Fibroblast Migration Is Regulated by Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein

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Abstract

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitously expressed substrate of protein kinase C (PKC) that is involved in reorganization of the actin cytoskeleton. We hypothesized that MARCKS is involved in regulation of fibroblast migration and addressed this hypothesis by utilizing a unique reagent developed in this laboratory, the MANS peptide. The MANS peptide is a myristoylated cell permeable peptide corresponding to the first 24-amino acids of MARCKS that inhibits MARCKS function. Treatment of NIH-3T3 fibroblasts with the MANS peptide attenuated cell migration in scratch wounding assays, while a myristoylated, missense control peptide (RNS) had no effect. Neither MANS nor RNS peptide treatment altered NIH-3T3 cell proliferation within the parameters of the scratch assay. MANS peptide treatment also resulted in inhibited NIH-3T3 chemotaxis towards the chemoattractant platelet-derived growth factor-BB (PDGF-BB), with no effect observed with RNS treatment. Live cell imaging of PDGF-BB induced chemotaxis demonstrated that MANS peptide treatment resulted in weak chemotactic fidelity compared to RNS treated cells. MANS and RNS peptides did not affect PDGF-BB induced phosphorylation of MARCKS or phosphoinositide 3-kinase (PI3K) signaling, as measured by Akt phosphorylation. Further, no difference in cell migration was observed in NIH-3T3 fibroblasts that were transfected with MARCKS siRNAs with or without MANS peptide treatment. Genetic structure-function analysis revealed that MANS peptide-mediated attenuation of NIH-3T3 cell migration does not require the presence of the myristic acid moiety on the amino-terminus. Expression of either MANS or unmyristoylated MANS (UMANS) C-terminal EGFP fusion proteins resulted in similar levels of attenuated cell migration as observed with MANS peptide treatment. These data demonstrate that MARCKS regulates cell migration and suggests that MARCKS-mediated regulation of fibroblast migration involves the MARCKS amino-terminus. Further, this data demonstrates that MANS peptide treatment inhibits MARCKS function during fibroblast migration and that MANS mediated inhibition occurs independent of myristoylation.

Introduction

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a ubiquitously expressed protein kinase C (PKC) substrate that binds both actin and calmodulin (CaM) and regulates actin dynamics. MARCKS is cooperatively tethered to cell membranes by insertion of its myristoylated amino-terminus as well as electrostatic interactions between the basic effector domain of MARCKS and acidic phospholipids of the plasma membrane [1,2]. Phosphorylation of MARCKS by PKC, or CaM binding, results in the release of MARCKS from the plasma membrane into the cytosol in a process called the “myristoyl-electrostatic switch” mechanism [3]. Dephosphorylation or release of CaM results in the ability of MARCKS to return to the plasma membrane. This membrane to cytosol shuttling, or bi-lateral translocation of MARCKS, has been associated with the reorganization of the actin cytoskeleton [4,5], with various cellular processes regulated by MARCKS, including: endothelial cell migration [6], pericellular matrix assembly [7], and phagocytosis [8,9], as well as cell migration [10,11].

MARCKS is involved in regulation of motility in various cell types including fibroblasts [12], myoblasts [13], human embryonic kidney cells [14], human hepatic stellate cells [10], vascular smooth muscle cells [15], neutrophils [16], macrophages [17], mesenchymal stem cells [18] and various cancer cells [11,19,20]. One of the initial steps during cell migration is
adherence of cells to the extracellular matrix, and a role for MARCKS in regulating such cell adhesion has been established. Expression of a mutated MARCKS in which the myristoyl-electrostatic switch mechanism is altered (thus inhibiting MARCKS bi-lateral translocation) resulted in abrogated cell adhesion and spreading [12,13]. Glioblastoma multiforme cells that express a constitutively active variant of the epidermal growth factor receptor (EGFR) underwent decreased adhesion, spreading and invasion when transfected with a siRNA targeting MARCKS [20]. Additionally, MARCKS is localized to focal adhesions during α2 integrin myoblast attachment and spreading and silencing of MARCKS resulted in decreased myoblast spreading [21].

Recently, a unique reagent called MANS, a myristoylated cell permeant peptide corresponding to the first 24-amino acids of MARCKS, has been used to demonstrate a role for MARCKS, specifically its myristoylated amino-terminus, in regulating the migration of neutrophils [16], macrophages [17] and mesenchymal stem cells [18]. These results raised the question as to which aspect(s) of the MANS peptide, as well as the amino-terminus of MARCKS, could be involved in regulation of cell migration, with particular interest in amino-terminal myristoylation, given its role in membrane attachment [22,23]. Fibroblasts, as opposed to neutrophils as previously described [16], were utilized in these experiments for two reasons. First, to determine if myristoylation of MANS is involved in regulating cell migration, a genetic structure-function analysis was performed. Fibroblasts are more suitable for these studies as they are a migratory cell type that are easily transfected, unlike terminally differentiated and difficult to transfect neutrophils. Second, fibroblasts were used in these experiments because they solely express MARCKS [23,34] while neutrophils, a phagocytic leukocyte similar to macrophages, may express MARCKS-like protein (MLP); also called MARCKS-related protein (MRP) or MacMARCKS in addition to MARCKS [24–26]. Both MARCKS and MLP have a myristoylated amino-terminus with approximately 50% homology [22] and are involved in the regulation of cell migration [12,13,27]. Thus, our previous work demonstrating that MANS peptide treatment inhibits neutrophil migration [16] does not rule out the possible involvement of MLP.

Herein, the MANS peptide was utilized to demonstrate that MARCKS is involved in the regulation of directed fibroblast migration, as measured by scratch wounding and PDGF-BB–induced chemotaxis assays. Further, siRNAs that target MARCKS were used to demonstrate that MARCKS expression is not essential to cell migration and that the MANS peptide specifically inhibits MARCKS function. Additional genetic structure-function analysis revealed that MANS peptide mediated inhibition of NIH-3T3 cell migration does not require the presence of the myristic acid moiety on the amino-terminus, as expression of MANS or unmyristoylated MANS (UMANS) C-terminal EGFP fusion proteins resulted in similar levels of attenuated migration. Taken together, the results of these studies support our previous findings that MARCKS regulates cell migration, although MARCKS expression is not essential to the process. These studies also demonstrate that the MANS peptide alters MARCKS function and that myristoylation of the MANS peptide is not required for MANS peptide-mediated inhibition of fibroblast migration.

Results

MANS Peptide Attenuates Migration of Fibroblasts on Fibronecin or Collagen Substrates

MANS peptide treatment significantly decreased NIH-3T3 fibroblast migration on either fibronecin (Figures 1A & B) or collagen (Figure 1C) substrates in a concentration-dependent manner. Treatment of the cells with the myristoylated missense scrambled control (RNS) peptide did not alter the ability of fibroblasts to migrate on either fibronecin or collagen (Figure 1). As a positive control for inhibition of migration, fibroblasts were incubated with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin, which has been shown previously to inhibit NIH-3T3 fibroblast migration [28]. As expected, wortmannin treatment resulted in significantly decreased fibroblast migration compared to non-treated or RNS treated cells, while wortmannin and MANS treated cells had a similar level of inhibition (Figure 1). Taken together, these results are the first to demonstrate that the MANS peptide inhibits fibroblast migration and suggest that the amino-terminus of MARCKS may be involved in regulating this process. Based on these results, additional studies utilized a concentration of 50 μM MANS or RNS.

To confirm that the results observed in our scratch-wounding assay were not due to altered cell proliferation, we performed carboxyfluorescein succinimidyl ester (CFSE) proliferation assays. Briefly, we incubated CFSE-labeled fibroblasts on fibronecin coated plates for 18 hours (duration of scratch assay) in the presence of 50 μM MANS, 50 μM RNS, vehicle control (VC; PBS) or wortmannin. As shown in figure 2, no difference was observed in NIH-3T3 proliferation in MANS or RNS treated cells compared to non-treated, wortmannin or VC treated cells. These results confirm that MANS peptide treatment does not alter cell proliferation within the context of our scratch assay and demonstrates that the results observed in Figure 1 are due to altered cell migration and not proliferation.

MANS Inhibits PDGF-BB Stimulated Fibroblast Chemotaxis

Fibroblasts migrate by both cell-contact cues, as demonstrated in the wound healing process, as well as by directional chemotaxis towards chemoattractants such as PDGF-BB [29–31]. In the past, PI3K has been thought to be the main mediator of cell migration, and several reports have established that PI3K is involved in regulating PDGF-BB mediated fibroblast migration [32–34]. However, PDGF-BB stimulation of Swiss 3T3 fibroblasts and human hepatic stellate cells also results in phosphorylation and membrane to cytosol translocation of MARCKS [10,35–37], suggesting a role for MARCKS in PDGF-BB induced motility. To determine if MANS peptide treatment inhibits PDGF-BB mediated fibroblast migration, a Boyden chamber approach was utilized with fibronecin-coated transwells and PDGF-BB or vehicle control (VC; sterile water) placed in the bottom chamber. A concentration of 1 nM PDGF-BB was used for these studies as we have previously demonstrated this concentration to be optimal for inducing directional chemotaxis in NIH-3T3 fibroblasts [32]. As shown in Figure 3, pretreatment of cells with the MANS peptide attenuated fibroblast migration towards PDGF-BB compared to RNS peptide or PBS (VC) treatment. Similar to the scratch wounding assays, MANS peptide inhibition of migration was comparable to treatment with wortmannin (Figure 3). Interestingly, unstimulated migration in wells containing VC (sterile water) was not affected by MANS treatment, suggesting that MARCKS protein specifically regulates directed migration of fibroblasts.
Figure 1. MANS peptide treatment attenuates migration of NIH-3T3 fibroblasts. NIH-3T3 fibroblasts were grown to confluency on fibronectin (A&B) or collagen (C) coated coverslips and scratch assays were performed with increasing concentrations (1, 50 or 100 mM) of MANS or RNS, VC (PBS) or 100 nM wortmannin. (A) Photos are representative of experiments on fibronectin-coated coverslips; bar is equivalent to 500 μm in length. The average percent wound closure is shown on fibronectin (B) or collagen substrates (C), with four individual experiments for both substrates performed. Statistical 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 reduced ability to migrate back into the wound relative to 1 μM, 50 μM and 100 μM RNS, respectively.
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Figure 2. Fibroblast proliferation is not altered by MANS peptide treatment. CFSE labeled NIH-3T3 fibroblasts (5 x 10^5 cells) were plated on fibronectin coated plates and allowed to adhere. Cells were incubated in DMEM with 2% FBS plus antibiotics in the presence of wortmannin (WORT; 100 nM), PBS (VC), 50 μM MANS or 50 μM RNS for 18 hours and cell proliferation was evaluated by flow cytometry. Data is representative of three independent experiments. NT denotes CFSE-labeled cells that were not treated.
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MANS Peptide Reduces Fibroblast Chemotaxis as Monitored by Live-cell Imaging

To further examine how MANS peptide treatment inhibits MARCKS function and attenuates fibroblast chemotaxis, we used live-cell imaging by total internal reflection fluorescence (TIRF) microscopy. NIH-3T3 fibroblasts expressing GFP-AktPH, a fluorescent biosensor for PI3K signaling, were used to simultaneously monitor cell movement and polarity of intracellular signaling in response to PDGF-BB gradients [32]. Whereas cells treated with RNS control peptide exhibited normal morphology and migration response, cells treated with MANS peptide tended to exhibit smaller contact areas and reduced motility (Figures 4A & B). Chemotactic fidelity was quantified as the fraction of time the cell was moving towards the PDGF gradient (angle within $90^\circ$) less the fraction of time moving away from the gradient (angle between $120^\circ$ and $180^\circ$); by this measure, the population of cells treated with MANS was underrepresented in cells exhibiting high fidelity (Figure 4C). Interestingly, the MANS-treated population was also underrepresented in cells moving predominantly away from the gradient, suggesting a general defect in cell migration persistence. Further analysis showed that, among the cells exhibiting the highest chemotactic fidelity in each population, the MANS-treated cells exhibited markedly less displacement from their original starting positions (Figures 4D & E). Interestingly, there were no differences observed in PI3K signaling as measured by the GFP-AktPH biosensor in either MANS or RNS treated cells. Taken together, these results confirm our Boyden chamber experiments (Figure 3) and demonstrate that MARCKS is involved in the directional migration of NIH-3T3 fibroblasts.

Expression of MARCKS is not Required for NIH-3T3 Fibroblast Migration

To determine whether MARCKS expression is essential to NIH-3T3 fibroblast migration, we transfected cells with two separate siRNAs targeting MARCKS and performed scratch-wounding assays on a fibronectin substrate. MARCKS knockdown was observed 48 hours after transfection with either MARCKS A siRNA or MARCKS B siRNA relative to control siRNA transfected or non-transfected cells (Figure 6A). Equal expression of beta-actin was observed in non-treated cells as well as control and MARCKS siRNA treated cells (Figure 6A). As shown in Figure 6B, no difference in cell migration was observed in non-transfected cells or cells transfected with control siRNA, MARCKS A siRNA or MARCKS B siRNA, suggesting that expression of MARCKS is not essential for migration of NIH-3T3 fibroblasts.

MANS Peptide does not Alter PDGF-BB-induced MARCKS or AKT Phosphorylation

PDGF-BB stimulation results in phosphorylation of MARCKS in Swiss 3T3 fibroblasts [34–36] as well as in human hepatic stellate cells [10]. Given that phosphorylation of MARCKS and subsequent membrane to cytosolic translocation is associated with MARCKS function, we asked if MANS peptide inhibition of PDGF-BB mediated chemotaxis was due to altered MARCKS phosphorylation. To address this question, we performed initial experiments to determine the conditions for optimal PDGF-BB induced MARCKS phosphorylation in fibronectin adherent NIH-3T3 fibroblasts. First, we performed a dose response assay by stimulating cells with 100, 10, 1, or 0.1 nM PDGF-BB for 1 minute and found 10 nM to be the optimal concentration of PDGF-BB to stimulate MARCKS phosphorylation (Figure 5A). Next, we performed a kinetics analysis by stimulating cells with 10 nM PDGF-BB for 1, 5, 10, or 20 minutes and found a 1 minute stimulation with 10 nM PDGF-BB resulted in optimal MARCKS phosphorylation (Figure 5B). To address if MANS peptide treatment alters PDGF-BB induced MARCKS phosphorylation, fibronectin adherent NIH-3T3 fibroblasts were pretreated with MANS, RNS or PBS (VC) for 30 min and then stimulated with 10 nM PDGF-BB for 1 min. As shown in Figure 5C, MANS pretreatment did not alter MARCKS phosphorylation in either VC or PDGF-BB stimulated NIH-3T3 cells, as no difference in MARCKS phosphorylation was observed in MANS, RNS or PBS treated cells. This demonstrates that MANS peptide inhibition of PDGF-BB induced fibroblast motility is not due to alterations in MARCKS phosphorylation.

To determine whether MANS peptide treatment interferes with PDGF-BB mediated PI3K signaling, we evaluated phosphorylation of Akt in cells stimulated with PDGF-BB that were treated with MANS or RNS. As shown in Figure 5C, no differences in Akt phosphorylation were observed in MANS, RNS or PBS treated cells upon PDGF-BB or VC stimulation. These results support the findings of the GFP-AktPH biosensor live-cell imaging experiment (Figure 4) and demonstrate that MANS peptide treatment does not interfere with PDGF-BB induced PI3K signaling.
transfected cells that were treated with or without MANS peptide (Figure 6B). Interestingly, reduced cell spreading was observed in control siRNA transfected cells treated with MANS peptide, whereas normal spreading was observed in MARCKS siRNA transfected cells with or without MANS peptide treatment (data not shown). Further, no difference in cell proliferation was observed within the parameters of the scratch assay in control cells treated with or without MANS peptide (Figure 6B).

Figure 4. MANS inhibition of chemotaxis analyzed by live-cell TIRF microscopy. (A) Time-course montage of a GFP-AktPH-expressing NIH 3T3 mouse fibroblast migrating chemotactically in response to a PDGF-BB gradient emanating from an alginate microsphere (open circle). The cell was treated with RNS peptide and monitored by TIRF microscopy, with localization of PI3K signaling displayed using a pseudo-color intensity scale; scale bar = 100 μm. (B) Montage as in part A, except that incubation was in the presence of MANS peptide, which inhibited productive cell movement. Multiple microspheres in this field are indicated by filled circles; scale bar = 100 μm. (C) Chemotactic fidelity was quantified as the fraction of time intervals during which cell movement was aligned with the PDGF gradient (angle within 60°) minus the fraction of time intervals during which movement was misaligned (angle between 120–180°). The histogram compares cells incubated with RNS (n = 32) versus MANS (n = 27) at peptide concentrations of 10 μM (gray, yellow) and 50 μM (black, red). (D&E) For each of the 4 treatment conditions compared in part C, chemotactic fidelity values were sorted into high, medium, and low subpopulations for RNS (D) and MANS (E). Cell centroid translocation paths are plotted with the initial centroid positions located at the origin and the initial PDGF-BB gradient vector aligned along the positive x-axis. DOI: 10.1371/journal.pone.0066512.g004

Figure 5. MANS pretreatment does not alter PDGF-BB mediated MARCKS phosphorylation or PI3K signaling. (A and B) Adherent fibroblasts were stimulated with the indicated concentrations of PDGF-BB for the indicated times and Western Blot analysis for phosphorylated MARCKS (p-MARCKS), total MARCKS (MARCKS) and beta-actin was performed. (A) Representative dose-response (1 min stimulation) experiment. (B) Representative kinetics analysis (10 nM PDGF-BB) experiment. (C) Adherent fibroblasts were pretreated with 50 μM MANS, 50 μM RNS or PBS (VC for MANS or RNS peptides) for 30 minutes prior to stimulation with 10 nM PDGF-BB or VC (sterile water) for 1 minute. Western blot analysis was performed to determine the expression of phosphorylated MARCKS (p-MARCKS), total MARCKS (MARCKS), phosphorylated AKT1 (p-AKT1), total AKT (AKT) and beta-actin. NT denotes no treatment and VC denotes vehicle control (sterile water); data is representative of three separate experiments. DOI: 10.1371/journal.pone.0066512.g005

Figure 6. MARCKS protein expression is not essential to NIH-3T3 fibroblast migration. (A) NIH-3T3 cells were transfected with control, MARCKS A or MARCKS B siRNAs and total MARCKS and beta-actin expression was determined by Western blot 48 hours post transfection. (B) Scratch-wounding assays on non-transfected, control siRNA, MARCKS A siRNA or MARCKS B siRNA transfected cells were performed with or without 50 μM MANS treatment. Statistical analysis (p<0.05) was performed where “a” denotes a significantly decreased ability to migrate back into the wound relative to non-transfected cells and “b” denotes significantly decreased ability to migrate back into the wound relative to control siRNA transfected cells. Data is representative of three independent experiments. DOI: 10.1371/journal.pone.0066512.g006
siRNA, MARCKS A siRNA or MARCKS B siRNA transfected cells with or without MANS peptide treatment (data not shown). Taken together, this data demonstrates that MARCKS expression is not essential for NIH-3T3 fibroblast migration and further demonstrates that the MANS peptide specifically inhibits the function of MARCKS.

Myristoylation of MANS is not Required for Inhibition of Fibroblast Migration

The MANS peptide is identical to the amino-terminus of MARCKS, including the presence of a myristic acid moiety. Myristoylation can enhance the ability of peptides to translocate across cell membranes (likely promoting the efficacy of the MANS peptide approach to alter MARCKS function in living cells) and contribute to peptide membrane attachment, which may be involved in the mechanism by which MANS affects MARCKS function. We could not determine the role for the myristic acid moiety in the latter mechanism without altering for former, so the role of myristoylation in MANS ability to alter MARCKS function and fibroblast function was addressed using a genetic structure-function approach. We generated pEGFP-N1 expression plasmids encoding C-terminal EGFP fusion proteins of either the MANS sequence or a G2A point mutant that eliminates the myristoylation signal, resulting in unmyristoylated MANS (UMANS). The parent pEGFP-N1 vector served as a control plasmid for these experiments rather than RNS::EGFP. Since RNS is a myristoylated peptide with the same amino acid composition as MANS but in a random scrambled sequence, cloning the RNS sequence into pEGFP-N1 using traditional methods would be challenging. Additionally, myristoyl-CoA:protein N-myristoyltransferase myristoylates proteins at the amino acid consensus sequence is NH2-GXXX(S). While both MANS and RNS peptides are commercially synthesized with an amino-terminal myristic acid, it is likely that only MANS would be myristoylated upon epigenetic expression as it has the sequence NH2-GAQFS. RNS, with a sequence of NH2-GTAPA, would likely not be myristoylated upon epigenetic expression as it lacks a serine in the in the fifth position of the myristoylation consensus sequence [30,31]. Thus, we chose to use the parent pEGFP-N1 vector for the control of these experiments as there is a chance that RNS::EGFP would likely function differently than the RNS peptide.

Western blot analysis using an anti-EGFP antibody demonstrated equal expression of EGFP, MANS::EGFP and UMANS::EGFP proteins 24-hours post transfection (Figure 7A). Western blot analysis also revealed equal expression of MARCKS in non-transfected, EGFP, MANS::EGFP and UMANS::EGFP transfected cells (Figure 7A). Subcellular fractionation revealed that MANS::EGFP appears to be predominantly targeted to the membrane fraction (with some cytosolic localization) while EGFP and UMANS::EGFP are predominantly localized to the cytosol (Figure 7B). Interestingly, in spite of the fact that UMANS::EGFP does not contain a myristoylation signal, the fusion protein did appear to associate with the membrane fraction, albeit less so than MANS::EGFP. As a control for fraction purity and equal protein loading, the expression of p38 MAPK (cytosolic fraction only) and beta-actin were also determined, respectively.

We next determined whether expression of MANS::EGFP affected fibroblast migration in a similar manner to MANS peptide treatment. Evaluation of NIH-3T3 cell migration using the scratch-wounding assay demonstrated that expression of MANS::EGFP significantly decreased migration compared to non-transfected cells and cells expressing EGFP alone. Further, expression of MANS::EGFP attenuated migration to a level approximately equal to that of 50 μM MANS peptide treatment (Figure 8A). Interestingly, expression of UMANS::EGFP also significantly inhibited NIH-3T3 fibroblast migration, similar to MANS peptide treated cells or cells expressing MANS::EGFP (Figure 8A). Further, expression of either MANS::EGFP or UMANS::EGFP in NIH-3T3 cells significantly inhibited migration in response to PDGF-BB in Boyden chamber chemotaxis assays compared to untreated cells and cells expressing EGFP alone (Figure 8B). As in the scratch assay, expression of MANS::EGFP and UMANS::EGFP fusion proteins inhibited PDGF-BB mediated fibroblast migration comparably to 50 μM MANS peptide treatment. There was no difference in unstimulated migration in non-treated, MANS treated or transfected (EGFP, MANS::EGFP or UMANS::EGFP) cells. These results confirmed that the MANS peptide, whether delivered by a cell permeable peptide approach or by epigenetic expression, significantly inhibits fibroblast migration. This data further demonstrates that myristoylation is not required for the ability of the MANS peptide to inhibit fibroblast migration and that other
aspects of the MANS peptide, related specifically to the amino acid sequence, are involved in regulating MARCKS function related to fibroblast migration.

Discussion

Herein, a role for MARCKS in regulation of NIH-3T3 fibroblast migration was demonstrated. Treatment with the MANS peptide, a myristoylated peptide corresponding to the first 24-amino acids of MARCKS that has been shown to inhibit MARCKS function, decreased migration of NIH-3T3 cells in scratch-wounding, PDGF-BB transmembrane chemotaxis assays and live-imaging chemotaxis assays. This finding is in accordance with previous studies from this laboratory demonstrating a role for MARCKS in migration of neutrophils [16], macrophages [17].
mediated neutrophil adhesion is regulated by MARCKS function

no difference in cell proliferation when NIH-3T3 fibroblasts were

Here suggest that MANS peptide mediated attenuation of NIH-

include a substrates is demonstrated. Integrins that recognize collagen

involved in the mechanism by which the peptide inhibits fibroblast

Previously, we demonstrated that isolated neutrophils are

PtACE::EGFP or UMANS::EGFP (Figure 8). Taken together,

et al. observed that increased PDGF-BB induced chemotaxis

observed in human vascular smooth muscle cells

To address if MANS peptide treatment or decreased protein

and stem cells [18]. Given these results, we hypothesize that the amino-terminus of MARCKS is required for regulating MARCKS function during fibroblast migration, given that either the MANS peptide or epigenetically expressed MANS similarly attenuated NIH-3T3 migration. Further experiments are underway in our laboratory is necessary to address this hypothesis.

To begin to address the structural features of the MANS peptide involved in the mechanism by which the peptide inhibits fibroblast migration, we utilized a genetic structure-function approach to express MANS and unmyristoylated MANS (UMANS) C-terminal EGFP fusion proteins in NIH-3T3 cells. Similar expression of EGFP, MANS::EGFP and UMANS::EGFP was observed in transfected cells, as measured by EGFP expression. Further, equal expression of MARCKS was also observed in non-transfected cells and cell transfected with either EGFP, MANS::EGFP or UMANS::EGFP. It should be noted that in some instances, transformation of 3T3 cells results in decreased expression of MARCKS relative to non-transformed cells due to transcriptional down-regulation [40–42].

Previously, we demonstrated that isolated neutrophils are permeable to both the MANS and RNS peptides, presumably because of the amino-terminal myristoyl moiety. Further, subcellular fractionation studies demonstrated that MANS peptide displaces MARCKS from cell membranes in untreated neutrophils, whereas the RNS peptide allows for MARCKS to remain localized to the membrane [16]. Similarly, MANS peptide treatment resulted in displaced MARCKS binding from mucin granules in human bronchial epithelial cells, which correlated with decreased mucin secretion in asthma models [43]. Herein, we demonstrate that MANS::EGFP fusion proteins are preferentially targeted to the membrane fraction of NIH-3T3 fibroblasts, with some cytosolic localization. Conversely, EGFP and UMANS::EGFP are primarily localized in the cytosol, with some localization in the membrane fraction. It should be noted that the exact cellular membrane that MANS::EGFP and UMANS::EGFP localize to and how this affects MARCKS membrane localization have not been determined. However, we hypothesize that neither the MANS::EGFP nor UMANS::EGFP constructs are being retained in the endoplasmic reticulum or Golgi apparatus since we observed similar levels of attenuated cell migration in MANS peptide treated cells as cells that were transfected with MANS::EGFP or UMANS::EGFP (Figure 8). Taken together, these results suggest that the MANS peptide localizes to cell membranes and supports our previous hypothesis that MANS acts by competing with MARCKS for membrane binding sites within cells. Interestingly, the results also suggest that other aspect(s) of the MANS peptide besides the myristic acid moiety may be involved in tethering it to the plasma membrane as we observed low expression levels of UMANS::EGFP in membrane fractions.

Results of previous studies demonstrated that β1-integrin mediated neutrophil adhesion is regulated by MARCKS function although MARCKS does not affect expression of β2-integrins on neutrophils [16]. In the present study, a role for MARCKS in regulating fibroblast migration on both fibronectin and collagen substrates is demonstrated. Integrins that recognize collagen include α1β1 and α2β1 [44] while α1β1 and α2β1 are integrins that recognize fibronectin [45,46]. NIH-3T3 cells express both fibronectin and collagen integrins [45,47] and the results reported here suggest that MANS peptide mediated attenuation of NIH-3T3 migration occurs independent of the specific integrins that mediate adhesion to the substratum.

Herein, we also demonstrate that MANS peptide treatment does not alter the proliferation of NIH-3T3 fibroblasts as there was no difference in cell proliferation when NIH-3T3 fibroblasts were not treated or treated with MANS, RNS, PBS (VC) or wortmannin (Figure 2). This confirms that the decreased migration observed in the scratch-wounding assay (Figure 1) is due to altered cell migration and not cell proliferation. It should be noted that studies have revealed that MARCKS negatively regulates fibroblast proliferation as quiescent Swiss 3T3 cells express high levels of MARCKS mRNA and protein that are down-regulated upon cell proliferation [49]; similar observations have also been observed in other cell types [49,50]. As shown in Figure 5, MANS peptide treatment does not alter MARCKS expression as equal MARCKS protein was observed in non-treated cells or cells treated with MANS, RNS or PBS (VC). Further, siRNA knockdown of MARCKS did not alter cell proliferation in comparison to non-treated or control siRNA transfected cells and MANS peptide treatment did not alter these observations (data not shown). Thus, while MANS may inhibit MARCKS function in regards to cell migration, MANS peptide treatment does not alter MARCKS expression or cell proliferation within the parameters of an 18-hour scratch-wounding experiment. Further experimentation, which does not fall within the scope of this study, is needed to determine if MANS peptide treatment alters the proliferation of NIH-3T3 fibroblasts in experiments lasting longer than 18 hours.

As previously stated, we have demonstrated that MANS peptide treatment of neutrophils, macrophages and mesenchymal stem cells results in decreased chemotaxis [16–18]. Additionally, MARCKS phosphorylation and perinuclear translocation is observed in microglial cells stimulated by the chemoattractant amyloid beta protein, suggesting a role for MARCKS in microglial cell chemotaxis [51–53]. Further, modifications of MARCKS protein expression in hHSCs results in altered PDGF-BB chemotaxis [10]. Herein, we used a Boyden chamber and live-cell imaging approach [32] to show that MANS peptide treatment interferes with directional migration of fibroblasts. In the live-cell imaging experiments, MANS peptide treated cells did not exhibit high fidelity of chemotaxis and had a lesser degree of displacement from their original starting position in comparison to RNS treated cells. This demonstrates that MARCKS functions downstream of the chemokine receptor, possibly assisting in remodeling the actin cytoskeleton and/or relaying signals from the chemokine receptor to integrins that are essential for cell migration.

To address if MANS peptide treatment or decreased protein expression of MARCKS results in similar levels of attenuated migration, we knocked down the expression of MARCKS by siRNA treatment (Figure 6). Using a scratch-wounding assay, we observed similar levels of migration in non-treated, control siRNA treated with MANS, RNS, PBS (VC) or MANS A or B siRNA treated cells. Interestingly, Rombouts, et al. observed that increased PDGF-BB induced chemotaxis occurred in human hepatic stellate cells (hHSCs) that were transfected with a siRNA targeting MARCKS, while decreased chemotaxis was observed in hHSCs overexpressing MARCKS [10]. Further, alterations of human endothelial cell migration were not observed in cells transfected with a MARCKS siRNA [15]. Thus, our data supports those of others and demonstrates that MARCKS expression is not essential for migration of NIH-3T3 fibroblasts. Our data also suggests that an alternative, unidentified mediator compensates for MARCKS function in fibroblasts expressing decreased MARCKS levels. MARCKS is likely preferred over this unidentified mediator during the cell migration process as cells with inhibited MARCKS function due to MANS peptide treatment haveameliorated cell migration while cells with decreased MARCKS expression are not affected by MANS peptide treatment (Figure 6). It should be noted, however, that MARCKS knockdown in human vascular smooth muscle cells
does result in ameliorated cell migration [15], suggesting that the mechanism by which MARCKS regulates cell migration may be cell type specific.

The results in Figure 6 also demonstrate that the MANS peptide specifically inhibits the function of MARCKS during fibroblast migration as MANS peptide treatment does not alter the migration of cells with decreased MARCKS protein expression. As previously stated, our lab has shown that the MANS peptide displaces MARCKS from the plasma membrane of neutrophils as well as from mucin granules of human bronchial epithelial cells [16,43], resulting in decreased cell migration and mucin secretion, respectively. While these studies demonstrate that MANS specifically interferes with MARCKS membrane localization and presumably function, these studies do not rule out the possibility that MANS peptide treatment may also have off-target effects. Given that we did not observe any difference in cell migration in MARCKS A or B siRNA transfected cells that were also treated with the MANS peptide, we can conclude that the MANS peptide specifically interferes with MARCKS function and does not have any off-target effects.

The exact mechanism by which the MANS peptide inhibits MARCKS function has yet to be determined, although it is hypothesized that MANS may compete with the ability of MARCKS to interact with other mediators involved in the cell migration process. At the present time, it is unclear as to the specific MARCKS interactions that the MANS peptide may interfere with, although a possibility could be MARCKS and phosphatidylinositol 4, 5-bisphosphate (PIP2) interactions. The unphosphorylated effector domain of MARCKS binds to PIP2-rich regions in the plasma membrane, thus clustering and sequestering PIP2 molecules. This MARCKS mediated sequestration of PIP2 has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP2. Upon chemotaxtractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP2 and thereby allowing PLC-gamma to hydrolyze PIP2 [54–56]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP2, as the concentration of PIP2 interferes with, although a possibility could be MARCKS and phosphatidylinositol 4, 5-bisphosphate (PIP2) interactions. The unphosphorylated effector domain of MARCKS binds to PIP2-rich regions in the plasma membrane, thus clustering and sequestering PIP2 molecules. This MARCKS mediated sequestration of PIP2 has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP2. Upon chemotaxtractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP2 and thereby allowing PLC-gamma to hydrolyze PIP2 [54–56]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP2, as the concentration of PIP2 interferes with, although a possibility could be MARCKS and phosphatidylinositol 4, 5-bisphosphate (PIP2) interactions. The unphosphorylated effector domain of MARCKS binds to PIP2-rich regions in the plasma membrane, thus clustering and sequestering PIP2 molecules. This MARCKS mediated sequestration of PIP2 has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP2. Upon chemotaxtractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP2 and thereby allowing PLC-gamma to hydrolyze PIP2 [54–56]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP2, as the concentration of PIP2 interferes with, although a possibility could be MARCKS and phosphatidylinositol 4, 5-bisphosphate (PIP2) interactions. The unphosphorylated effector domain of MARCKS binds to PIP2-rich regions in the plasma membrane, thus clustering and sequestering PIP2 molecules. This MARCKS mediated sequestration of PIP2 has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP2. Upon chemotaxtractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP2 and thereby allowing PLC-gamma to hydrolyze PIP2 [54–56]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP2, as the concentration of PIP2 interferes with, although a possibility could be MARCKS and phosphatidylinositol 4, 5-bisphosphate (PIP2) interactions. The unphosphorylated effector domain of MARCKS binds to PIP2-rich regions in the plasma membrane, thus clustering and sequestering PIP2 molecules. This MARCKS mediated sequestration of PIP2 has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP2. Upon chemotaxtractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP2 and thereby allowing PLC-gamma to hydrolyze PIP2 [54–56]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP2, as the concentration of PIP2 interferes with, although a possibility could be MARCKS and phosphatidylinositol 4, 5-bisphosphate (PIP2) interactions. The unphosphorylated effector domain of MARCKS binds to PIP2-rich regions in the plasma membrane, thus clustering and sequestering PIP2 molecules. This MARCKS mediated sequestration of PIP2 has been shown to inhibit phospholipase C-gamma (PLC-gamma) mediated hydrolysis of PIP2. Upon chemotaxtractant stimulation, MARCKS becomes phosphorylated or bound by CaM and dissociates from the plasma membrane, releasing its sequestration of PIP2 and thereby allowing PLC-gamma to hydrolyze PIP2 [54–56]. Actin cytoskeletal dynamics are regulated by the concentration of free PIP2, as the concentration of PIP2

that myristoylation of MARCKS is not essential to it’s function [13]. Given this, we can be fairly certain that unmyristoylated MARCKS would likely not affect cell migration, as adhesion and spreading are key steps to the process of cell motility.

Results of the studies reported here indicate that myristoylation of the MANS peptide itself is not required for MANS peptide-mediated inhibition of MARCKS regulated fibroblast migration. Epigenetically expressed unmyristoylated MANS (UMAN-5-EGFP), which was localized to both the membrane and cytosol, was capable of inhibiting cell migration in a similar manner to MARCKS peptide treatment or epigenetic expression of MAN-5-EGFP. These findings demonstrate that myristoylation of the MANS peptide is not essential for inhibition of cell migration. This data further suggests that membrane localization of the MANS peptide is not essential to disrupting MARCKS function and other aspects of the MANS peptide may be involved in regulating MARCKS function. There are specific amino acids within the MANS peptide (and the amino-terminus of MARCKS) that could potentially be involved in regulating MARCKS function. Candidate amino acids within the amino-terminus of MARCKS that may be involved in regulating cell migration are Lys6 and Thr7, as it is known that proteolytic cleavage occurs at this site by an unidentified protease [63]. Calpain, a potential candidate for the unidentified protease [64,65], is a calcium-activated protease that is localized to the leading edge of polarized neutrophils and is involved in pseudopod formation and chemotaxis [66,67]. It is also involved in lymphocyte function-associated antigen-1 (LFA-1) mediated T-lymphocyte adhesion as well as focal adhesion formation in bovine aortic endothelial cells [68,69]. Calpain is known to cleave MARCKS in myoblasts, resulting in a 55 kDa fragment [64] and inhibition of calpain activity resulted in decreased myoblast migration associated with an accumulation of membrane bound MARCKS [64,70–72]. Further, adenosine triphosphate (ATP)-mediated activation of calpain results in amino-terminal MARCKS cleavage products in virally transduced human bronchial epithelial cells (HBE-1) [73]. Thus, we hypothesize that generation of the six amino acid fragment by proteolytic cleavage of MARCKS between Lys6 and Thr7 may be involved in regulating cell migration, with further experimentation needed to confirm this hypothesis.

PDGF-BB, a known mitogen and chemotaxtractant for fibroblasts, signals through a receptor tyrosine kinase, PDGFR. Signaling through PDGFR increases intracellular Ca²⁺ concentrations and activates PKC, both of which mediate MARCKS function [31,74]. In Swiss 3T3 cells, PDGF-BB stimulation results in phosphorylation of MARCKS and subsequent membrane to cytosol translocation [35–37]. MARCKS can be phosphorylated by PKCζ, PKCε and PKCθ isomorfs in NIH-3T3 cells [75] and PDGF-BB is known to activate PKC-α in various fibroblast lines [76,77]. PDGF-BB mediated MARCKS phosphorylation is dependent on both PKC-α and –e during the migration of hHSCs [10]. Thus, it is likely that PKCζ is involved in PDGF-BB induced MARCKS phosphorylation in the studies reported here, although identification of the exact PKC isoform(s) involved was not performed. Interestingly, MANS peptide treatment did not alter PDGF-BB mediated phosphorylation of MARCKS in adherent NIH-3T3 fibroblasts (Figure 5), demonstrating that MANS peptide mediated attenuation of fibroblast migration is not due to abnormalities in MARCKS phosphorylation. Additionally, there were no observed abnormalities in PI3K activity in MANS or RNS treated cells, as measured by an EGFP-AktPH biosensor (Figure 4) or phospho-Akt Western Blot analysis (Figure 5). PDGF-BB signaling in fibroblasts results in PI3K localization to the polarized leading edge, and inhibition of PI3K...
activity results in attenuated cell migration [28,32]. Given that abnormal PI3K signaling in MANS treated cells was not observed, these results indicate that the MANS peptide-attenuated cell migration occurs independently of PI3K activation. However, PI3K signaling is known to be involved in PKC activation and subsequent MARCKS phosphorylation [76,79], so it appears that while PI3K may affect MARCKS function, MARCKS does not appear to affect PI3K activity.

In summary, these studies have indicated an important role for MARCKS in regulating NIH-3T3 fibroblast migration, although MARCKS expression is not essential to the process. Further, treatment of cells with the MANS peptide, which specifically inhibits MARCKS function, results in ameliorated cell migration. MANS peptide mediated inhibition of cell migration occurs regardless of amino-terminal myristoylation. Given that the MANS peptide has also been shown to inhibit neutrophil, macrophages and mesenchymal stem cell migration [16–18], the results reported here provide further evidence for the concept that inhibiting MARCKS function may be a valuable novel therapeutic approach for various diseases associated with exacerbated cell migration, such as inflammation, injury/repair and metastatic disease.

Materials and Methods

Reagents and Cell Culture

MANS and RNS peptides were synthesized as previously described [39] 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 and were resuspended in sterile water or PBS, respectively. Type II rat-tail collagen and fibronectin (Sigma) 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

MANS and UMANS inserts were PCR amplified from pCDNA4/TO wt MARCKS plasmid with glycine (GCT) to alanine (GCT) point mutation in the second amino acid position for UMANS. MANS and UMANS were cloned into the EcoRI and BamHI restriction sites of pEGFP-N1 (Clonetech, Mountain View, CA); colonies were screened by colony PCR using pEGFP-N1 sequencing primers and positive colonies were sequenced (MWG, Huntsville, AL). Qiagen’s EndoFree Maxi Kit (Qiagen, Valencia, CA; and 0.2% Penicillin (10,000 U/ml) Streptomycin (10,000 µg/ml) solution (Gemini Bio-Products).

Transmembrane Chemotaxis Assay

Fibroblast chemotaxis assays were performed as described [81]. 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 (1 x 10^6 cells/nucleofection or 2 x 10^6 cells/scratch assay) with scratch assays starting 18-24 hours after transfection. For scratch assays on siRNA knockdown cells, one siRNA reaction per scratch was performed, with scratch assays occurring 48 hours after transfection. Two parallel scratches that were consistent in width were made in the monolayer using a standard sterile 200 µl pipette tip. The coverslips were washed with sterile PBS and replaced with DMEM containing 2% FBS and antibiotics. In some experiments, media was supplemented with MANS, RNS, sterile PBS (VC) or 100 nM wortmannin. The T = 0 coverslip was immediately removed and processed prior to incubating the remainder of the plate for 18 hours at 37°C, 5% CO2. Coverslips were processed by fixing in 10% neutral buffered formalin solution (Fisher) and stained with Harris Hematoxylin or Diff-Quick following standard procedures. Coverslips were mounted onto microscope slides and an ocular micrometer was used to measure the wound distance at ten random locations along the scratch under at 40 x. Wound closure distance for each sample was determined by subtracting the average wound closure for each sample from the average initial T = 0 wound distance with data represented as average percent wound closure ± standard error of the mean (SEM). Photographs of the scratches were obtained using a Nikon AZ100 microscope (Nikon, Melville, NY) under bright field conditions.

CFSE Proliferation Assay

NIH-3T3 fibroblasts were loaded with 2 µM CFSE (eBioscience, San Diego, CA) and plated (5 x 10^5 cells/well) on fibronectin (10 µg/ml) coated 6-well plates in complete media (DMEM with 10% FBS and antibiotics). Cells were allowed to adhere to the plate for 2 hours at 37°C, 5% CO2 and then washed twice with sterile PBS. DMEM with 2% FBS and antibiotics was added to each well and cells were treated with either 50 µM MANS, 50 µM RNS, PBS (VC) or 100 nM wortmannin before incubating for 18 hours at 37°C, 5% CO2. Cells were then harvested and fixed in 1% paraformaldehyde in PBS and cell proliferation was evaluated by flow cytometry using an Accuri C6 flow cytometer (30,000 total events collected) with data analysis performed using FlowJo software.

Transmembrane Chemotaxis Assay

Fibroblast chemotaxis assays were performed as described [81]. 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 and washed in PBS. Transfected or non-transfected NIH-3T3 cells were washed and resuspended in sterile serum-free DMEM at a concentration of 5 x 10^6 cells/ml. In some experiments, cells were pretreated with 50 µM MANS, 50 µM RNS, sterile PBS (VC) or 100 nM wortmannin for 30 minutes at 37°C. Chemotaxis buffer consisted of serum free DMEM containing 1 mg/ml fatty acid free BSA; 1 nM PDGF-BB or VC (sterile water) was added to the chemotaxis buffer prior to addition to a 24-well plate with

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fibronectin coated transwells 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, 5% CO₂. 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 or Diff-Quick prior to removing the filters from the transwell and mounting on glass microscope slides. The number of cells on the bottom of the filter was counted in 10 randomly selected high-powered fields (400×) of a light microscope.

Western Blotting and PDGF Stimulation

For PDGF stimulation of adherent cells, NIH-3T3 fibroblasts were seeded and grown to 90% confluence on 6-well plates that were coated with 10 µg/mL fibronectin. 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 50 µM MANS, RNS or VC (PBS) for 30 minutes at 37°C and cells were stimulated with PDGF-BB for the indicated time and plates were immediately placed on ice. Cells were washed with ice cold sterile PBS prior to the addition of RIPA buffer containing protease inhibitors (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50 mM sodium fluoride) 1 mM phenylmethanesulphonylfluoride (PMSF) and 1:100 dilution of Sigma protease inhibitor cocktail) were separated by 4–12% SDS-PAGE. Phospho-MARCKS (Ser 152/156), phospho-AKT1 (Ser 473), total AKT1 and beta-actin antibodies were purchased from Cell Signalling Technology (Danvers MA) and total MARCKS (M-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western Blots were developed by ECL enhanced chemiluminescence (Thermo Scientific, Rockford, IL) and exposed to radiographic film.

Subcellular Fractionation

Subcellular fractionation was performed using the Subcellular Protein Fractionation Kit (Thermo Scientific) 24 hours after transfection according to the manufacturer's instruction. Equal protein concentrations were loaded onto a 4–12% SDS-PAGE and Western blots were performed with the following antibodies: EGFP polyclonal (Santa Cruz Biotechnology), p38 MAPK (Cell Signaling) and Western blots were performed with the following antibodies: EGFP polyclonal (Santa Cruz Biotechnology), beta-actin (Cell Signaling).

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Statistical Analysis

Statistical analysis was performed by Sigma Stat Software (Systat Software, Inc, Chicago, IL) using Student's t-test with P-values less than or equal to 0.05 considered statistically significant.

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Author Contributions

Conceived and designed the experiments: LEO EJS ATM JMH SLJ. Performed the experiments: LEO EJS ATM MKS. Analyzed the data: LEO EJS ATM JMH SLJ. Conceived and designed the experiments: LEO EJS ATM JMH SLJ. Performed the experiments: LEO EJS ATM MKS. Analyzed the data: LEO EJS ATM JMH SLJ. Contributed reagents/materials/analysis tools: JMH KBA SLJ. Wrote the paper: LEO EJS JMH KBA SJL. Edited and reviewed manuscript: ATM MKS.
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