) 5’ CTG TTT TTG TGA ATT GCG CTC CCA T; Marcksb translation blocking (MBT) 5’ TTT TGG AGA TTT GTG CTC CCA TGC T. MOs were suspended to 100 nM with sterile water and stock solutions were stored at room temperature. Prior to loading MOs into calibrated needles, MOs were diluted in 0.5% phenol red. For 0.5 and 1 ng injections, MOs were diluted 1:10 and one or two pumps were injected into each embryo, respectively. For 2 ng injections, MOs were diluted 1:1 and one pump was injected into each embryo. Injected embryos were transferred to a petri dish containing egg water and incubated at 28°C with egg water being changed daily with removal of dead embryos.

Flow cytometry analysis

For flow cytometric analysis samples were performed in duplicate or triplicate with one sample containing five normal or mild phenotyped embryos. Wild type EKK embryos were used as negative controls for GFP expression. Embryos were euthanized in 0.17% Tricaine methanesulfonate (Finquel MS-222, Argent Chemical Laboratories, Redmond, VA) and embryos were transferred to eppendorf tubes and resuspended in sterile PBS containing 5% fetal bovine serum (FBS). Embryos were passed through a 40 μm cell strainer (Becton

Dickinson, Franklin Lakes, NJ) into a 50 mL conical vial with a 1 cc syringe plunger and the mesh strainer was rinsed with sterile PBS containing 5% FBS. Cells were transferred to polystyrene flow cytometry tubes and centrifuged for 10 minutes at 12,000 rpm and washed once in sterile PBS containing 5% PBS. Flow cytometry analysis was performed by gating on GFP+ (using wild type embryos as a reference) cells using a FACSCalibur flow cytometer and Cellquest Pro software (Becton Dickinson, San Diego, CA) with all events in the tube collected for each sample.

Tail wounding assay and real time video microscopy

Tail wounding of MPO:GFP embryos were performed as previously described (22, 35). Briefly, 3 dpf embryos were embedded in 35 x 10 mm petri dishes in 1% low melting point agarose (Genesee Scientific, San Diego, CA) in egg water containing 0.017% Tricaine. Embryos were positioned on the bottom of the petri dish and the agarose was allowed to solidify. Once solidified, embryos were wounded on the tailfin by a 25-guage needle and egg water containing 0.017% tricaine was gently placed on top of the agarose. Pictures were obtained immediately after tail wounding and 4 hours later on an AZ100 microscope (Nikon, Melville, NY) with a Ri1 camera and NIS-Elements AR 3.1 software (Nikon).

For real time video microscopy, embryos were embedded as described above. Real time images were obtained on an AZ100 microscope (Nikon) using the DQC-FS Digital Camera (Roper Scientific, Sarasota, FL) camera and NIS-Elements AR 3.1 software (Nikon).

Images were captured at 60X magnification and images were obtained every 45 seconds for duration of 80 minutes with a total of 107 frames per embryo. Stacked TIFF files were generated and total distance, velocity and persistence (distance from origin at the last frame/total distance traveled) were determined by Image J software (National Institute of Health, Bethesda, MD) with 3-4 cells per embryo analyzed per movie.

Statistical Analysis

Statistical Analysis was performed by using one-way ANOVA and Sigma Stat Software (Systat Software, Inc., Chicago, IL) with $P<0.05$ considered statistically significant.

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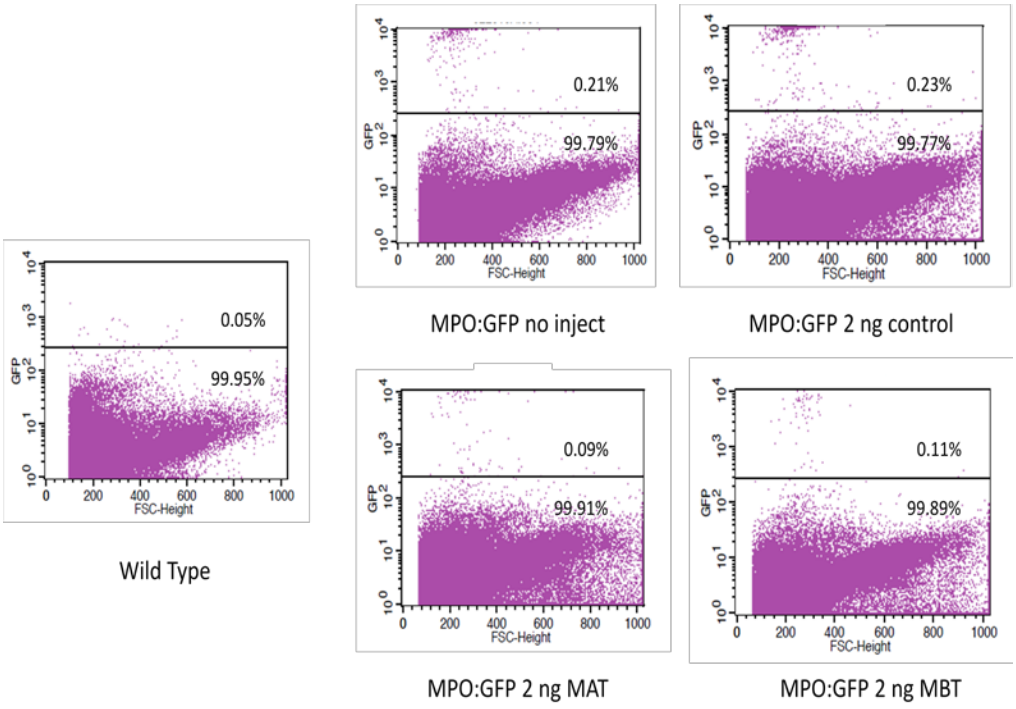
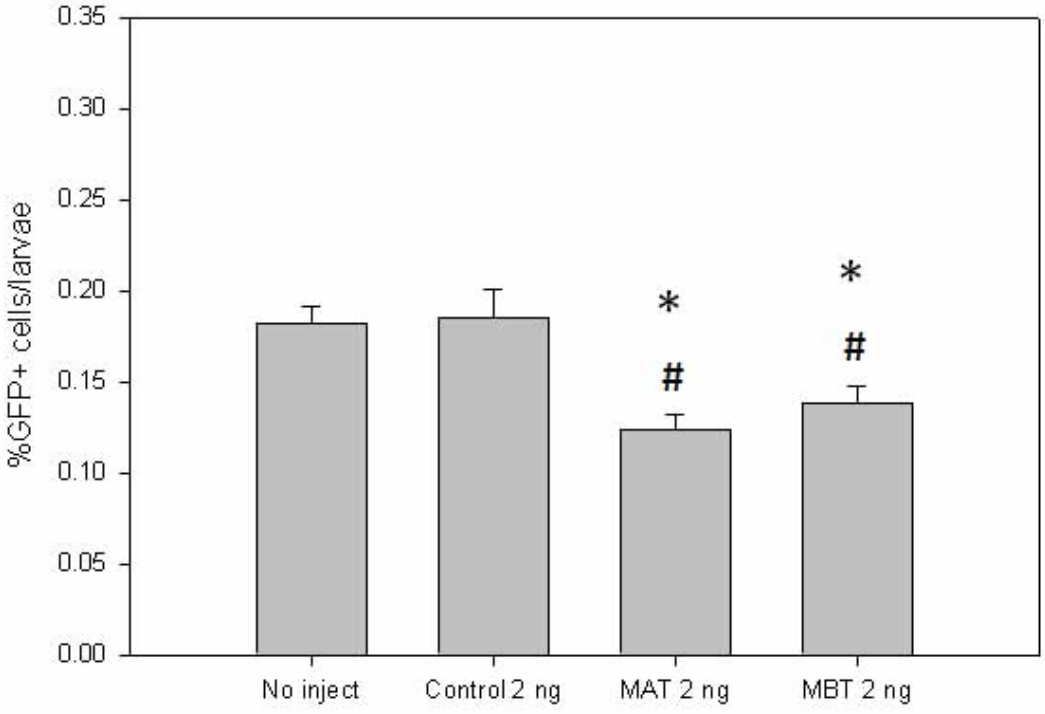
Figure 1: MARCKS deficiencies result in decreased neutrophils in zebrafish. MPO:GFP embryos were injected in the single cell stage with 2 ng (A and B), 1 ng (C) or 0.5 ng (D) MAT, MBT or control MOs and 72 hour later, embryos were processed for flow cytometric analysis as described in Materials and Methods. Representative dot plots are depicted from non-injected or 2 ng injected MAT, MBT and control MPO:GFP embryos (A) and quantification of 2ng (B), 1 ng (C) and 0.5 ng (D) injected embryos are shown with data reported as % GFP+ cells/larvae. MAT (2ng and 1 ng) and MBT (2, 1 and 0.5 ng) injection results in a statistically significant decrease in % GFP+ cells/larvae relative to control injected and non-injected embryos, as designated by * and $^{\#}$, respectively ($p>0.05$). The number of replicates per sample are as follows: no inject (A, B, C), n=24; control 2 ng (A), n=10; MAT 2 ng (A), n=9; MBT 2 ng (A), n=10; control 1 ng (B), n=10; MAT 1 ng (B), n=9; MBT 1 ng (B), n=10; control 0.5 ng (C), n=10; MAT 0.5 ng (C), n=10; MBT 0.5 ng (C), n=10.

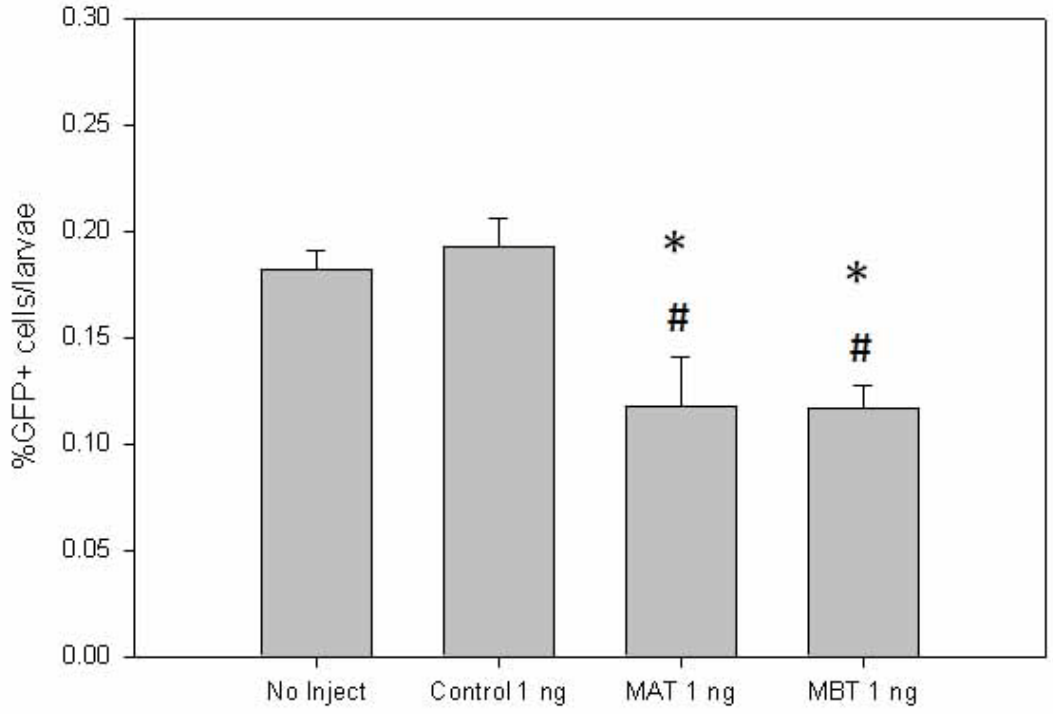
A.


0.05%
99.95%
GFP
FSC-Height
Wild Type
0.21%
99.79%
MPO:GFP no inject
0.23%
99.77%
MPO:GFP 2 ng control
0.09%
99.91%
MPO:GFP 2 ng MAT
0.11%
99.89%
MPO:GFP 2 ng MBT

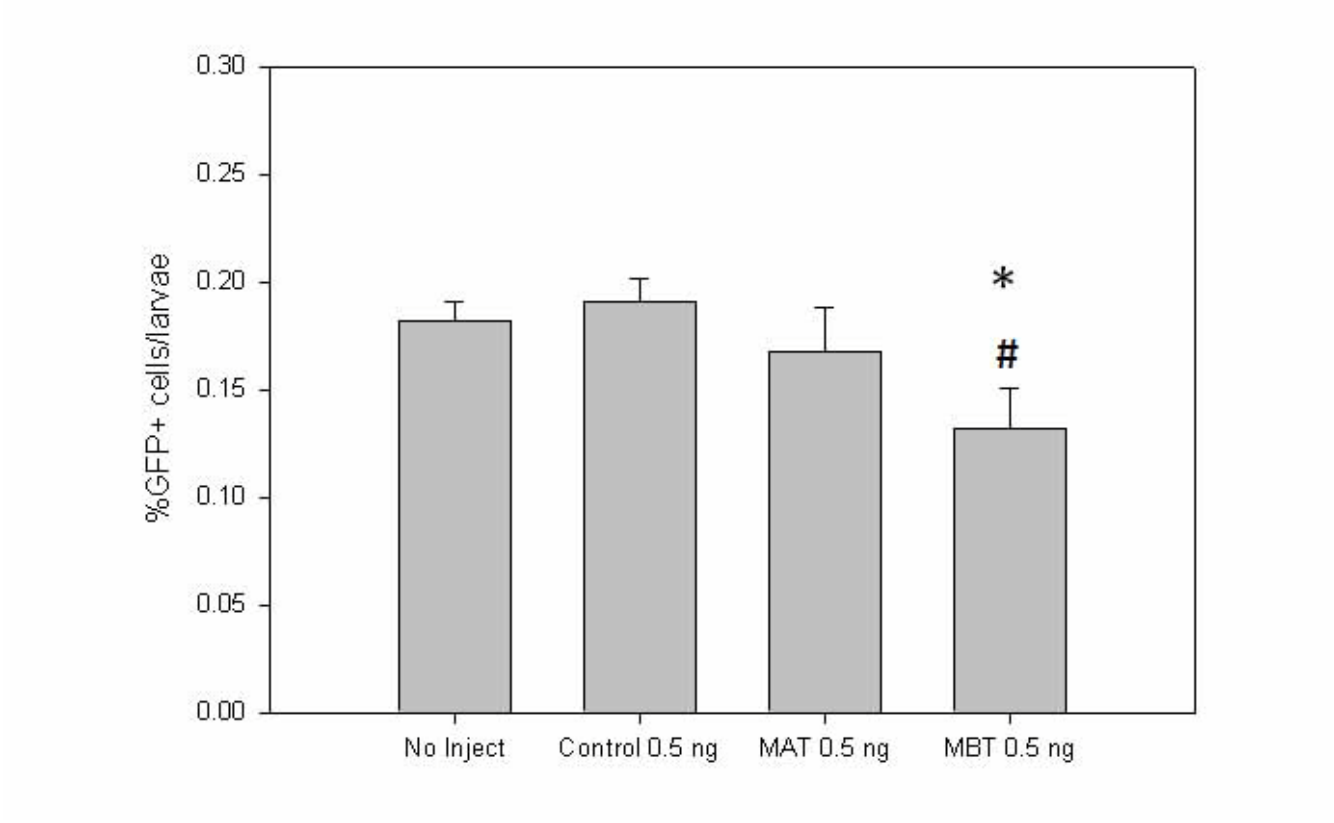
B.


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
%GFP+ cells/larvae
*
#
*
#
No inject
Control 2 ng
MAT 2 ng
MBT 2 ng

C.

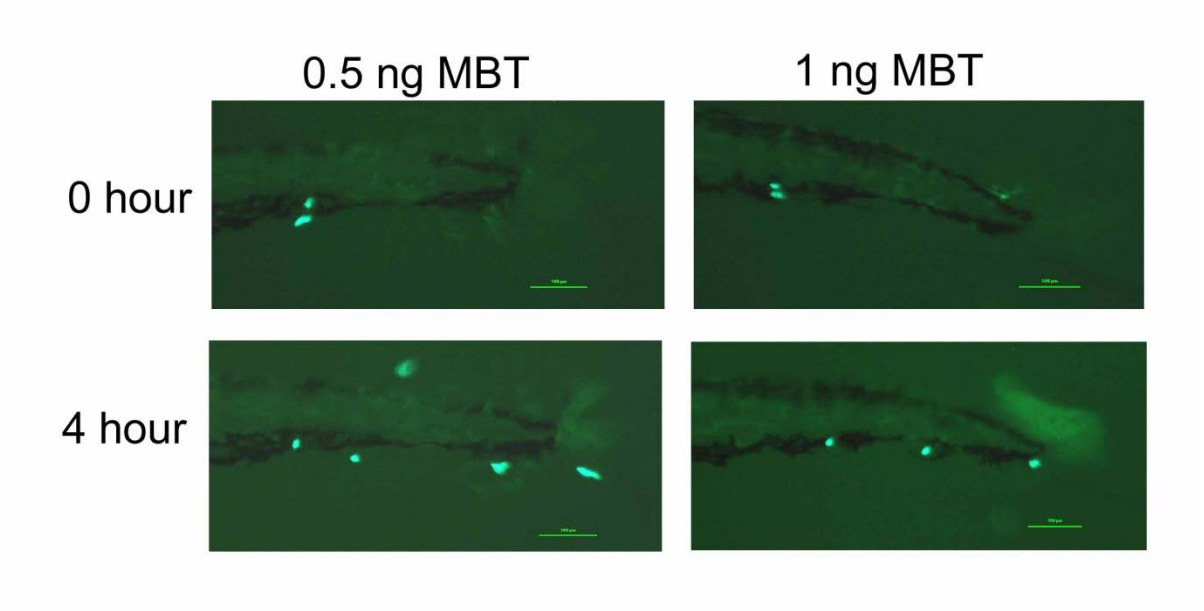

0.30
0.25
0.20
0.15
0.10
0.05
0.00
%GFP+ cells/larvae
*
#
*
#
No Inject
Control 1 ng
MAT 1 ng
MBT 1 ng

D.


0.30
0.25
0.20
0.15
0.10
0.05
0.00
%GFP+ cells/larvae
*
#
No Inject
Control 0.5 ng
MAT 0.5 ng
MBT 0.5 ng

<u>Figure 2:</u> Marcksa and Marcksb are involved in the regulation of neutrophil migration in zebrafish. MPO:GFP embryos were injected with 2, 1 or 0.5 ng MAT, MBT, or control (CONT) MOs and tail wounding assays were performed. The numbers of cells within 100 μm of the wound were quantified initially after wounding (0 hour) and 4 hours later. (A) Representative pictures of 0.5 ng control and MBT injected embryos at 0 and 4 hours are depicted. (B) Quantification of the number of GFP+ neutrohpils at 0 and 4 hours are shown in graphical representation. Embryos injected with 2 ng MAT have a statistically decreased number of neutrophils within 100 um of the wound at 4 hours post wounding relative to 2 ng control injected embryos, as depicted with an $^{\#}$ ($p<0.05$). Embryos injected with 2, 1 or 0.5 ng MBT have significantly decreased number of GFP+ neutrophils within 100 μm of the wound 4 hours post wounding relative to non-injected or respective control injected embryos, as depicted by an * or $^{\#}$, respectively ($p<0.05$). The number of embryos per sample are as follows: no inject, n=17; control 2 ng, n=10; control 1 ng, n=12; control 0.5 ng, n=9; MAT 2 ng, n=7; MAT 1 ng, n=8; MAT 0.5 ng, n=7; MBT 2 ng, n=14; MBT 1 ng, n=14; MBT 0.5 ng, n=10.

A.



B.

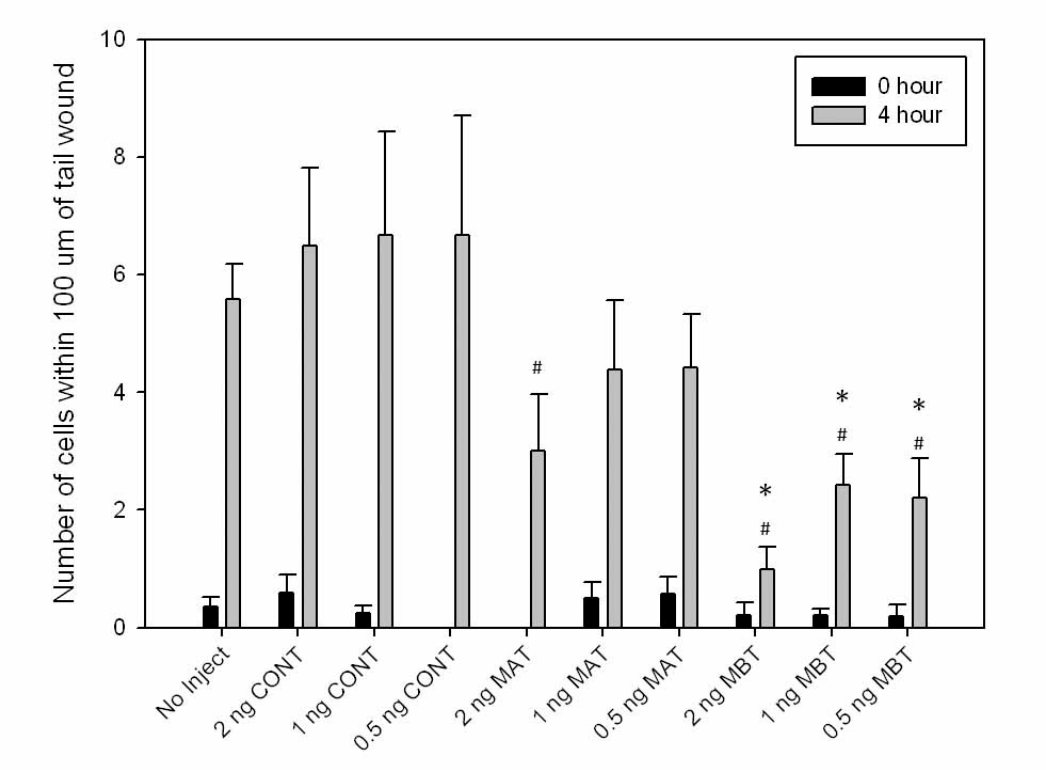
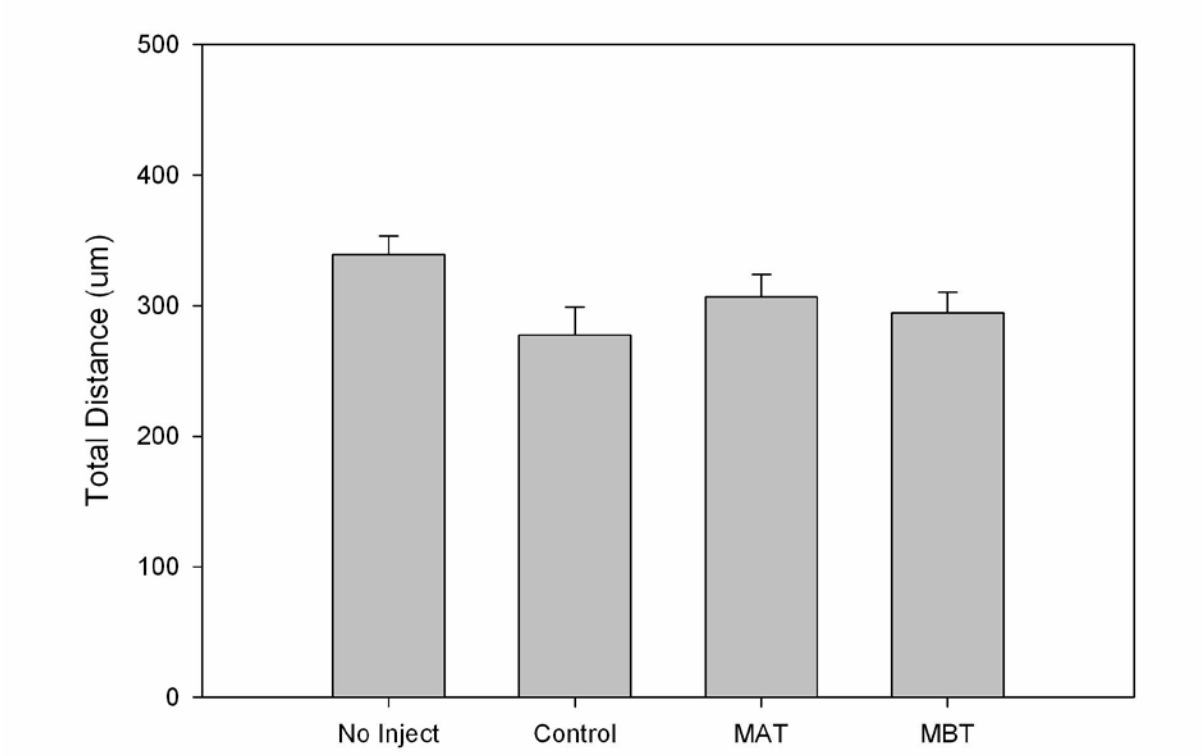
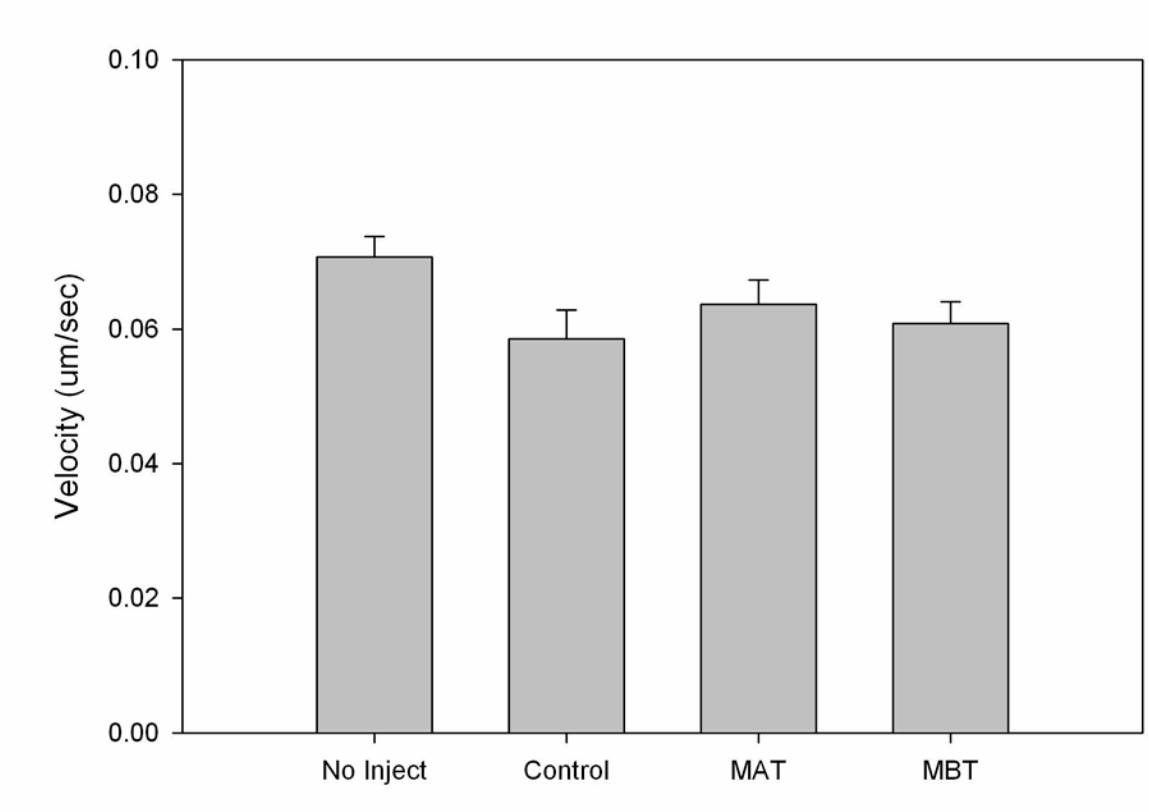

Number of cells within 100 um of tail wound
10
8
6
4
2
0
0 hour
4 hour
#
*
#
*
#
*
#
No Inject
2 ng CONT
1 ng CONT
0.5 ng CONT
2 ng MAT
1 ng MAT
0.5 ng MAT
2 ng MBT
1 ng MBT
0.5 ng MBT

Figure 3: MARCKS deficiencies do not result in decreased neutrophil migration as measured by total distance, velocity and persistence. The tails of normal or mild phenotyped non-injected (n=13 cells) or injected [2 ng MAT (n=17 cells), 2 ng MBT (n=17 cells) or 2 ng control (n=13 cells)] MPO:GFP embryos were wounded at 72 hpf. Single cell tracking of cells were performed and total distance (A), velocity (B) and persistence (C) were determined.

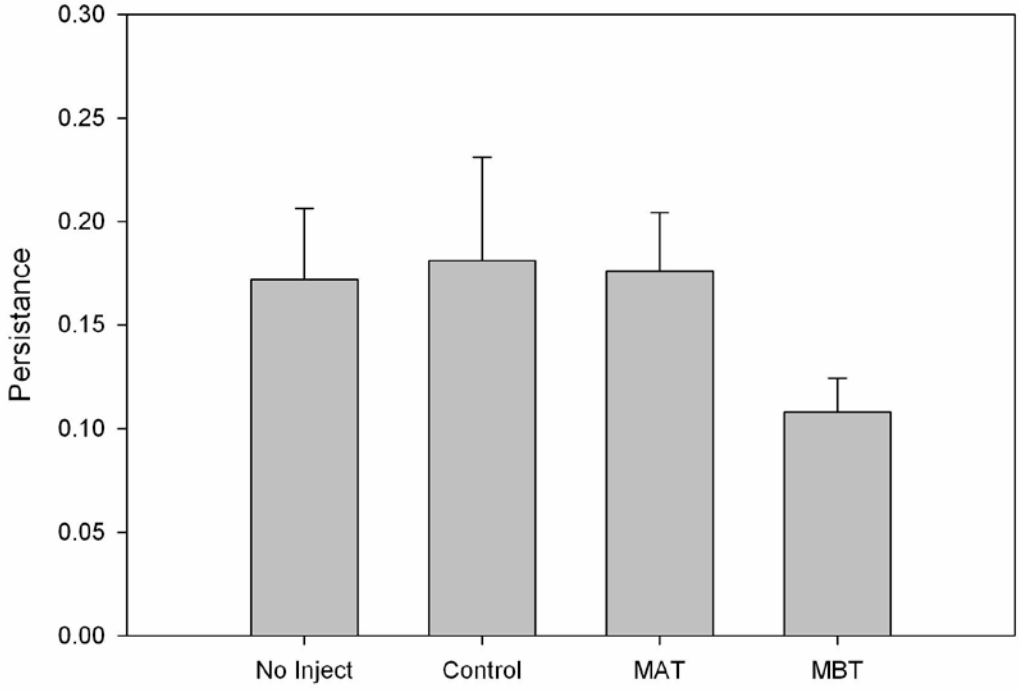
A.


Total Distance (um)
500
400
300
200
100
0
No Inject
Control
MAT
MBT

B.


0.10
0.08
0.06
0.04
0.02
0.00
Velocity (um/sec)
No Inject
Control
MAT
MBT

C.


0.30
0.25
0.20
0.15
0.10
0.05
0.00
Persistance
No Inject
Control
MAT
MBT

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APPENDIX

ROLE OF p38 MAPK IN LPS INDUCED PRO-INFLAMMATORY CYTOKINE AND CHEMOKINE GENE EXPRESSION IN EQUINE LEUKOCYTES

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Abstract

Endotoxemia occurs when bacterial lipopolysaccharide (LPS) in the blood induces a dysregulated inflammatory response, resulting in circulatory shock and multi-organ failure. Laminitis is a common complication in endotoxemic horses and is frequently the cause of death. Blood leukocytes are a principal target of LPS in endotoxemia leading to activation of multiple signal transduction pathways involved in the induction of a number of pro-inflammatory genes. In other animal models, the p38 mitogen activated protein kinase (MAPK) pathway has been associated with induced expression of tumor necrosis factor-α (TNFα), interleukin (IL)-1β, IL-6 and IL-8. The goal of this study was to determine the role of the p38 MAPK pathway in the induction of these pro-inflammatory cytokine and chemokine genes in LPS stimulated equine leukocytes. Stimulation of equine peripheral blood leukocytes resulted in an increase in TNFα, IL-1β, IL-6, and IL-8 mRNA levels. Pharmacological inhibition of p38 MAPK activity with SB203580 or SB202190 reduced the ability of LPS stimulation to increase mRNA levels for all four genes. However, only SB203580 pre-treatment significantly reduced LPS-stimulated IL-1β and IL-8 mRNA expression and only pre-treatment with SB202190 significantly reduced LPS-stimulated TNFα and IL-6 mRNA expression. From this study we conclude TNFα, IL-1β, IL-6 and IL-8 are induced upon LPS stimulation of equine leukocytes and that this induction of gene expression is dependent on the p38 MAPK pathway. However, there are differences in the efficacy of the p38 inhibitors tested here that may be explained by differences in specificity

or potency. This study provides evidence for the use of selective p38 MAPK inhibitors as potential therapeutics for the treatment of equine endotoxemia.

Keywords: Endotoxemia, LPS, p38, equine, leukocytes, inflammation

Abbreviations: cDNA; complementary DNA, COX; cyclooxygenase, Ct; threshold cycle, DEX; dexamethasone, IL; Interleukin, LPS; lipopolysaccharide, LRP; leukocyte rich plasma, MAPK; mitogen activated protein kinase, NF-κB; nuclear factor-κB, PGE_2; prostaglandin E_2, TLR; toll-like receptor, TNFα; tumor necrosis factor α, Thr; threonine, Tyr; tyrosine, VC; vehicle control.

1. Introduction

Endotoxemia is a frequent complication of gastrointestinal disorders and gram-negative bacterial infections and accounts for many deaths each year among horses (Werners et al., 2005). Laminitis is a complication of endotoxemia that contributes significantly to the mortality rate. Although the precise mechanism of laminitis pathogenesis remains unknown, there is increasing evidence that a systemic inflammatory response, such as that seen in endotoxemia, and subsequent inflammation of the hoof laminae are key features of the disease (Belknap et al., 2007; Fontaine et al., 2001; Rodgerson et al., 2001; Waguespack et al., 2004).

Endotoxemia is a clinical syndrome that occurs when bacterial endotoxin (lipopolysaccharide (LPS)), from gram-negative bacteria, enters the blood stream and causes a systemic inflammatory response (Moore and Barton, 2003; Moore et al., 1981; Werners et al., 2005). LPS triggers inflammation by binding to and activating its receptor complex on cellular membranes, comprised of CD14, toll-like receptor-4 (TLR-4), and MD-2. Ligation of the LPS receptor leads to the activation of multiple intracellular signaling pathways, including the nuclear factor-κB (NF-κB) and the mitogen activated protein kinase (MAPK) pathways (Dauphinee and Karsan, 2006; Fitzgerald et al., 2004; Palsson-McDermott and O'Neill, 2004; Zhong and Kyriakis, 2007) The activation of multiple intracellular signaling pathways due to LPS leads to one of the key characteristics of the pathophysiology of endotoxemia; induction of a dysregulated systemic inflammatory response (Moore and Barton, 2003). This dysregulated systemic inflammatory response results in the induction of

an array of pro-inflammatory mediators: enzymes, eicosanoids, cytokines and chemokines (Adrie and Pinsky, 2000; Alluwaimi, 2004; Janicke et al., 2003; Lohmann et al., 2003). These pro-inflammatory mediators alter endothelial permeability and vasoregulation, activate platelets, increase neutrophil influx into a number of tissues, and activate the coagulation cascade. Ultimately, endotoxemia can lead to hypotensive shock, multi-organ failure and even death (Adrie and Pinsky, 2000; Brady and Otto, 2001).

Some pro-inflammatory genes that are upregulated during endotoxemia include the cytokines tumor necrosis factor-alpha (TNFα), interleukin (IL)-1β (IL-1β), IL-6, the chemokine IL-8 and the enzyme cyclooxygenase-2 (COX-2) (Alluwaimi, 2004; Janicke et al., 2003; Laan et al., 2005; Zhu et al., 2007). A recent study demonstrated that LPS from *Rhodobacter sphaeroides* induces the expression of TNFα in equine whole blood and equine monocytes (Lohmann et al., 2003). In a model of equine endotoxemia, where equine smooth muscle cells were stimulated with LPS, there was an increase in COX-2 mRNA and protein levels (Janicke et al., 2003). In another study, sow mammary glands were inoculated with *Escherichia coli* (*E. coli*) and there was upregulation of TNFα, IL-1β, IL-6 and IL-8 mRNA within 24 hours of inoculation (Zhu et al., 2007). These studies indicate that there is a rapid induction of pro-inflammatory genes upon LPS stimulation in horses and other species, and that these genes play a major role in the pathophysiology of endotoxemia.

The p38 MAPK pathway is a member of MAPK signal transduction pathway, which has a common feature of phosphorylation of both threonine (Thr) and tyrosine (Tyr) residues in the motif Thr-X-Tyr. The middle amino acid in the Thr-X-Tyr motif depends on the

MAPK isoform, with p38 MAPK having a glycine (Gly) residue at this site (Herlaar and Brown, 1999). There are 4 isoforms of p38 MAPK, α, β, γ and δ, and the expression of these isoforms varies depending on the cell type. Inflammatory cells, including neutrophils, monocytes, macrophages and $CD4^+$ T cells, predominantly express p38α and δ, whereas endothelial cells express all isoforms of p38 MAPK (Hale et al., 1999).

Many pro-inflammatory events are mediated by the p38 MAPK pathway, including cytokine and chemokine production, integrin expression, chemotaxis and generation of oxidative burst (Detmers et al., 1998; Nick et al., 1999; Read et al., 1997; Ridley et al., 1997). Our lab and others have demonstrated that LPS stimulation of equine leukocytes results in the activation of the p38 MAPK pathway. It was also demonstrated that upon LPS stimulation of equine leukocytes, COX-2 expression increases and COX-2-dependent prostaglandin E_2 (PGE_2) production is upregulated and this upregulation is dependent on the p38 MAPK pathway (Brooks et al., 2007; Eckert et al., 2007; Laan et al., 2005). We analyzed the expression of COX-2 in LPS stimulated equine leukocytes and showed that SB203580 and SB202190, specific inhibitors of p38 MAPK in the pyridinyl imidazole family of compounds (Frantz et al., 1998; Young et al., 1997), significantly decreased both protein and mRNA expression of this pro-inflammatory enzyme (Eckert et al., 2007). These studies suggest that the p38 MAPK pathway is involved in pro-inflammatory gene expression in horses suffering from endotoxemia.

In other animal models besides the horse, the importance of the p38 MAPK pathway in pro-inflammatory gene expression has been demonstrated (Kotlyarov et al., 1999; Lappas et al., 2007; Lee et al., 1994; Lee and Young, 1996; van den Blink et al., 2001; Zhu et al., 2000). In one study, researchers deleted one of the downstream targets of p38 MAPK, MAPKAP kinase 2 (MK2), in mice and showed that there was a decrease in LPS dependent stimulation of TNFα protein expression. This same group also showed that there was a decrease in IL-1β and IL-6 protein levels in these knock out mice when stimulated with LPS compared to the wild type control mice (Kotlyarov et al., 1999). This study indicates that the p38 MAPK pathway plays a pivotal role in pro-inflammatory gene expression during endotoxemia. This study also indicates that the p38 MAPK pathway may be a suitable target for therapeutics designed for the treatment of endotoxemia and other pro-inflammatory diseases. *In vivo* and *in vitro* studies have been performed in human models indicating that the use of p38 MAPK inhibitors in the treatment of endotoxemia would be a beneficial therapeutic (Branger et al., 2002; Fijen et al., 2001).

In the current study, we assessed the role of p38 MAPK in the mRNA expression of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and the pro-inflammatory chemokine IL-8 in equine peripheral blood leukocytes that have been stimulated with LPS. We hypothesized that mRNA expression of TNFα, IL-1β, IL-6 and IL-8 will be upregulated in LPS stimulated equine leukocytes. We also hypothesized that this increase in expression is dependent on the p38 MAPK pathway. We tested these hypotheses by using a quantitative real time PCR approach

2. Materials and Methods

2.1. Leukocyte Collection

Fresh whole blood was collected via the jugular vein from 10 healthy horses. A 60-cc luer tip syringe was pre-coated with Heparin sodium salt (Baxter, Deerfield, IL) and an 18-gauge polypropylene hub hyperdermic needle (Kendall, Mansfield, MA) was used to collect 60 mL of blood. Blood was then transferred to two sterile endotoxin free 50 mL conical tubes (Sarstedt, Newton, NC) and blood was allowed to settle for 40 minutes at room temperature. The leukocyte rich plasma (LRP) was then collected and placed into a fresh sterile endotoxin free 50 mL conical tube.

2.2. Pretreatment with p38 inhibitors and LPS stimulation

Three mL of LRP was added to sterile, endotoxin free 15 mL conical tubes (Sarstedt). The LRP was pretreated with 10 μM SB203580 or SB202190 (Invitrogen, Carlsbad, CA) or vehicle control (VC), dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). In some experiments, the LRP was pretreated with 10 μM Dexamethasone (Sigma, St. Louis, MO) or VC, ethanol (Fisher Scientific, Pittsburg, PA). Pretreatment was at 37˚C for 30 minutes. LRP was then stimulated for either 2 or 4 hours with 10 ng/ml LPS from E. coli O55: B5 (Sigma, St. Louis, MO) or phosphate buffered saline (PBS) at 37˚ C.

2.3. RNA isolations and complementary DNA (cDNA) synthesis

After the LPS stimulation, the reaction was stopped by adding 60 μL of 0.5 M Ethylenediaminetetraacetic acid (EDTA) (Fisher, St. Louis, MO). The cell suspension was gently mixed and then centrifuged for 8 minutes at 1200 rpm. The supernatant was then aspirated and the cells were resuspended in 500 μL of ice cold sterile PBS and transferred to a sterile RNase/DNase free microcentrifuge tube. The cells were then centrifuged for 5 minutes at room temperature and at 1500 rpm.

Cells were homogenized using the RLT buffer supplied in Qiagen's RNeasy Protect mini kit and QIAshredder™ columns (Qiagen, Valencia, CA). β-mercaptoethanol was added to the RLT buffer, and the RNA isolations were performed according to the kit manufacturer's guidelines. An RNase-Free DNase Set (Qiagen, Valencia, CA) was used for an on column DNase digestion step that was suggested by the manufacturer of the RNeasy Protect Mini Kit. RNA was eluted into 30 μL of RNase/DNase free water (Fisher) and the concentration was determined using an Eppendorf BioPhotometer and RNase/DNAase/protein-free cuvettes (Eppendorf, Westbury, NY). Samples were stored at -80˚C until reverse transcriptase PCR was performed.

Complementary DNA (cDNA) synthesis was performed using 1 μg of RNA. The PCR reaction was set up with the appropriate amount of RNase/DNase free water, 2 μL of 10 mM dNTPs (Applied Biosystems, Foster City, CA), 2 μL of random hexamers (Applied Biosystems) and the appropriate amount of RNA to total 30 μL. The samples were then incubated for 10 minutes at 70˚C. Using Superscript II RT enzyme kit (Invitrogen), a master

mix was made. The volume for one reaction of the master mix is as follows: 10 µL 5X First Strand Buffer, 5 µL 0.1 M DTT, 2.5 µL RNase inhibitor (Applied Biosystems) and 2.5 µL Superscript II RT. Twenty µL of the master mix was added to each sample and the thermocycling parameters were as follows: 25˚C, 10 minutes; 42˚C, 60 minutes; 42˚C, 60 minutes; 75˚C, 15 minutes; 4˚C, infinity. The thermocycler used was MyIQ single color real time PCR detection system (Bio-Rad, Hercules, CA). cDNA was stored at -20°C until further use.

2.4. Quantitative Real Time PCR

The optimal annealing temperature was determined for each primer pair (IDT, Coralville, IA) by the temperature gradient aspect of the MyIQ thermocycler. A 20X volume master mix was made comprising of 124 µL PCR water, 12 µL of 5 µM forward primer, 12 µL of 5 µM reverse primer, and 252 µL of 2X SYBR Green PCR Master Mix (Applied Biosystems). A separate master mix was made for each primer pair and 1µL of cDNA was added to each master mix. Twenty µL of the master mix/cDNA mixture was added in duplicate to the PCR plate and the plate was sealed with an adhesive plastic plate sealer (Bio-Rad) and placed into the thermocycler. The thermocycling conditions are as follows: 95˚C for 10 minutes, 50 cycles of [95˚C for 15 sec, gradient temperature for 60 sec], 90 cycles increasing the temperature 0.5˚C every 0.05 sec starting at 50˚C, and 4˚C for infinity. The temperature gradient was: 65°C, 64.5°C, 63.5°C, 61.4°C, 58.9°C, 57.1°C, 55.8°C and 55°C. Refer to table 1 for annealing temperatures used for each primer pair.

Quantitative Real Time PCR was performed in triplicate wells by adding 1.2 μL cDNA into wells containing a master mix for each of the primers used. TNFα, IL-1β, IL-6, IL-8 and GAPDH primer sequences have been published (Bogaert et al., 2006; Garton et al., 2002) and are listed in table 1. The primer validation was performed as described (Trivedi and Arasu, 2005). For one reaction, the master mix was as follows: 15 μL of RNase/DNase free water, 0.6 μL of 5 μM forward primer, 0.6 μL of 5 μM reverse primer and 12.6 μL of 2X SYBR Green Master Mix (Applied Biosystems). The thermocycling conditions for the real time PCR reaction were: 95˚C for 10 minutes, 50 cycles of [95˚C for 15 sec, appropriate annealing temperature for 60 sec], 90 cycles increasing the temperature 0.5˚C every 0.05 sec starting at 50˚C, and 4˚C for infinity. The MyIQ single color Real Time PCR Detection System Thermocycler was used (Bio-Rad) and each primer pair was assayed on a separate plate. The results were then analyzed using the MyIQ software and the ΔΔCt method of analysis.

2.5. Statistical Analysis

Statistical analysis was preformed using a paired t-test with p-value (p<0.05) considered significant.

3. Results

3.1. p38 MAPK activity is required for LPS induction of TNFα expression

First, we analyzed the expression of TNFα mRNA after 2 hours of LPS stimulation and observed a significant increase in mRNA levels in LPS stimulated equine leukocytes compared to PBS alone (Figure 1). To determine whether p38 MAPK activity was necessary for LPS-stimulated increase in TNFα mRNA levels, we pretreated the cells with the specific p38 MAPK inhibitors, SB203580 or SB202190 (10 μM of each). We chose this concentration based on the reported potency of each compound in cells from other species as well as from our own data demonstrating that 10 μM maximally inhibits LPS-induced increase in COX-2 mRNA and protein levels in equine leukocytes (Eckert et al., 2007). Inhibition of p38 MAPK with either compound reduced TNFα levels in LPS-stimulated equine leukocytes (Figure 1). However, this reduction was significant only for SB202190 treatment when compared to LPS-stimulated VC treated cells (Figure 1). Dexamethasone treatment was used as a control for the ability to inhibit LPS-stimulated TNFα mRNA. As expected, dexamethasone treatment significantly reduced TNFα mRNA levels in LPS-stimulated cells compared to LPS-stimulated VC treated cells (Figure 1). There was no different between the cells treated with PBS alone and the PBS treated cells that were pretreated with SB203580, SB202190 or dexamethasone.

3.2 LPS induced IL-1β and IL-6 expression is dependent on p38 MAPK signaling

Next, we analyzed the expression of IL-1β and IL-6 after 4 hours of LPS stimulation and observed a significant increase in mRNA levels for both genes in LPS-stimulated equine leukocytes compared to PBS alone (Figure 2 and Figure 3). Pretreatment with both SB203580 and SB202190 inhibited the LPS-stimulated increase in IL-1β mRNA levels, but this decrease was only significant for SB203580 treatment compared to LPS-stimulated VC treated cells (Figure 2). Pretreatment with both SB202190 and SB203580 also inhibited LPS-stimulated elevation in IL-6 mRNA levels, but this decrease was only significant for SB202190 compared to VC treated LPS-stimulated cells (Figure 3). As with TNFα, there was no significant difference in IL-1β or IL-6 mRNA levels in cells treated with PBS with or without SB203580 or SB202190 pretreatment (Figure 2 and Figure 3, respectively).

3.3 Increased IL-8 chemokine expression is dependent on p38 MAPK signaling in LPS stimulated equine leukocytes

Finally, we analyzed the expression of IL-8 after 4 hours of LPS stimulation. We observed a significant increase in IL-8 mRNA levels in LPS-stimulated cells when compared to PBS alone (Figure 4). Both SB202190 and SB203580 inhibited LPS-induced IL-8 mRNA levels, but this was only significant for SB203580 when compared to LPS-stimulated cells treated with VC (Figure 4). We found no difference in IL-8 mRNA levels in cells treated with PBS with or without pretreatment with the p38 MAPK inhibitors.

4. Discussion

In this study, we investigated the role of the p38 MAPK pathway in pro-inflammatory cytokine and chemokine expression in equine leukocytes stimulated with LPS. The purpose of this study was to determine whether p38 MAPK activity was essential for induction of key pro-inflammatory genes in LPS-stimulated equine leukocytes. We used mRNA as a measure of gene expression because of the utility of this method to screen for expression of multiple genes and because of the strong correlation between mRNA and cellular protein levels for these genes. While mRNA levels may not ultimately be equivalent to the secretion of active products, particularly for genes such as TNFα and IL-1β that undergo post-translational modifications dictating physiological activity, mRNA is a sensitive indicator of gene expression that is suitable for this mechanistic study defining the role for p38 MAPK in the upregulation of inflammatory genes in leukocytes stimulated by LPS.

This study demonstrated that the pro-inflammatory genes, TNFα, IL-1β, IL-6 and IL-8, are induced in equine leukocytes stimulated with LPS. Pretreatment with the selective p38 MAPK inhibitors, SB203580 or SB202190, inhibited the increase in gene expression induced by LPS. However, the ability of each compound to inhibit gene expression was not statistically significant for every gene analyzed. SB203580 significantly inhibited LPS induction of IL-1β and IL-8 and SB202190 significantly inhibited LPS induction of TNFα and IL-6. These results suggest that there may be subtle p38 MAPK isoform requirements for each set of genes. It is notable that both SB203580 and SB202190 significantly inhibited

the LPS induction of COX-2 expression in equine leukocytes, demonstrating that for some genes, at least, both inhibitors are effective (Eckert *et al*., 2007).

Other possible explanations for the differential effects of SB203580 and SB202190 include potential effects on other kinases or differences in potency. Although both inhibitors are relatively specific for p38 MAPK at the concentrations used in this study, they have been found to inhibit, albeit less potently, stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs) leukocyte-specific protein tyrosine kinase (Lck), glycogen synthase kinase 3β (GSK3β) and protein kinase B α (PKBα) (Clerk and Sugden, 1998; Davies et al., 2000; Lali et al., 2000). The concentrations of both SB203580 and SB202190 used in this study were those that maximally inhibit the expression of a number of genes in our system by each respective compound. Further, we found that the pattern of inhibition was different, depending on the gene. Together, these observations argue that differences in potency do not *a priori* explain the differential effects of the compounds in this study. However, differences in potency amongst the various cell types in this mixed leukocyte population remain possible if the various cells in our preparation (e.g. neutrophils and monocytes) contribute differently to the overall production of each cytokine or chemokine measured here.

Our lab and others have demonstrated the involvement of the p38 MAPK pathway in production of eicosanoids in endotoxic horses. These studies indicated that COX-2 dependent upregulation of prostaglandin E_2 is dependent on the p38 MAPK pathway (Brooks et al., 2007; Eckert et al., 2007). This dependency on p38 MAPK lies at the level of induction of COX-2 gene expression. This study demonstrates that p38 MAPK is essential

for the upregulation of a panel of key pro-inflammatory genes that are important in the pathophysiology of equine endotoxemia.

The p38 MAPK pathway can regulate gene expression by one of two mechanisms, activation of transcription and stabilization of mRNA transcripts (Ono and Han, 2000). Messenger RNA transcripts that contain an ARE region, consisting of multiple copies of the overlapping AUUUA motif, are destabilized by deadenylation (Dean et al., 1999; Dean et al., 2003; Xu et al., 1997). Activation of the p38 MAPK pathway leads to inhibition of ARE induced mRNA destabilization, thus stabilizing the mRNA transcript and increasing the levels (Lasa et al., 2000; Winzen et al., 1999). The p38 MAPK pathway is involved in stabilization of multiple pro-inflammatory genes that contain ARE sequences, including COX-2, IL-6, TNFα, IL-1β and IL-8 (Dean et al., 1999; Dean et al., 2003; Lasa et al., 2000; Miyazawa et al., 1998).

Our studies suggest that p38 inhibitors may be an effective treatment for equine patients with endotoxemia that have a high risk for developing laminitis. In this study, we pretreated equine leukocytes with the p38 MAPK inhibitors prior to LPS stimulation. While this pretreatment protocol is not clinically relevant since treatment of endotoxemia in patients is usually initiated when clinical signs appear after the onset of LPS-stimulated gene expression has already occurred, our results provide proof of principle that inhibition of p38 may be a viable therapeutic strategy for treating systemic inflammation associated with endotoxemia in equine patients. Further support for this strategy is provided by studies in endotoxic rodents where pharmacological inhibition of the p38 MAPK pathway decreases

TNFα levels in myocardial tissue (Peng et al., 2003) and decreases TNFα protein levels in plasma (Badger et al., 1996; Underwood et al., 2000). Moreover, two human studies in which p38 MAPK inhibitors, BIRB 796 BS (Branger et al., 2002) and RWJ-67657 (Fijen et al., 2001), were given orally to healthy human patients before intravenous injection of LPS. Both of these studies reported a decrease in the clinical symptoms associated with endotoxemia in patients who received the oral p38 MAPK inhibitors in comparison to patients who received placebo. These studies also demonstrated that these p38 MAPK inhibitors decrease the protein concentrations of TNFα, IL-6 and IL-8 in human plasma (Branger et al., 2002; Fijen et al., 2001).

Our current study, along with the human data, indicates the importance of the p38 MAPK pathway in pro-inflammatory cytokine production during endotoxemia. The human studies support the potential therapeutic benefit of p38 MAPK pathway inhibitors for the treatment of horses suffering from endotoxemia. Moreover, our results suggest that p38 MAPK inhibitors may reduce the induction of inflammatory gene expression in other tissues, such as the hoof lamina where several inflammatory genes are increased during laminitis (Belknap et al., 2007; Fontaine et al., 2001; Rodgerson et al., 2001; Waguespack et al., 2004). However, the effects of p38 MAPK inhibitor treatment on the equine lamina in horses with or without endotoxemia must be examined. It is notable that inhibition of p38 MAPK adversely affects mucosal repair in a model of intestinal ischemia (Shifflett et al., 2004). Thus, it remains to be determined whether unwanted tissue affects will limit the usefulness of p38 MAPK inhibitors in practice.

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Table 1. Primer sequences and annealing temperature used for quantitative real time PCR experiments.

Gene	Accession#	Forward primer	Reverse primer	Annealing temperature (°C)
GAPDH	DQ403064	GGC AAG TTC CAT GGC ACA GT	CAC AAC ATA TTC AGC ACC AGC AT	55
TNFα	NM_001081819	GCTCCA GAC GGT GCT TGT G	GCC GATCAC CCC AAA GTG	55.8
IL-1β	NM_001082526	TGA AGG GCA GCT TCC AAG AC	GGG AGA ATT GAA GCT GGA TGC	58.9
IL-6	U64794	CCC CTG ACC CAA CTG CAA	TGT TGT GTT CTT CAG CCA CTC A	55
IL-8	AF062377	CGG TGC CAG TGC ATC AAG	TGG CCC ACT CTC AAT CAC TCT	55

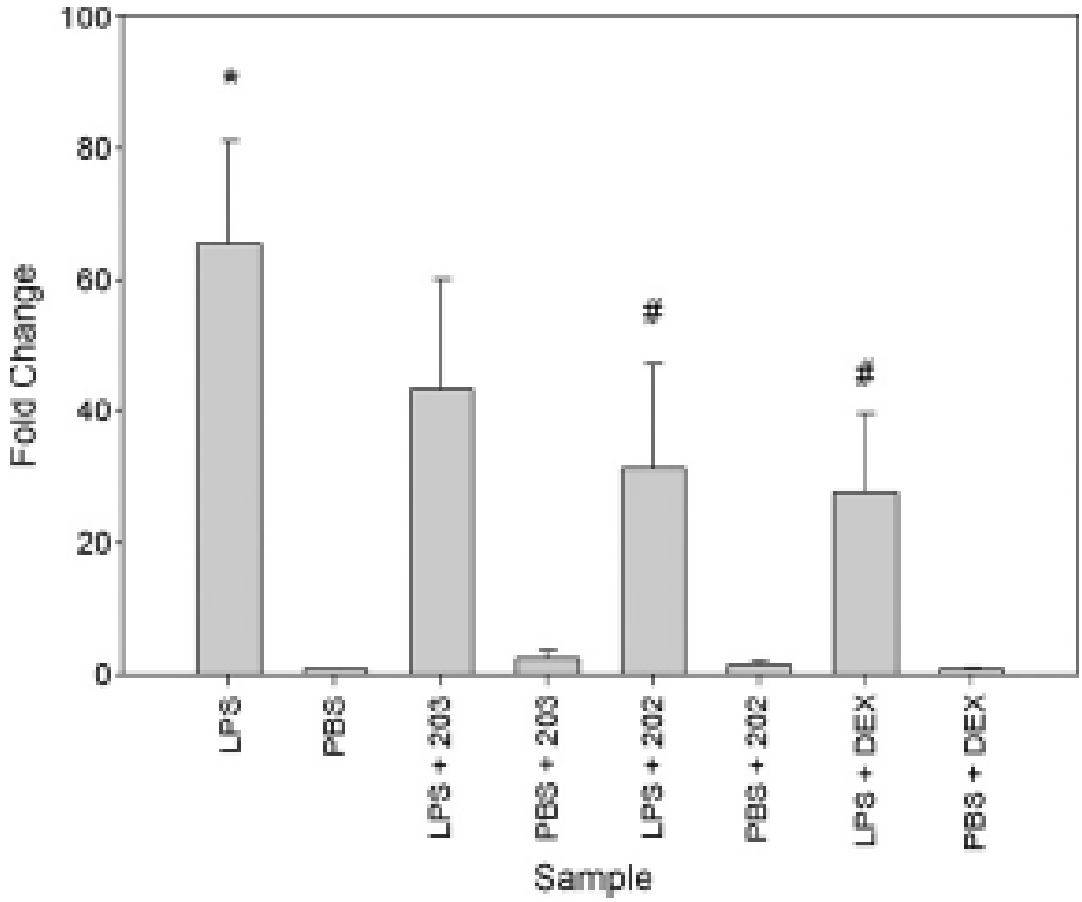

Figure 1. LPS induces a p38 MAPK dependent increase in TNFα mRNA expression. Equine peripheral blood leukocytes were pretreated with 10 μM SB203580 (203), 10 μM SB202190 (202) or 10 μM Dexamethasone (DEX) for 30 minutes at 37°C. Leukocytes were then stimulated with 10 ng/ml LPS for 2 hours at 37°C and mRNA was subsequently isolated. The mRNA was then synthesized into cDNA, which was then used in quantitative real time PCR with equine GAPDH as the housekeeping gene. Data points are mean (±S.E.M) of 7 horses, normalized to the PBS (vehicle control) sample. The asterisk denotes that upon LPS stimulation, there was a significant increase in TNFα mRNA expression relative to PBS treated samples ($p<0.05$). The pound sign indicates a significant decrease in TNFα mRNA expression in SB202190 and DEX pretreated samples relative to LPS stimulation alone ($p<0.05$).

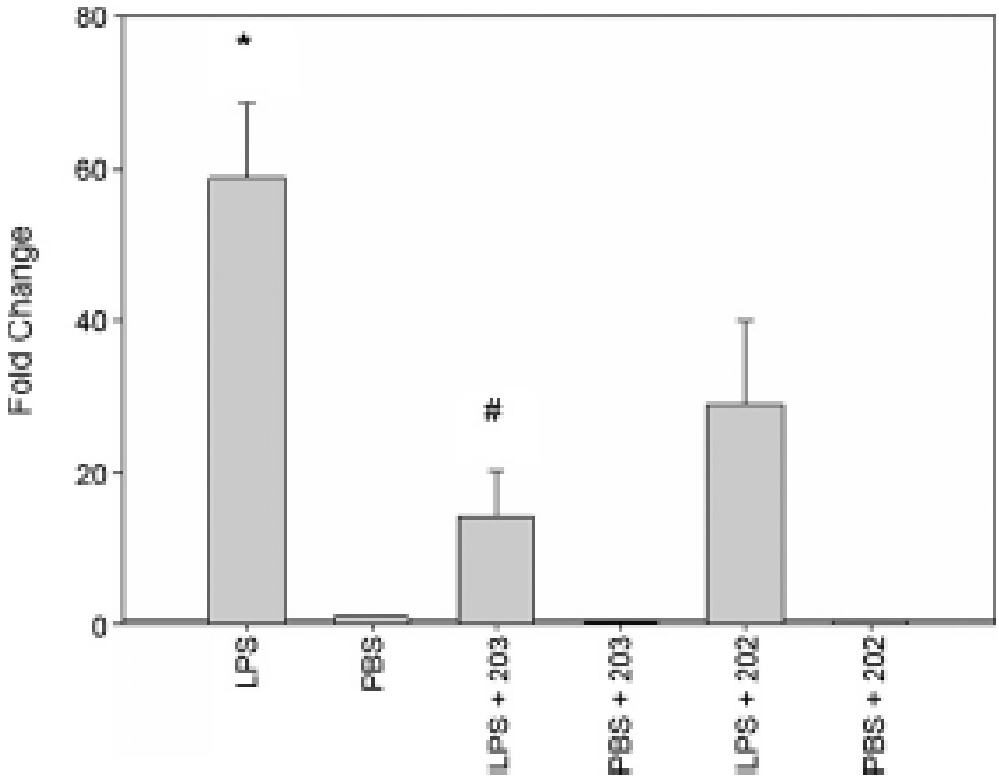

Figure 2. Equine peripheral blood leukocytes stimulated with LPS express IL-1β mRNA and this expression is inhibited by the p38 MAPK inhibitor SB203580. Peripheral blood leukocytes were pretreated with 10 μM SB203580 (203) or 10 μM SB202190 (202) for 30 minutes and then stimulated for 4 hours with 10 ng/ml LPS all at 37˚C. Quantitative real time PCR was performed on mRNA isolated from the samples using GAPDH as the housekeeping gene. Data points represent the mean (±S.E.M.) of 6 horses, normalized to the PBS sample. The asterisk denotes a significant increase in IL-1β mRNA expression LPS stimulated leukocytes relative to PBS treatment ($p<0.05$). The pound sign denotes a significant decrease in IL-1β mRNA expression in SB203580 pretreated samples stimulated with LPS relative to LPS stimulated samples without pretreatment ($p<0.05$).

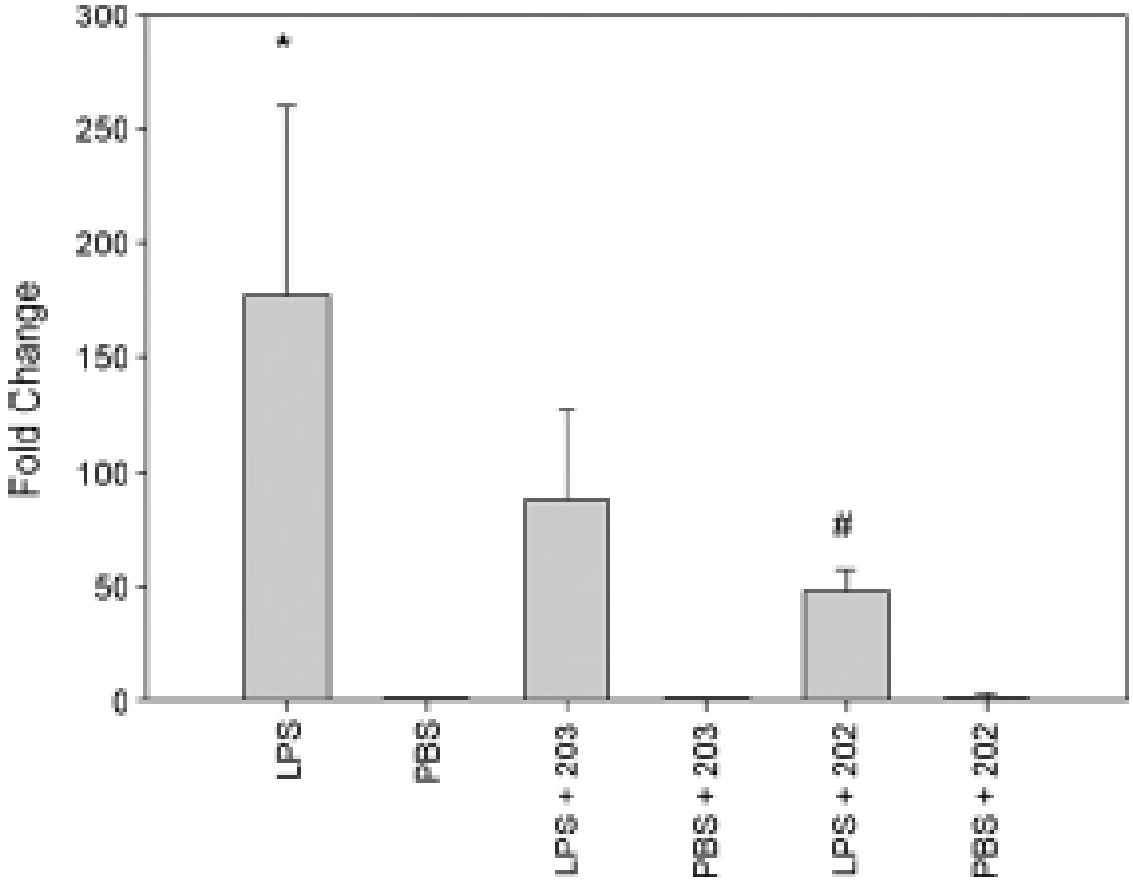

Figure 3. The p38 MAPK inhibitor SB202190 significantly decreases LPS-induced IL-6 mRNA expression in equine peripheral blood leukocytes. Leukocytes were pretreated with 10 μM SB203580 (203) or 10 μM SB202190 (202) for 30 minutes at 37°C. Leukocytes were then stimulated with 10 ng/ml LPS for 4 hours at 37°C and quantitative real time PCR was performed on mRNA isolated from the samples with GAPDH as the housekeeping gene. Data points represent the mean (±S.E.M.) of 8 horses, normalized to the PBS sample. The asterisk denotes a significant increase in IL-6 mRNA expression in LPS stimulated samples relative to PBS treated samples ($p<0.05$). The pound sign denotes significant decrease in IL-6 mRNA expression in SB202190 pretreated samples stimulated with LPS relative to LPS stimulated samples without pretreatment ($p<0.05$).

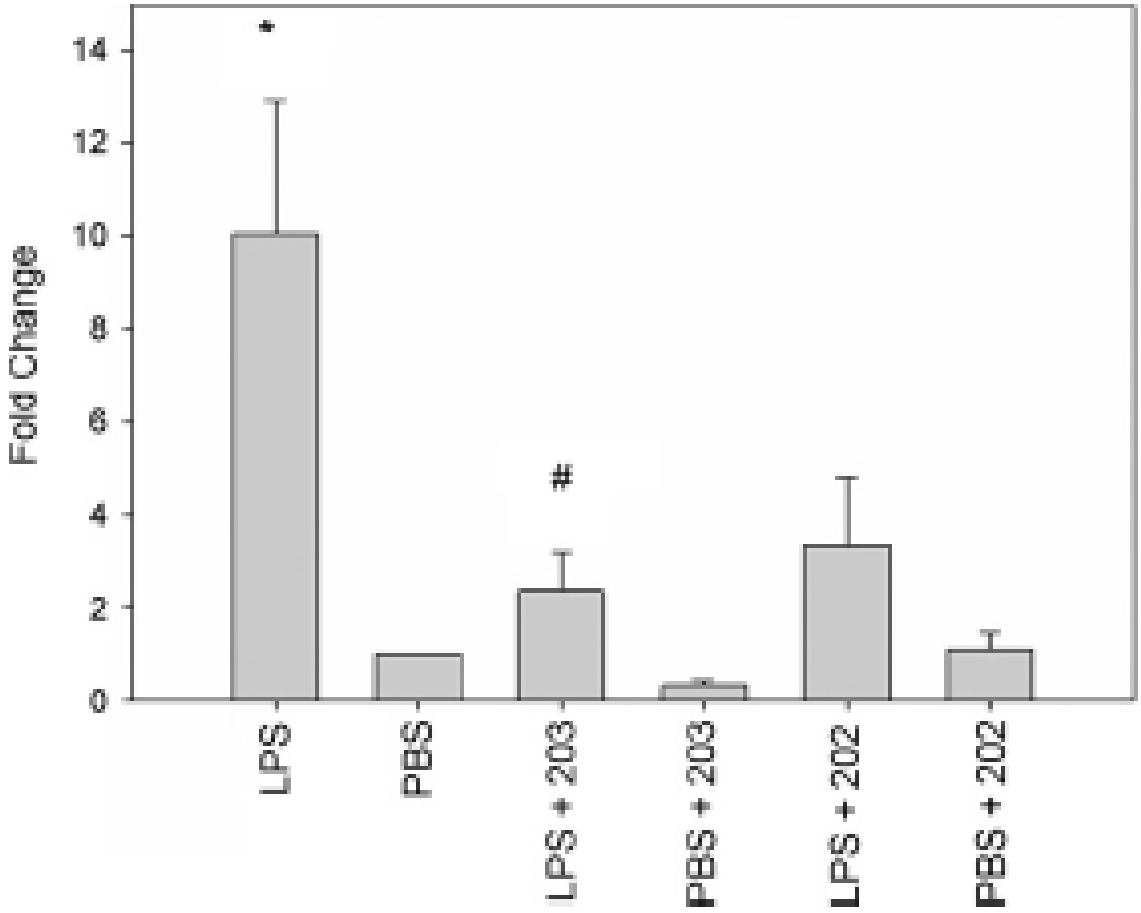

Figure 4. LPS stimulation induces the expression of IL-8 mRNA in equine peripheral blood leukocytes and this expression is inhibited by the p38 MAPK inhibitor SB203580. Peripheral blood leukocytes were pretreated with 10 μM SB203580 (203) or 10 μM SB202190 (202) for 30 minutes at 37°C and then stimulated for 4 hours with 10 ng/ml LPS. Quantitative real time PCR was performed on mRNA isolated from the leukocytes and GAPDH was used as the housekeeping gene. Data points represent the mean (±S.E.M.) of 7 horses, normalized to the PBS sample. The asterisk indicates a significant increase in IL-8 mRNA expression in LPS stimulated leukocytes relative to PBS control ($p<0.05$). The pound sign indicates a significant decrease in IL-8 mRNA expression in SB203580 pretreated samples with LPS stimulation relative to LPS stimulation without pretreatment ($p<0.05$).

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