PROTEIN KINASE Cδ REGULATES THE RHYTHM OF CONTRACTILITY DURING GROWTH FACTOR INDUCED MIGRATION

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The skin is an important barrier essential for immune response. When the skin is wounded, the initial step of fibroblast migration into the tissue is required for subsequent restored and normal integrity of the skin. Fibroblasts involved in this process of wound healing require proper signaling critical for cell motility and contractions of the extracellular matrix (ECM). Furthermore, regeneration of dermal tissue represents one of the most intense anabolic processes, requiring a robust vascular system to deliver nutrients and remove dead debris. Endothelial cells are also influenced by the regulation of a provisional ECM by fibroblasts. As a reservoir of growth factors/cytokines for motility/regression, it functionally serves as a dynamic scaffold for vessels to graft into the wound to mediate angiogenesis. Both systems of angiogenesis and fibroblasts remodeling require growth factor induced regulation of motility. During growth factor induced motility, PLCy1 is activated via EGFR/VEGFR/other RTK signaling and produces diacylglycerol for coactivation of PKCδ to regulate MLC-2 contractility in fibroblast and endothelial cells. However, the functional regulation of PKCδ mediated contractile signaling has not been investigated fully, as it specifically relates to upstream signaling. Therefore, it is hypothesized PLCy1 dependent regulation of PKCδ mediates force signaling in these cell types. To test this hypothesis we first investigated the consequence of PKCδ translocation to the membrane for activation and whether localization is implicated in force regulation. To determine whether PKCδ activation during force signaling is mediated at

the membrane, PKCδ was targeted to the membrane by engineering a K-ras farnesylation motif of the c-terminus of PKCδ. Membrane tethering of PKCδ led to increased directional force exertion onto the ECM/substrate, as upstream regulation is mediated by PLCy1. In addition, we also investigated, whether PKCδ regulation was involved in endothelial capillary retraction mediated by initial dissociation signals during wound healing. Endothelial cells were allowed to form cords on Matrigel, and cords were dissociated with CXCR3 ligand, CXCXL-4 (PF4). During this cord dissociation, we found that motile contractility mediated by VEGFR signaling is partially dependent on PKCδ. This study further supports PKCδ as a key regulator of contractility in cell migration.

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PREFACE

Ever since childhood, I have always had a natural disposition to question nature and the world around me, which I perpetuated by my own exploration. The completion of this graduate project required both my heart and soul as I dug deeper into scientific questions involving cell motility. This was also done by concurrently discovering more of myself and reconciling my learning with the *invisible* mirror image of truth that is in the essence of discovery and exploration. Essentially, this project would not have been made possible without the guidance and support of Dr. Alan Wells. Furthermore, the support and help of Dr. Wells' research team, thesis committee, and various other individuals in the school of medicine have also been integral in my development. In addition, I would like to thank my wife, Patrice Thorpe-Jamison for guiding me throughout the graduate process and my parents and my sister for guiding my development from youth into adulthood. In all, I am very thankful for this experience and the support in my pursuit in science.

1.0 INTRODUCTION

Cell migration is essential for organism development, wound healing, immune response, and cancer invasion (Germain et al., 2012; Lauffenburger and Horwitz, 1996; Roca-Cusachs et al., 2013; Stossel, 1993; Wells et al., 1998). As cell migration is a basic necessity to maintain life, cells rely on directional cues to govern successive actions for normal processes in the body, which could be either further migration, contraction, secretion of growth factors/chemokines, etc. The mechanism of cell migration, as governed by extracellular signals has been intensely investigated (Roca-Cusachs et al., 2013; Wells et al., 1998). However, exact predictive and therapeutically targetable mechanisms have remained elusive in many disease models due to dynamic, heterogeneous, and noisy cellular environments in the body. As migration is key to how cells function in many of these disease models such as cancer and developmental disorders, it is also essential in maintaining proper wound healing (Wu et al.; Yates et al., 2007). Deficiencies in wound healing contribute to 6 to 20 billion (USD) in health care cost. These diseases in wound healing, although not as life threatening as other human diseases, affects a significant number of the population estimated to (1%) and (15%) of the elderly (Markova and Mostow, 2012). In efforts to decrease morbidity of defective wound healing, much investigation has occurred in elucidating the precise mechanisms of how cells migrate to regenerate the wound. It is known that cell migration is directed by cellular sensing of local concentration gradients of chemical factors (Roca-Cusachs et al., 2013; Wells et al., 1998). In wound healing,

for cells to migrate into the wound, growth factors such as EGF, PDGF, HB-EGF, VEGF, PDGF, and others are utilized for multiple cell types for regeneration and repair (Roca-Cusachs et al., 2013; Wells et al., 1998). To further understand cell migration mediated by growth factor mediated motility, we explored the role of a downstream target of EGFR/VEGFR signaling, PKCδ, and how it mediates contractility after growth factor stimulation. But more importantly, this study further explores the mechanism and impact of cell migration, as it is an underlying function of individual cells to promote both disease and life.

1.1 BIOPHYSICAL PROCESS OF CELL MIGRATION

From characterizing fibroblasts locomotion, cell migration can be separated into four distinct processes that often function in synchrony. These events during cell migration are: lamellipod extension, leading edge adhesion to the substratum, contraction of the cell body towards the leading edge, and rear end release. To begin cell movement, the lamellipod extension is often the first step to establish cell asymmetry as the cell situates itself for active motility. As initiation occurs, multiple lamellipodia and/or filopodia can emanate from the cell body in which subsequently one lamellipod becomes dominant as the other lamellipodia and filopodia are retracted. Furthermore, additional extensions are suppressed as this dominant lamellipod emerges (Bailly et al., 1998). Persistence of this lamellipodium occurs as long as the original direction is maintained (Wells et al., 1998). Molecularly, these cell protrusions consisting of lamellipodia/filopodia are primarily mediated by the assembly of actin filaments. Neighboring the plasma membrane, the increased polymerization of actin monomers to actin

crosslinks is thought to mediate blebbling which overcomes membrane tension leading to a visible protrusion (Pollard and Borisy, 2003). After a dominant lamellipodium extends, it adheres to the substratum through ECM binding via integrin and non-integrin receptors (Faassen et al., 1992; Huttenlocher et al., 1995). Focal adhesions are formed at or near the leading edge and remain until the cell rear detaches, as the process is repeated (Lauffenburger and Horwitz, 1996). Being linked consequentially to these actions, the cell body is pushed forward through cytoskeletal regulation of contraction in which tension signaling is initially generated at the leading edge. The forces necessary for this cell body contraction towards the extended lamellipodia is primarily generated by myosin, actin, and crosslinking proteins (Goeckeler and Wysolmerski, 1995; Lauffenburger and Horwitz, 1996). The last step requires rear end release where both force mediated by this retraction and calpain mediated cleavage of focal adhesions (Leloup et al.; Shao et al., 2006) releases the rear end towards the cell body for eventual net movement that is based on leading edge direction. As a result of chemotactic signaling, cytoskeletal reorganization shifts sessile cells to an asymmetric, motile morphology. Furthermore, these processes may be synchronous or asynchronous of each other depending on molecular signaling, environment, etc., but in either case each process influences all other processes (Wells et al., 1998).

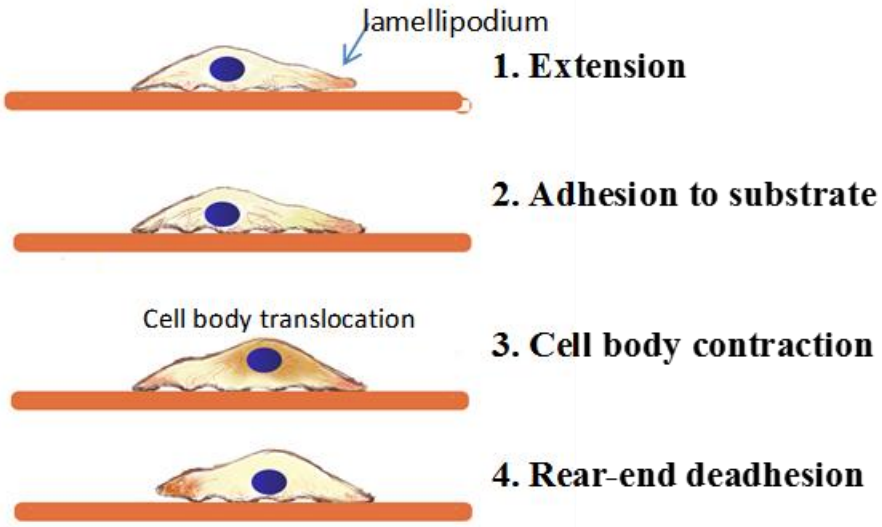

Figure 1. The process of cell migration

This is a schematic of cell migration on a 2D substrate. The first step requires extension of lamellipodium directed schematically to the right, in which net movement is projected to occur. This occurs through adhesions to the substrate, increased actin dynamics at the leading edge with increased crosslinking, and increased turnover of adhesions at the leading edge to obtain a dominant leading edge. The lamellipodium adheres to the substrate, as (EGFR activation of PLCy1 is primarily at the leading edge). Growth factor chemotactic signaling also stabilizes the lamellipodium extension. Cell body contraction towards the extended lamellipodium is needed for most of the cell to productively move. Cytoskeleton reorganization is required to mediate this process, but PLCy1 also mediates this contraction by activating PKCδ that can activate contractility. Finally there is rear release of adhesions through both increased contractility and increased calpain cleavage.

1.1.1 DUROTAXIS (MECHANICAL STIMULIM MODULATING CELL MOTILITY

The body is mostly made of compliant tissue that deforms from a certain amount of exerted force. In addition, human body tissue is also heterogeneous and dynamic, in which the cells are receptive of compliance or stiffness in the cell. As cells respond to the ECM environment in this manner, the cells migrate via durotaxis. Durotactic migration occurs in fibroblasts where cells migrate preferentially from softer to stiffer regions of substrate (Baker and Chen, 2012). As conventional 2D motility occurs on a hard and consistent substrate, studies utilizing soft 2D gels have shown that durotaxis affects cell adhesions, cell differentiation, and cell survival and functionality (Engler et al., 2006; Paszek et al., 2005).

Furthermore, cells can mediate signal transduction during durotaxis as ECM bound proteins or proteins linked to scaffolds connected to the ECM undergo conformational modifications as a result of changes to the ECM (Roca-Cusachs et al., 2012; Roca-Cusachs et al., 2013). More specifically, focal adhesion complexes are modulated by force, as a previous study found that modulation of the focal adhesions through extracellular force causes talin to expose a vinculin binding site (del Rio et al., 2009). This results in vinculin binding as through mechanotransduction signaling of talin. Durotaxis is even more complicated, due to the cell's response to force. Not only does the cell detect externally applied force, the cell must respond appropriately to this force by actively exerting force on the substrate as a result of the deformation that it caused to the substrate. This process as a positive feedback loop, requires cell migration to mediate the function of durotaxis both actively and passively simultaneously.

1.1.2 Theoretical mechanisms of durotaxis

There are two major hypotheses in how cells responsively mediate durotactic migration (Roca-Cusachs et al., 2013). One theory is based on the cytoskeletal structure of the cell, in which the cell has a centripetal flow of actin polymerization toward the cell center. During durotactic movement, forces and deformations that are experienced by the cell through ECM linked proteins modulate the dynamic flow of actin cytoskeleton (Chan and Odde, 2008). An alternative hypothesis indicates that the cell during migration is trying to maintain constant substrate deformation. As substrate stiffness increases, the cell exerts more force to maintain constant deformation onto the substratum (Plotnikov et al., 2012; Wang, 2009). Furthermore, the cell senses and interprets the amount of force required to exert a certain level of substrate deformation. The transmission of force to the substratum could be isolated to individual focal adhesions connected to contractility (Ghassemi et al., 2012; Plotnikov et al., 2012) or the whole cell. For increased net migration of the cell in both cases, focal adhesions linked to the substratum must be broken by force (Chan and Odde, 2008) and calpain protease activity (Leloup et al.; Shao et al., 2006). Furthermore, cell migration that occurs on stiffer substrates requires increased force to break cell to ECM bonds. This partly explains evidence of pronounced stress fibers occurring in cells on stiff substrates such as plastic or glass, as increased force is required to break cell to ECM bonds. Although there is much work to validate these theoretical propositions on the mechanism of how durotaxis is occurring, it is essential to take these theories into consideration during cell migration.

1.2 3D CELL MIGRATION

As new technologies are better able to mimic the *in vivo* microenvironment, increasing consideration of the differences between 2D migration and 3D migration have been observed in the field. Moreover, these differences should be considered to comprehensively extrapolate how cell motility/cell migration occurs irrespective of dimension. One of these differences is that 3D environments have lower substrate stiffness that may impact the regulation of durotaxis (Baker and Chen, 2012). *In vivo* ECM has fibrous, structurally heterogeneous, and anisotropic characteristics (Pathak and Kumar, 2011). Size, shape, and organization of the ECM as well as how the cell is constrained by the ECM impacts the manner of force impacting the cell in 3D. Furthermore, neighboring cells localized within fibrocartilage were found to experience stretch differently based on their proximity and extent of adhesions to the collagen fibrils in ECM (Upton et al., 2008). Additional parameters during 3D migration are cell morphology and orientation to the direction of applied forces (Kurpinski et al., 2006). These parameters have also been shown experimentally to differentially influence gene expression (Heo et al., 2011). Furthermore, durotaxis as discussed previously is modulated differently in 3D environments compared to 2D. Cells in the 2D environment are allowed to deform free of mechanical or physical constraint dorsally in the cell, in which the ventral portion is constrained and attached to the substrate (Baker and Chen, 2012). In addition to constraint in all dimensions, 3D environments force cells to narrow as they elongate under stretch as this action generates stress in all planes of the cell (Baker and Chen, 2012). Moreover in 2D environments, cell migration occurs in the orientation of force in respect to focal adhesions that is tangential (in the same path) to the cell surface (Baker and Chen, 2012). Therefore force is transmitted towards the basal surface of the cell along the stress fibers (Dembo and Wang, 1999; Tan et al., 2003). In contrast

to 2D migration, cells involved in 3D migration are embedded in a matrix and apply force within that matrix mostly perpendicular to the membrane. This force persists through the midline of the cell as it is propelled forward through the matrix (Baker and Chen, 2012).

Differences in the dimension of cell migration have been an important aspect in investigating motility. This study utilized both 2D assays and 3D assays, where the experimental variables involved in the regulation of chemokinectic signaling was not changed in the 3d environment according to our experimental input and output variables.

1.3 WOUND HEALING AND FIBROBLASTS MOTILITY/CONTRACTION

During wound healing fibroblasts and keratinocytes migrate into the wound bed after the fibrin clot is formed, as platelets degranualate releasing growth factors and cytokines as an initial phase for wound healing (Leibovich and Wiseman, 1988). These released growth factors elicit chemokine homing of fibroblasts to the wound and further matrikine signals to induce remodeling, contraction, and synthesis of a collagen rich wound bed (Singer and Clark, 1999). As the fibroblasts are contracting and synthesizing the provisional matrix, fast and responsive signaling cues are needed to induce the appropriate remodeling (Yates et al., 2011). As the provisional matrix matures into acellular, ECM rich dermis, retention of most of the original tensile strength is essential (Yates et al., 2011). Furthermore, the dermis receives and absorbs most of the mechanical load received in the skin, in a wound the ECM strength is immature because it is actively being remodeled by fibroblasts (Langrana et al., 1983; Lauritzen et al., 1981; Timmenga et al., 1991). The forces that are applied via fibroblasts for remodeling must be

concerted and connected as they coordinate for regeneration of the dermis. However, if there is too much contraction then fibroblast and myofibroblast contractility exacerbate the wound to a scar, with overabundant, disorganized ECM (Yates et al., 2011). This is also mediated by fibroplasia through hyper-proliferation with reduced cell death in fibroblasts exacerbate this phenotype (Yates et al., 2011).

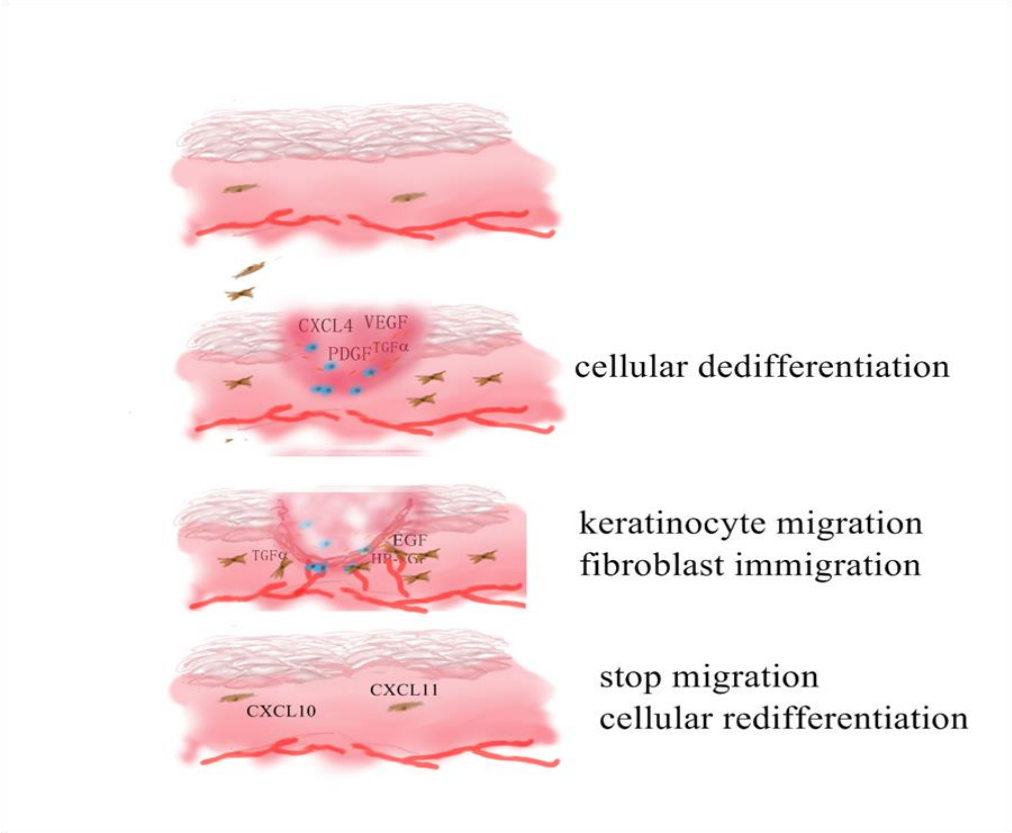

Figure 2. Schematic drawing of the skin wound healing model

Cellular dedifferentiation occurs after the fibrin clot has formed and where platelet degranulation releases both growth factors and cytokines. Afterwards, this partly initiates keratinocyte migration and fibroblast immigration into the wound for repopulation and for synthesis of a provisional and immature ECM for

restoration of tissue. However stop signals mediated by IP-10 and IP-9, CXCR3 ligands, are needed to halt proliferation of fibroblasts and keratinocytes. They are also needed to elicit contraction of the provisional matrix through myofibroblasts.

1.4 DYNAMICS OF ANGIOGENESIS DURING WOUND HEALING

In conjunction with fibroblast regulation of the ECM, increased vasculature through the formation of new blood vessels is required for wound healing. The developing capillary sprouts digest the basement membrane and then penetrate through it in order to invade into the stroma. As new capillaries have not yet formed, endothelial cells organize into tube-like structures that extend, branch, and network until vessels stabilize and quiesce for diapedesis. Proliferation also feeds this growing network as chemotaxis leads from the front (Clark et al., 1996; Madri et al., 1996). In the context of wound healing, after 4 days, capillary sprouts mediate this same process as previously described (McClain et al., 1996). Furthermore, the initiation of this process of invasion into the platelet plug precedes fibroblast invasion (Tonnesen et al., 2000). This timing also supports that capillary tips of angiogenic blood vessels are surrounded by plasma-derived fibrin and fibronectin, as the migrating wound fibroblast-derived ECM composed of fibronectin and hyaluronan appears slightly later (Tonnesen et al., 2000). The specific dynamics of angiogenesis where endothelial cells migrate from preexisting vessels to form new vessels is tightly regulated, in which VEGFR signaling is a central component to this regulation. Notch signaling allows for the differentiation between tip and stalk endothelial cells. The tip cells lead a group of endothelial cells to migrate away from preexisting vessels through downregulation of stalk cell VEGFR levels, as these cells are not leading the sprout (Hellstrom et al., 2007; Lanner

et al., 2007). This also is modulated by chemotaxis in which the individual motility of the tip cells is important for the initial sprout as they migrate towards increased VEGF/GF concentrations. In addition, growth factor induced motile signals disrupt quiescence, which also contribute to dissociation and the propulsion of the new sprout. Growth factor induced PKCδ regulation of contractility may also be involved in the process of endothelial retraction. Furthermore, inadequate angiogenesis has been shown to be a predominant pathology of chronic and poorly healing wounds influenced by the complications of venous stasis disease, diabetes, and aging (Bodnar et al., 2006; Yates et al., 2007) .

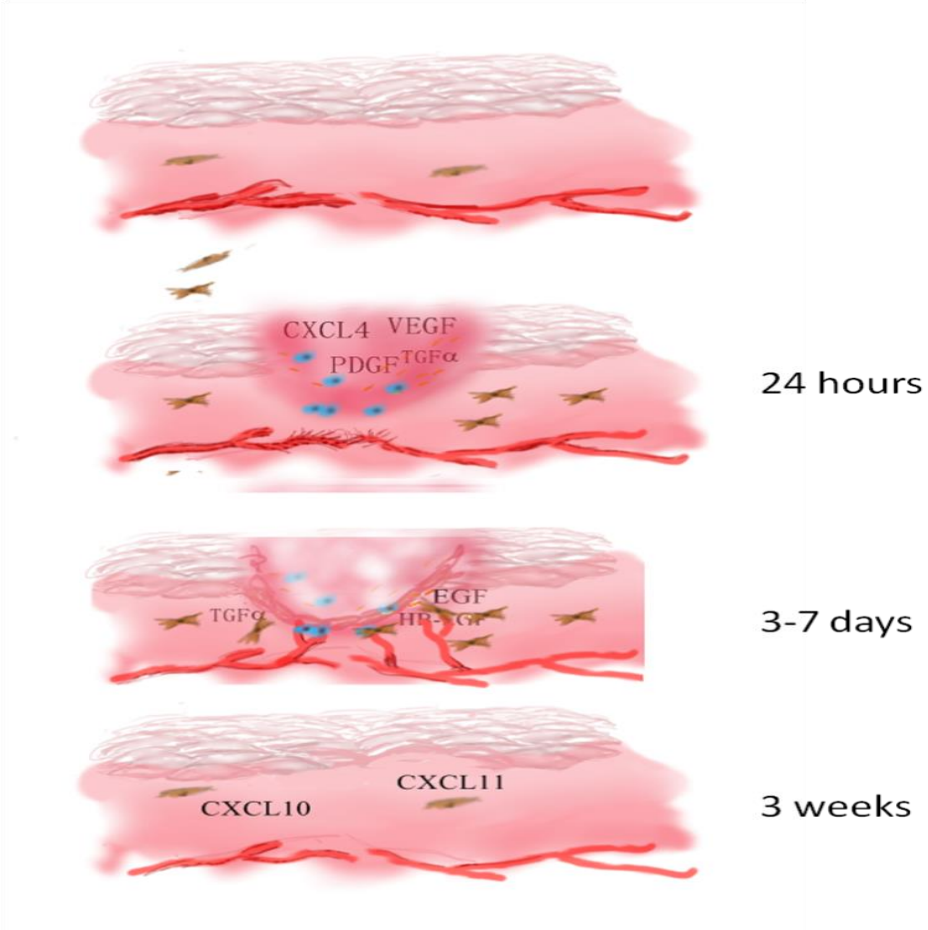

Figure 3. Angiogenesis during Wound healing.

In the initial 24 hours, platelets have migrated into the wound to form a fibrin clot. In addition macrophages are also localized in this early wound phase digesting cellular debris, as they also combined with platelet release of chemokines and growth factors. After 4 days, neoangiogenesis is driven by increased proliferation and migration of endothelial cells into the wound. There they help vascularize the tissue for additional remodeling. After a few weeks, vascularization regresses from the dermis to leave an acellular matrix rich tissue that restores the skin close to its original integrity. CXCR3 chemokines, such as IP-10 are needed to cause regression of angiogenesis and stop proliferation and migration of endothelial cells

1.5 REGULATING CELL MIGRATION VIA RECEPTOR TYROSINE KINASES

Receptor Tyrosine Kinases (RTK) such as Epidermal Growth Factor Receptor (EGFR) and Vascular Endothelial Growth Factor Receptor (VEGFR2) are master regulators of signaling cascades involved in motility, proliferation, and survival of cells, especially during wound healing. They activate numerous molecules leading to mitogenic and motile signaling cascades in the cell. EGFR agonists such as HB-EGF, EGF, TGFα, and etc., are released in the tissue to promote juxtacrine, matrikine, and chemokine signaling for motility (Wells et al., 1998). Liberated and bound ligands bind to EGFR at the cell surface, where EGFRs dimerize and cross-phosphorylate cytoplasmic tails for further transactivation (Ferguson, 2004). The kinase activity of EGFR and other RTKs leads to phosphorylation on primarily tyrosine residues on numerous

substrates. Among these substrates phosphorylated by EGFR and VEGFR, phospo-lipase c γ-1 (PLCγ1) is a known substrate that is directly phosphorylated by RTKs (Bates et al., 2002; Margolis et al., 1990; Wells et al., 1999). This activation increases the activity of PLCγ1 to hydrolyze phosphoinositide bisphosphate (4, 5) (PIP2) into diaclyglycerol and IP3. IP3 regulates calcium channels which converges with other PKC regulation, but diaclyglycerol is known to mediate coactivation of novel PKC family members and augment further activation of kinase activity of these proteins through non-mitogenic signaling (Iwabu et al., 2004; Wells et al., 1999). Subsequent PKCδ kinase signaling converges with other growth factor induced signals to increase motility while other pathways through RTK signaling mediate survival and proliferation. In addition, PKCδ that is downstream from EGFR signaling has been shown to be an important regulator of cell speed through systems biology (Kharait et al., 2007).

1.6 PKC-FAMILY (SERINE/THREONINE) KINASES

PKCδ is part of the serine/threonine PKC family that makes up approximately 2% of the human kinome (Rosse et al., 2010). These kinases are conserved throughout eukaryotes from yeast, to fruit fly to mammals (Mellor and Parker, 1998). The kinase domain in all PKC isoforms is highly conserved and is located next to a hinge region which links it to a n-terminal regulatory domain. In many PKC family members, this n-terminal regulatory region is held inactive by a pseudosubstrate auto-inhibitory motif, which in the inactive state binds to the binding pocket for PKC substrates. Once activated, PKC is also modulated by the binding of second messengers and/or allosteric effectors at its regulatory domain, in which this regulation typically occurs close to the plasma membrane. All of these events disrupt conformational auto-regulation by

displacing the bound pseudosubstrate region from the active site of the kinase domain allowing activation of PKC (Oancea and Meyer, 1998).

This family of kinases have overlapping function and the PKC family can be divided into four distinct subgroups, mostly distinguished by their divergent regulatory domains. The most notable class is the typical or conventional PKCs (cPKCs), comprising of PKCα, PKCβ and PKCγ. They are activated allosterically by diacylglycerol and phospholipid binding at their conserved region 1 (C1) domains and are activated by Ca^{2+} -dependent phospholipid binding at their C2 domains. The novel PKCs (nPKCs) consisting of PKCδ, PKCε, PKCθ, and PKCη, are also allosterically activated by diacylglycerol, but their phospholipid binding is Ca^{2+} independent (Rosse et al., 2010).

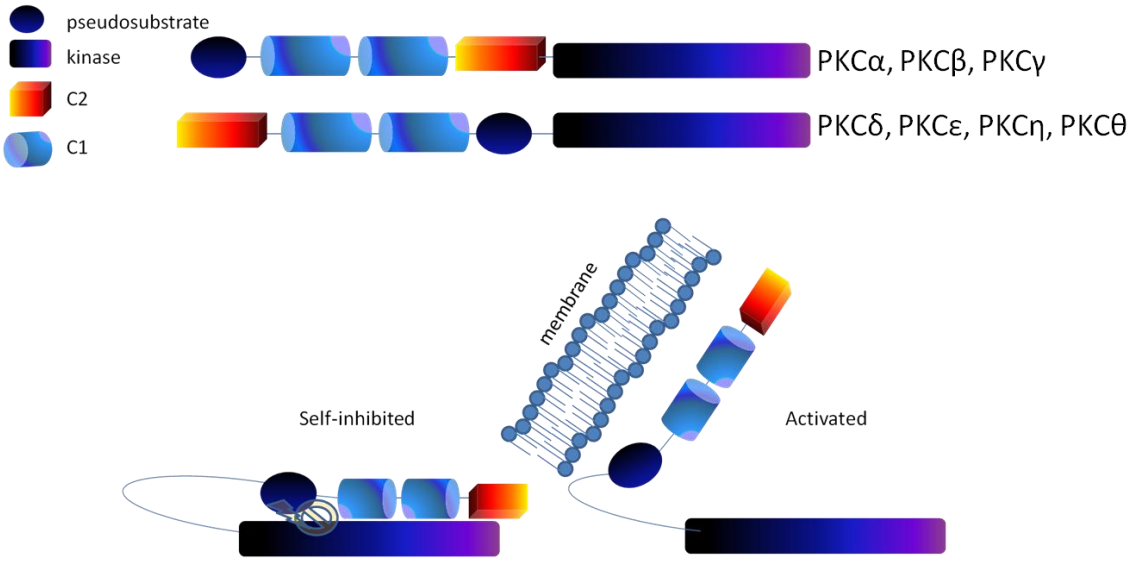

Figure 4. PKC family

This figure depicts a schematic of the protein structure of the PKC kinase family. The first group is the typical or conventional PKC family members which are calcium sensitive. The novel PKC family members have modifications in their regulatory regions rendering them calcium insensitive. b) Indicates the structure of the kinase when it is auto-inhibited by its pseudosubstrate region, upon

activation and membrane localization the pseudosubstrate inhibition is prevented, and its catalytic kinase domain is liberated to phosphorylate substrates for signal transduction.

1.6.1 Specific PKCδ structure and activation

PKCδ among other PKC isoforms contain an N-terminal regulatory domain, two membrane targeting region (C1, C2), and a conserved c-terminal catalytic domain that has catalytic kinase activity and ATP substrate binding (Stahelin et al., 2004). These motifs are contained within all the PKC family of proteins. However, the C1 domain of PKCδ, (and novel PKC family members) does not bind to calcium but only to diacylglycerol and phosphotidyl serine. PKCδ has two C1 domains comprised of two beta sheets and a short c-terminal alpha-helix (Kikkawa et al., 2002). Diacylglycerol (PMA) can bind to this region increasing hydrophobicity causing the n-terminus to be more hydrophobic promoting increased membrane targeting. These actions eventually result in conformational change releasing pseudosubstrate auto-inhibition (Stahelin et al., 2005; Stahelin et al., 2004). Eventually PKCδ in the open confirmation is targeted to the membrane where activation is stabilized by PDK1 phosphorylation (Newton, 2003), as PDK1 is stabilized by PIP3 anchoring at the membrane. Interestingly, EGFR activates PI3 kinase which phosphorylates PIP2 into PIP3 and possibly synergizing the anchoring of PDK1 (Choi and Jeong, 2005; Paradis et al., 1999; Toker, 2003). In addition to these factors, further auto-phosphorylation activates PKCδ further on the turn motif S643 residue (Ron and Kazanietz, 1999; Seki et al., 2005). However different mechanisms of phosphorylation to further activate PKCδ are based on cell type/stimulus. Due to a nuclear localization sequence, PKCδ has been found to actively translocate to the nucleus upon certain

stimuli. Non-receptor tyrosine kinase, Src, has also been found to directly phosphorylate PKCδ. Furthermore, Src kinase mediates phosphorylation on specific tyrosine residues on PKCδ in transformed mouse keratinocytes, as this contributes to alterations of tight junctions resulting in a differentiated phenotype (Joseloff et al., 2002). Additional data also associate Src signaling to force, as seen in cyclic uni-axial stretching of endothelial cells (Katanosaka et al., 2008; Wang et al., 2001b). Stretch induced activation of Src tyrosine kinase leads to increased phosphorylation of focal adhesion proteins including FAK, p130Cas, and paxillin (Joseloff et al., 2002; Katanosaka et al., 2008; Wang et al., 2001a).

1.6.2 PKCδ molecular function

PKCδ is expressed in numerous cell types and has been found to mediate numerous functions: cell motility, contraction, apoptosis, differentiation, anti-proliferation ((Bertram and Ley, 2011; Breitkreutz et al., 2007; Puceat and Vassort, 1996; Saito, 1995; Soltoff et al., 1998; Weinstein, 1991). In mouse studies with PKCδ deletion, PKCδ-KO mice had a normal life-span and normal fertility. However, mice displayed increased B-lymphocytes due to decreased cell death, which led to an autoimmune phenotype (Miyamoto et al., 2002). In other organ systems such as the bone, PKCδ-KO mice embryos showed decreased ossification and increased chondrocyte maturation (Tu et al., 2007). In addition, further studies have also indicated that PKCδ is involved in vessel formation and angiofactor formation in diabetic limbs, kidneys, and retinas (Geraldes et al., 2009; Lizotte et al., 2013; Mima et al., 2012). These studies suggest PKCδ involvement in cell proliferation and cytoskeleton function. As a kinase, PKCδ has been found to interact with numerous substrates in many disease models. Moreover, its translocation to many cellular compartments such as the cytosol, nucleus, mitochondria, membrane, vesicles,

and numerous cellular sites for protein scaffolds have been documented in the literature. For cell motility, numerous interactions were found in different systems that impacted migration. EGFR signaling can be attenuated by PKC agonist, increasing phosphorylation at its c-terminus and directly inhibiting EGF binding to EGFR (Wells et al., 1998). Furthermore, PKCδ can localize and suppress junctions by direct phosphorylation of overexpressed E-cadherin (Chen and Chen, 2009) or direct interaction with p190RhoGaP and FAK to increase multicellular permeability (Fordjour and Harrington, 2009; Grinnell and Harrington; Harrington et al., 2005). Although direct cytoskeletal regulation through PKCδ was found, these studies did not fully investigate implications to the mechanism of cytoskeletal regulation. A previous study investigated how EGFR dependent regulation mediates phosphorylation of an intermediate kinase, MLCK (Iwabu et al., 2004). This activation of MLCK is mediated through activation of PKCδ through conventional membrane targeting is augmented by EGFR signaling/DAG regulation (Iwabu et al., 2004). However, direct interaction has not been found that suggests the role of an intermediate kinase which is beyond the scope of this study. At increased kinase activity, MLCK can directly phosphorylate myosin light chain which then allows for active MLC-2 to regulate the affinity of myosin heavy chain to actin by treadmilling actin/myosin interactions important for contraction (Pasapera et al., 2010; Totsukawa et al., 2000).

1.7 EGFR REGULATION OF ASYMMETRIC SIGNALING FOR MOTILITY

EGFR phosphorylation of PLCy1 is localized at the leading edge membrane (Wells et al., 1999), as phosphoinositides such as PIP_2 are prevalent throughout the membrane and organelle compartments. Furthermore, phosphatidylinositol (3,4,5)-triphosphate (PIP3) is distributed at

the leading edge and has been implicated for its activity in directionality and persistence in cell motility (Chen et al., 1996; Turner et al., 1996; Wells et al., 1998). Interestingly phosphatidylinositol (4,5)-bisphosphate (PIP2), that could also be generated from PIP3, has been found to be an important cofactor in many proteins involved in motility, notably for proteins that have PH domains causing targeting to the membrane. As shown in (Fig. 4), PLCy1 catalyzes PIP2 primarily at the leading edge during motility and generates diacylglycerol and IP3 (Insall and Weiner, 2001; Wells et al., 1999). This catalysis of PIP2 may be one of the many factors driving cell asymmetry, where amounts of PIP2 are at low stabilized levels at the leading edge (Shao et al., 2006).

1.8 PKCδ REGULATION OF ASYMMETRIC SIGNALING FOR MOTILITY THROUGH MLC ACTIVATION

During asymmetric signaling PKCδ is localized behind the leading edge but in front of the nucleus (Fan et al., 2006). PKCδ is also localized to membrane fractions that are at the leading and internalized endosomes that are being recycled during EGFR signaling (Llado et al., 2004; Wadsworth and Goldfine, 2002). PKCδ localization behind the leading edge is thought to facilitate PKCδ translocation to the membrane upon RTK stimulation as it remains activated in the cytoplasm to induce MLCK to MLC phosphorylation for contractility. This is presumed to coordinate with cell body contraction towards the lamellipod during active cell motility and other cellular functions not investigated. System analysis accurately predicts activation of PKCδ to correlate with increased cell speed and activation of MLC also correlates with increased 2D cell speed (Kharait et al., 2007). Taken altogether, these data indicate the spatiotemporal distribution

of PKCδ is important for growth factor induced motility. Furthermore from a previous investigation, PKCδ regulated force signaling in an EGFR/PLCy1 dependent manner (Iwabu et al., 2004). When EGFR signaling to PLCy1 was inhibited PKCδ activation and MLC activation was delayed (Iwabu et al., 2004). In addition delay in isometric force was also observed when this pathway was inhibited (Iwabu et al., 2004), suggesting PKCδ and MLC as major downstream mediators to this pathway. However, from these data it was not known how the intrinsic translocation of PKCδ affects activation and how asymmetric signaling of force was occurring. Correlating strongly with durotaxis, PKCδ and associated activation mediate signaling that regulate adhesions during basal motility as it indirectly affects cell-substratum interaction. Including this study, more evidence is attributing PKCδ specifically serving as a communication between adhesion receptors during cell migrations.

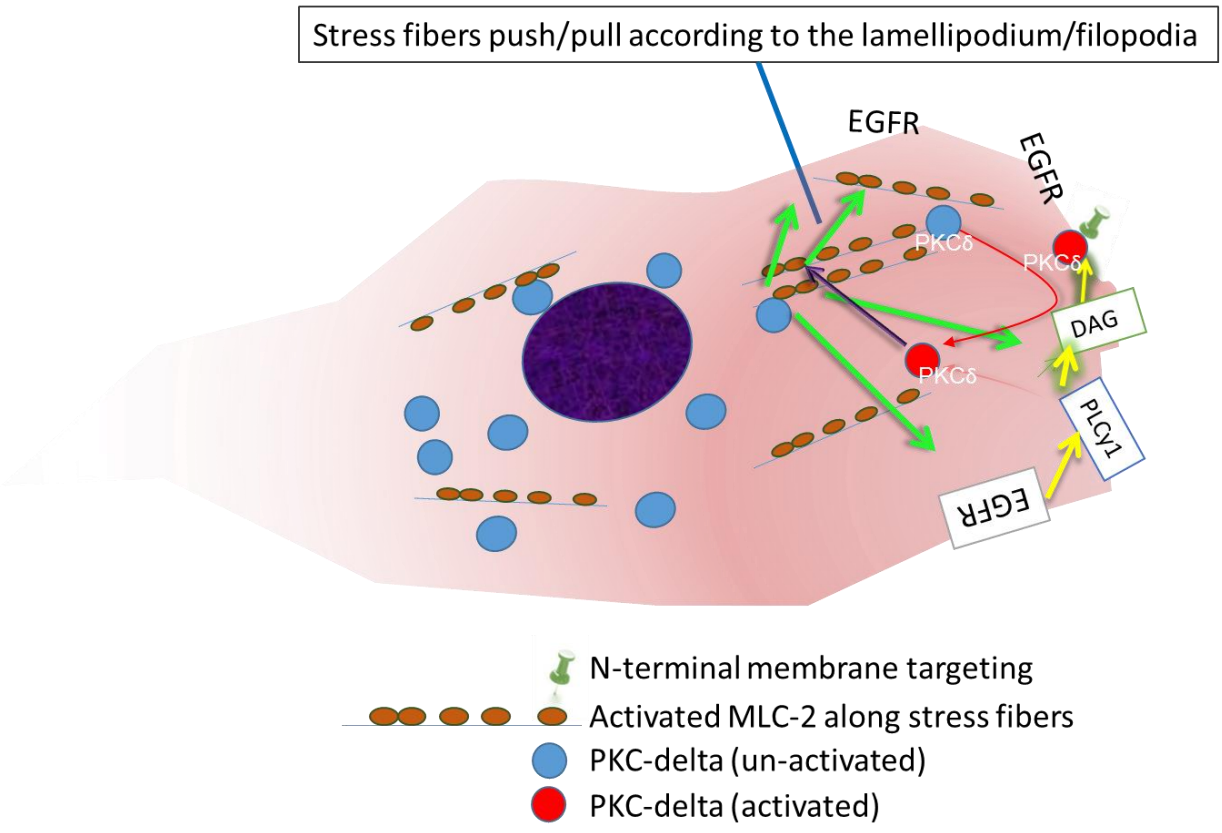

Figure 5. PKCδ regulation of stress fibers according to lamellipodium/filopodia

During growth factor induced motility, EGFR upregulate PLCy1 at the leading edge. This activation induces PLCy1 hydrolysis of PI 4,5 bisphophate into diacylglycerol and IP3. Resultant diacylglycerol and phosphotidyl serine as coactivators of PKCδ cause translocation of PKCδ to the membrane. Further activation occurs at the membrane to signal downstream for MLC-2 activation. MLC-2 then regulates the actin/myosin heavy chain interaction influencing contraction in motility as it is enriched near membrane protrusions and retraction areas

1.9 OTHER REGULATION OF CONTRACTILITY DURING MOTILITY

Motility is regulated through complex pathways mediating dynamic regulation of cytoskeleton and the connecting plasma membrane. Other divergent pathways also mediate contractility through Rho-GTPASE family of proteins, as these proteins regulate cytoskeleton actin nucleation and actin polymerization at the leading edge and through filopodia (Hall, 2012; Negishi and Katoh, 2002). RhoA, RhoB, and RhoC in the GTP-bound activated states regulate ROCK1 and ROCK2 kinase activity (Chaturvedi et al., 2011; Ridley, 2013; Shi et al., 2009; Vega et al., 2011). Furthermore, this activation causes ROCK1 to activate Lim kinase that would subsequently regulate cofillin (Vardouli et al., 2005), ROCK has been found also to phosphorylate cytoskeleton proteins such as MLC and Myosin phosphatase to regulate contractility (Hall, 2012; Totsukawa et al., 2004). PKCδ regulates contractility in conjunction with other cytoskeletal proteins but that PKCδ plays a more upstream role in chemotaxis/chemokinesis signaling. Furthermore, PI3 kinase regulation of PIP3 by EGFR to

stabilize lamellipodia extension also divergently connects to PIP2 hydrolysis through diacylglycerol linked to PKCδ that induces increased contractility as previously described. Increasedelucidation of molecular signaling duringcell motility implicates phosphoinositides at the membrane as major coordinators driving chemotaxis. These studies also suggest that these pathways converge to mediate motility, implying a simple straight forward model may not accurately depict the major driving forces of cell migration.

1.10 CXCR3 REGULATION OF CONTRACTION IN THE WOUND

As both motility and isometric force are involved in remodeling collagen or compacting a provisional wound matrix (Allen et al., 2002), key regulators of growth factor induced motility are essential in understanding how the wound bed contracts appropriately. During the resolving phase of wound healing, 'stop' signals (CXCL10 and CXCL11) are needed to cause regression of proliferation and migration into the wound by fibroblasts, keratinocytes, and endothelial cells. This regression in healing tissue is required and if not properly regulated, results in increased scarring due to fibroplasia and hyper-proliferation of keratinocytes (Yates et al., 2007). This results in decreased collagen organization and decreased tensile strength and integrity of the skin. Pathological phenotype is shown in CXCR3 KO mice where in mouse wounded skins there is hyper proliferation of fibroblasts with a disorganized less contracted collagen matrix (Yates et al., 2007). Furthermore, this results in a delay in wound healing in which wounds take longer to heal compared to mice with normal CXCR3 activity (Yates et al., 2007).

1.10.1 CXCR3 regulation in context of spatiotemporal regulation in fibroblast

Further investigation of individual cells reveals CXCR3 is a mediator of complex intercellular communication involved in migration/proliferation/contraction of the wound. CXCL11 (IP9) and CXCL10 (IP10) are secreted by macrophages and keratinocytes as keratinocytes regenerate the basal cellular layer of the epidermis (Satish et al., 2005). Furthermore, these cytokines were first shown to elicit signals that decreased cell motility and decrease proliferation (Yates et al., 2011). Motility is inhibited through CXCR3 mediated activation of PKA, which inhibits m-calpain activity (Leloup et al.). This is dominant over EGFR mediated upregulation of m-calpain activity. As m-calpain is involved in a critical step of motility for rear-retraction, CXCR3 activation by IP-10 and IP9 would inhibit rear retraction for productive motility (Leloup et al.). EGFR activation of ERK phosphorylation for m-calpain would also increase the activity in combination with m-calpain binding of PIP2 that has been shown to critically activate its protease activity (Leloup et al.). This spatio-temporal regulation also is in concert with contractility, at which IP-10 and IP-9 would cause inhibition of rear retraction but would potentiate the cell to increase isometric contraction onto the matrix directed from the lamellipodium. This potentiation of contraction was investigated in this study through an endothelial cell model. Furthermore, CXCR3 likewise modulated calpain activity that affected isometric contraction in the endothelial capillaries undergoing dissociation.

1.10.2 CXCR3 regulation of vessels

As a wound is formed, capillaries that are damaged induce angiogenic regenerative signaling of severed vessels. As pro-angiogenic growth factors are released by platelets and

macrophages, individual endothelial cells involved require initial signals to 'dedifferentiate' and dissociate from vessels. These signals are mediated primarily by growth factors VEGF/PDGF/HB-EGF/TGF. In addition, CXCL4 (PF4) also mediates dissociation through CXCR3 signaling (Bodnar et al., 2006). This was previously shown that CXCR3 'stop' signaling mediated by IP-10 and PF4 inhibits neoangiogenesis in the wound, if inhibited the wound vasculature would become leaky. PF4 activation of CXCR3 initiates vessel regression through increasing apoptosis, decreasing migration, and increasing capillary dissociation (Bodnar et al., 2006). This mechanism of capillary dissociation related to motility is through CXCR3 activation of PLCβ, which increases μ-calpain activity by increased calcium levels (Bodnar et al., 2009). These calpains were found to mediate dissociation through disrupting Integrin β3 bonds in the cytoplasm that translate to the ECM and cell to cell adhesions. As this regulation is occurring, force signaling is presumed to occur through RTK signaling of force through PKCδ.

1.11 POSSIBLE PKCδ DEPENDENT REGULATION OF ENDOTHELIAL DISSOCIATION THROUGH CXCR3

It has been well established that VEGFR signaling to PKCδ is involved in cord dissociation and increased capillary permeability when activated (Bates et al., 2002; Goeckeler and Wysolmerski, 1995; Rahman et al., 2001) and PKCδ inhibition results in reduced regulation of stress fibers and focal adhesions in endothelial cells (Joyce and Meklir, 1992; Rahman et al., 2001; Tinsley et al., 2004). When PKCδ is knocked-down in diabetic mice, endothelial dysfunction occurs as this results in less VEGF, TGFβ, and extracellular matrix proteins in

kidney glomeruli. This also caused less albuminuria and attenuation of kidney dysfunction (Mima et al., 2012). Additional *in vivo* data involving PKCδ in endothelial retraction and barrier function, indicated that PKCδ was responsible for decreased blood flow, capillary density, and number of capillaries in diabetic mice (Bai et al., 2010). Although these data are in the context of hyperglycemia, data from the literature on PKCδ strongly suggest its involvement in endothelial cell retraction and cell survival. Furthermore, PKCδ was also found at steady-state activation of cytoskeleton tension through junctions and actin fibers promoting endothelial cell barrier (Harrington et al., 2005; Yuan, 2002)]. Conversely activated PKCδ promotes decreased vascular permeability, as it alternatively provides signaling for structural function. In endothelial retraction, it has been found that MLCK, downstream of PKCδ (Goeckeler and Wysolmerski, 1995; Sheldon et al., 1993), regulates endothelial retraction via MLC activation. VEGF has been shown to induce increased cellular force to the ECM via CTFM (Ghosh et al., 2008; Yang et al., 2011). Furthermore, endothelial capillaries are also responsive to shear stress that induce VEGF and growth regulation (Gan et al., 2000; Masumura et al., 2009; Urbich et al., 2003; Wang et al., 2005). Capillaries must also dissociate in order for immune cell extravasation that requires force to break intra/intercellular bonds for cells to dissociate. Capillary dissociation for angiogenesis requires VCAM, VE-Cadherin bond breakage in order for the endothelial cells to dissociate. However, this breakage requires force at a molecular level, but also at a cellular level in which cell and tissue morphology applies to force dissociation. At the *in vitro* tissue level, it is not well understood how PKCδ is regulating force signaling in capillaries. Furthermore, the role of PKCδ mediating endothelial cord retraction has not been determined in the context of CXCR3 upregulation.

1.12 FIBROBLASTS AND ENDOTHELIAL CORDS REGULATE CONTRACTILITY THROUGH SIMILAR MECHANISMS OF PKCδ

Although these two cell types regulate contractility with different mechanisms and cellular functions, PKCδ regulates force signaling similarly with different spatiotemporal context for cell migration and isometric force exertion. In these experimental models, spatio-temporal regulation through EGFR/RTK signaling has been well elucidated in motility and contractions. However the spatiotemporal modulation of force signaling mediated by PKCδ has not been investigated. The regulation of PKCδ in the context of CXCR3 mediated dissociation has also not been investigated. It is possible that dynamic localization of PKCδ may impact dissociation of endothelial cords in the same way as fibroblasts mediate ECM compaction. Endothelial cords as a model system can be visualized systematically as multicellular force is generated. In contrast, impact of force of fibroblast in an ECM can only be measured through its effects on collagen compaction itself which is less direct.

1.13 MEMBRANE-TARGETING WITH FARNESYLTRANSFERASE

A technique previously established (Leloup et al.) would drive increased placement of PKCδ spatially close to the membrane. This involves splicing 6 contiguous lysines followed by c-terminus Kras-farnesylation motif (CaaX) onto PKCδ for increased membrane targeting. prenyl modification is covalently placed on the cysteine residue through a farnesyltransferase in which the aliphatic amino acids are replaced with a methyl group. This occurs through a biochemical process of 3 steps. The actual binding of this motif involves both electrostatic and

hydrophobic interactions to increase the affinity for the membrane (Leloup et al.). The K-ras protein instead of H-ras was also selected due to its constitutive activity in cells for prenylation, whereas the H-ras motif is prenylated in select systems and induced with select stimuli. Therefore, this robust technique was utilized to target PKCδ to the membrane.

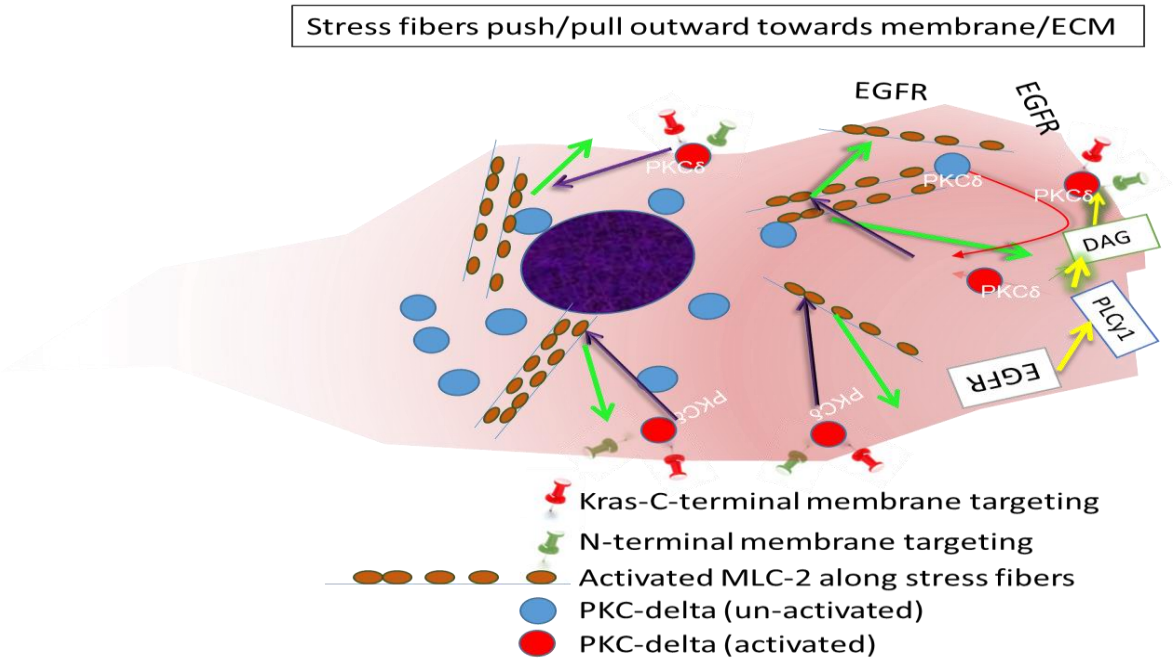

Figure 6. Membrane targeted PKCδ regulation of stress fibers that are directed towards PKCδ regulations at membrane/to ECM

K-ras membrane targeting (CaaX) constitutively places PKCδ to the membrane from its c-terminal. With this modification, the C1 domain of the n-terminal region isable to bind to DAG/PS that is generated at the leading edge independent of translocation. This localization would increase the probability that PKCδ could be activated by diacylglycerol and drive p-MLC-2 activity for stress fiber activity outwards towards the ECM and thus promote increased contractility in tissues through isometric contraction.

1.14 *HYPOTHESIS*

It is hypothesized that PKCδ modulates the distribution of force from VEGF/EGFR signaling. As an essential location where PKCδ mediated signaling is connected to this chemotactic response, activation at the membrane modulates force distribution. Furthermore, PKCδ also regulates the cytoskeleton in interconnected endothelial cells in the form of multi-cellular endothelial cord structures. It is also hypothesized that force signaling regulated by PKCδ aides in endothelial cord dissociation by signaling for motile contractile signaling that would disrupt cell to cell adhesions and initiate sprouting for angiogenesis. These aims will support the overall hypothesis that PKCδ is a dynamic and an essential mediator of contractility during cell migration, as this regulation is strongly connected and coordinated with VEGFR/EGFR signaling driving chemotaxis.

2.0 *PKCδ LOCALIZATION AT THE MEMBRANE INCREASES MATRIX TRACTION FORCE DEPENDENT ONPLCΓ1/EGFR SIGNALING*

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2.1 ABSTRACT

Introduction: During wound healing, fibroblasts initially migrate into the wound bed and later contract the matrix. Relevant mediators of transcellular contractility revealed by systems analyses are protein kinase c delta/myosin light chain-2 (PKCδ/MLC-2). PKCδ is activated by growth factor-driven PLCγ1 hydrolysis of phosphoinositide bisphosphate (PIP_2) hydrolysis when it becomes tranlocated to the membrane. This leads to MLC-2 phosphorylation that regulates myosin for contractility. Furthermore, PKCδ n-terminus mediates PKCδ localization to the membrane in relative proximity to PLCγ1 activity. However, the role this localization and the relationship to its activation and signaling of force is not well understood. Therefore, we investigated whether the membrane localization of PKCδ mediates the transcellular contractility of fibroblasts.

Methods: To determine PKCδ activation in targeted membrane locations in mouse fibroblast cells (NR6-WT), two PKCδ constructs were generated; PKCδ-CaaX with farnesylation moiety targeting PKCδ to the membrane and PKCδ-SaaX a non-targeting control.

Results: Increased mean cell force was observed before and during EGF stimulation in fibroblasts expressing membrane-targeted PKCδ (PKCδ-CaaX) when analyzed with 2D cell traction force and 3D compaction of collagen matrix. This effect was reduced in cells deficient in EGFR/PLCy1 signaling. In cells expressing non-membrane targeted PKCδ (PKCδ-SaaX), the cell force exerted outside the ECM (extracellular matrix) was less, but cell motility/speed/persistence was increased after EGF stimulation. Change in cell motility and

increased force exertion was also preceded by change in cell morphology. Organization of actin stress fibers was also decreased as a result of increasing membrane targeting of PKCδ.

Conclusion: From these results membrane tethering of PKCδ leads to increased force exertion on ECM. Furthermore, our data show PLCγ1 regulation of PKCδ, at least in part, drives transcellular contractility in fibroblasts.

2.2 INTRODUCTION

Fibroblasts require time- and context-specific signaling for motility and contraction of the matrix. In cells that undergo motility/contractions, the filopodia/lamellipodium first extends and eventually adheres to the substrate/target. The cell body then impels towards the lamellipodium with subsequent rear retraction. Subsequent cell retraction is modulated through disruption of adhesions at the rear of the cell. Similar migration and contraction in the wound are stimulated by release of growth factors such as epidermal growth factor (EGF), VEGF, PDGF. Interestingly, as wound healing resolves, CXCR3 cytokines such as CXCL4, CXCL9, and CXCL10 are released, with their subsequent signaling preventing rear retraction. This signaling eventually leads to channeling the motile phenotype into amplified trans-cellular contractions required to contract to restore tensile strength to the tissue (Allen et al., 2002).

Components of the cell contractility and motility pathway have been identified. Growth factor and matrikine signaling through the epidermal growth factor receptor (EGFR) initiates motility via phosphorylation and activation of PLCy1 at the membrane (Chen et al., 1994b). Activated PLCy1 then catalyzes the hydrolysis of PIP_2 primarily at the leading edge and generates diacylglycerol (DAG) and IP3 (Insall and Weiner, 2001; Wells et al., 1999). Increased levels of DAG at the leading edge (Shao et al., 2006) synergizes the effect of PKCδ localization to the membrane (Ron and Kazanietz, 1999). DAG subsequently stabilizes the activation of PKCδ through direct binding of its N-terminal C1 domain (Kikkawa et al., 2002; Seki et al., 2005; Stahelin et al., 2005). Furthermore, PKCδ localization behind the leading edge allows it

to propel the cell body towards the extended lamellipodium and also mediate isometric force concomitant with motility (Andujar et al., 1992).

We previously showed that the EGFR-induced activation of PKCδ modulates force through an intermediate kinase, myosin light chain kinase (MLCK). MLCK can directly phosphorylate (myosin-light-chain) MLC to induce cellular contractions (Iwabu et al., 2004). Furthermore, reduced activation of PLCy1 delayed subsequent activation of PKCδ and downstream MLC2. This caused inefficient contractions by the cells compared to normal PLCy1 signaling (Iwabu et al., 2004). These data indicate that EGFR triggers contractile responses efficiently and quickly through PLCy1/PKCδ pathway. However, how the spatial localization of PKCδ to upstream modulators mediates force signaling has not been demonstrated. Therefore, PKCδ regulation of contraction and force distribution was investigated through its membrane translocation to PLCy1 activity.

2.3 RESULTS

2.3.1 Membrane targeting of PKCδ increases extracellular force on substratum

To investigate whether membrane targeting is sufficient to initiate trans-cellular contractility, PKCδ was directed to the membrane by splicing the farnesylation site of K-ras to the C-terminus (Leloup et al.)(Fig. 7a). These PKCδ constructs in a bicistronic vector expressing GFP were then stably transfected into mouse fibroblast cells with either reconstituted full length EGFR (NR6-WT) or a truncated EGFR that fails to activate PLC□ (NR6-991). To

specifically investigate how membrane targeted PKCδ affects individual cell force that is exerted onto the substratum, contractility was assessed utilizing cell traction force microscopy.

Cells expressing PKCδ-CaaX exerted increased contraction of the substratum. This increased force was mainly localized at the front or rear of the cells with the cells appearing generally non-motile (Fig. 7b). Furthermore, PKCδ-CaaX expressing cells also exerted increased tension at non-peripheral parts of the cell possibly due to the ubiquitous expression of PKCδ localization at the membrane. The cells responded to EGF quickly with concerted forces being exerted on to the substratum before and after EGF treatment compared to PKCδ-SaaX (Fig. 7c).

PKCδ-CaaX localization in the cell would position it closer to PLCγ1 activity (hydrolysis of PIP2) after EGFR stimulation (Margolis et al., 1990) or simply move it to be activated constitutively by phosphotidyl serine on the inner membrane. To determine whether PLCγ1 signaling was required for force generation through membrane targeted PKCδ, cells that fail to activate PLCy1 signaling upon EGF exposure (NR6-991), were investigated for cell force generation. NR6-991 cells could not exert as much force as NR6-WT (Fig. 7c). Molecular signaling of PLCy1 was further investigated in membrane-targeted PKCδ expressing cells. Decreased phosphorylation of PKCδ in response to EGF was observed in cells challenged with PLCy1 deficient signaling, suggesting full PKCδ effects are PLCy1 mediated (Fig. 7d). In addition, knockdown of endogenous PKCδ and similar levels of protein expression from constructs were confirmed in stable cell lines (Fig. 7e, Fig. 7f). These results reinforce the rationale that EGFR stimulation of PLCy1 is key to PKCδ mediated fibroblast contractility.

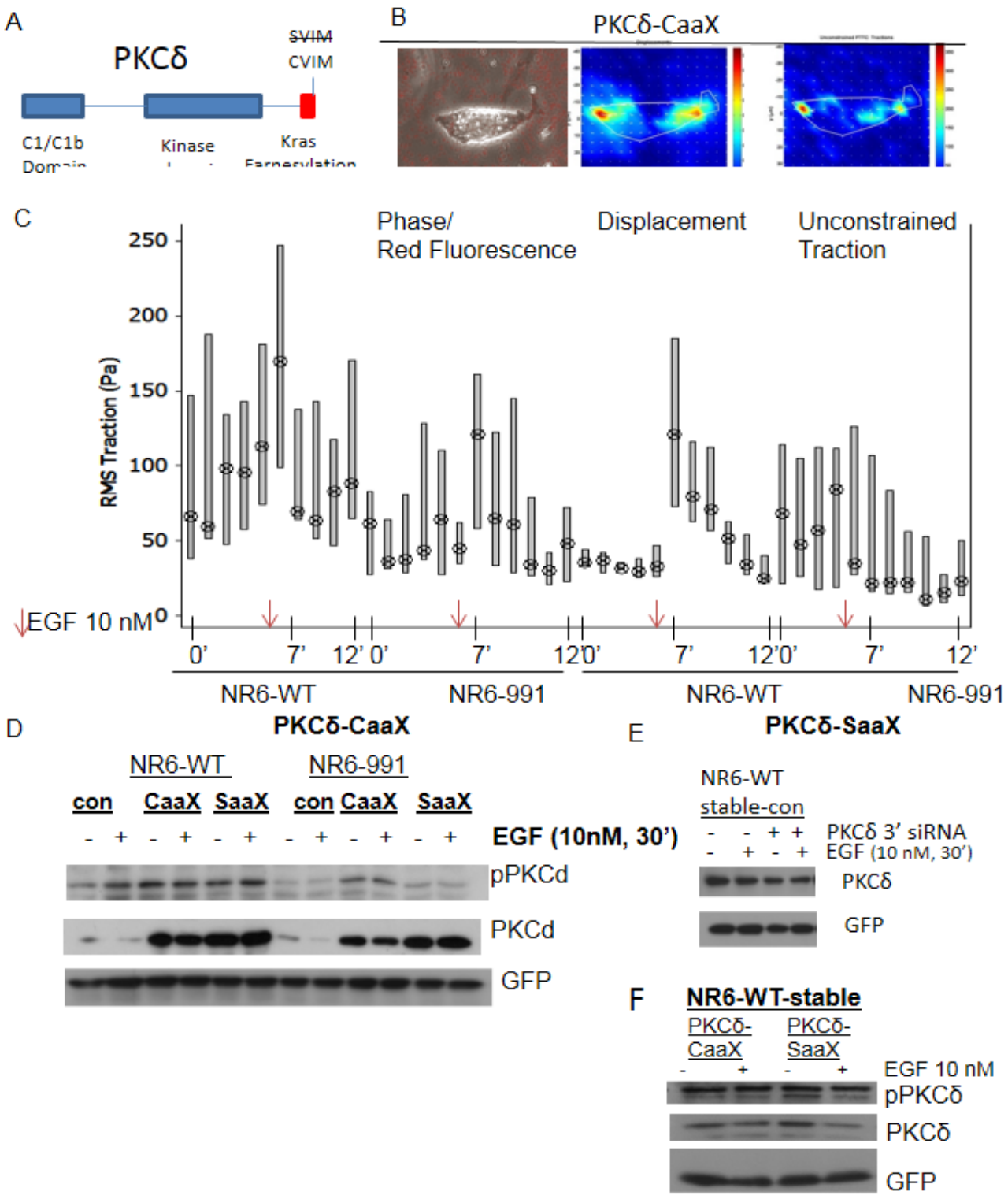


Figure 7. Membrane targeted PKCδ increases force of isometric contractions through EGFR/ PLCγ1 signaling *(Cell Traction Force Microscopy)*.

a) A schematic of PKCδ showing the kras farnesylation motif at the c-terminus of the protein. Membrane targeted PKCδ (PKCδ-CaaX) is represented with the CVIM domain and non-membrane targeted (PKCδ-SaaX) is represented by SVIM. bc) PKCδ-CaaX cells were placed on (0.5μm red beads) were prepared with 100 μg collagen cross-linked to PAG/beads. b) Cell traction was extrapolated through bead displacement as the cells exerted force. All forces exerted onto the substratum of each cell by bead displacement were computationally measured and analyzed using the software MatLab environment (Wang; Wang and Li, 2009; Wang et al., 2002). Unconstrained traction is force exerted by the cells in kPa that is derived from bead displacement on 3kPa PAG/bead gel and described previously in (25-27). Traction force output with unconstrained traction and absolute bead displacement from data extrapolation was gathered from all groups for each individual cell. Colorimetric indicators displays red as the most intense in traction force and dark blue displays minimal traction force. Images of cells were taken at 20X objective magnification. c) Boxplot of individual cell constrained force measurements between 25^{th} and 75^{th} percentile. Collective statistical analysis via Student's T Test was performed between NR6-WT PKCδ-CaaX and NR6-991-PKCδ-Caax after EGF treatment at p = 6.87821e-09). As indicated in results and methods, NR6-WT cell lines contain full length EGFR and NR6-991 cell lines contain truncated EGFR that is deficient in PLCy1 signaling. d) Immunoblot analysis of cells transiently transfected with (PKCδ-C/SaaX) and 50 μM of siRNA of mouse PKCδ siRNA into NR6-WT and NR6-991 fibroblast were then incubated in quiescent media overnight and treated with EGF for 1 hour prior to cell lysis. Western blot analysis of cell lysates was performed. GFP that is expressed with the vector was utilized as control for protein levels. e)

Lysates of siRNA knockdown of endogenous PKCδ of fibroblasts is represented in immunoblot. The non-linked GFP on the same vector were utilized for loading control. f) Immunoblot of cell lysates with stably transfected PKCδ-CaaX and PKCδ-SaaX. Non-linked GFP protein levels were utilized for loading control.

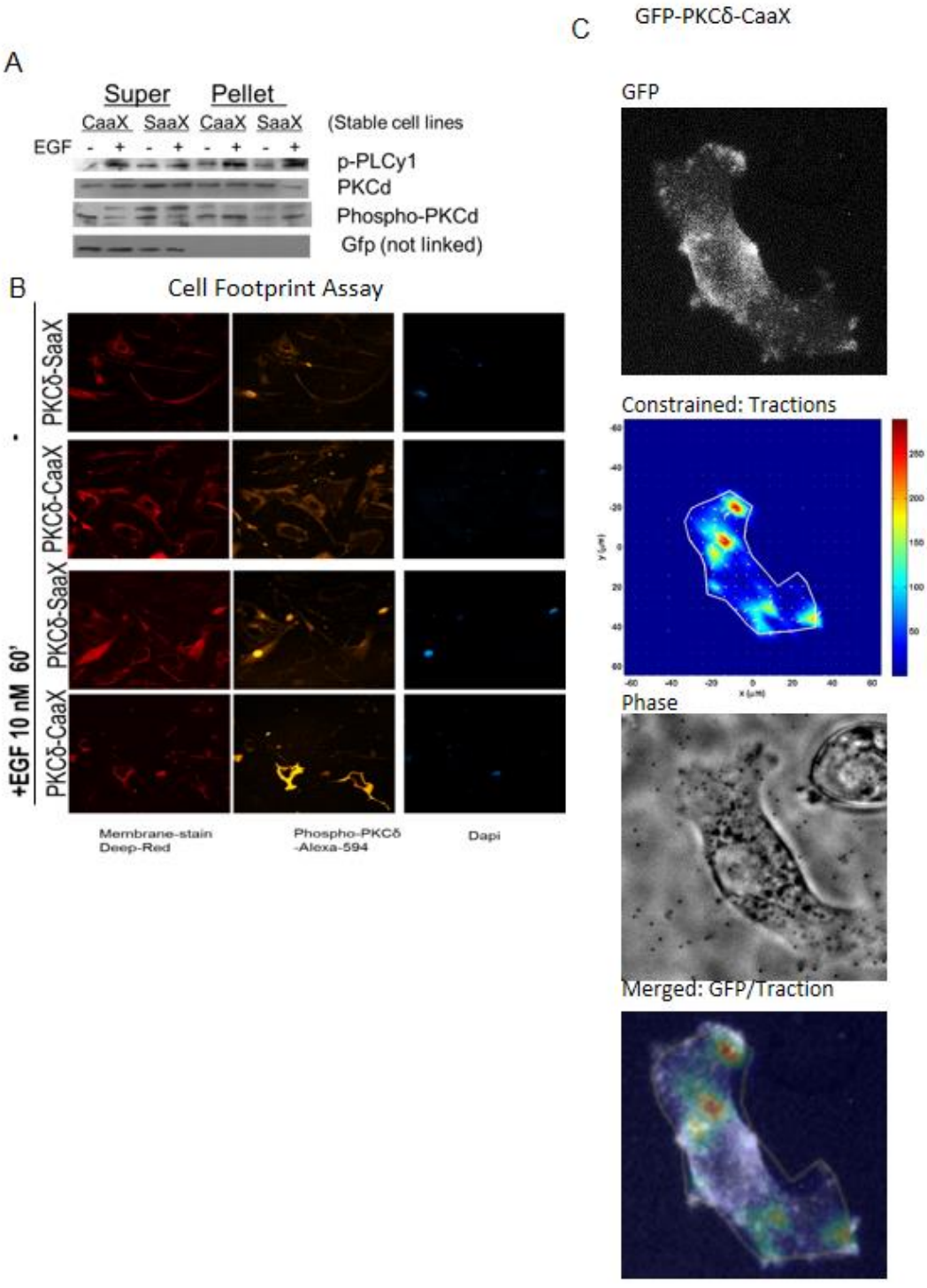
2.3.2 Membrane-targeted PKCδ localizes to cell membrane to induce force signaling

PKCδ membrane translocation is essential to regulation of its activity. To determine how increased membrane targeting affects PKCδ activation, membrane and cytosolic fractions of PKCδ were analyzed comparing the two constructs in stably transfected cell lines. From these data, there was increased total PKCδ in the membranes of PKCδ-CaaX stably transfected cells compared to PKCδ-SaaX expressing cells. EGF stimulation activated both PKCδ-CaaX and PKCδ-SaaX at membrane indicated by increased phosphorylated PKCδ fractions (Fig. 8a). In addition, depletion of cytosolic fractions of activated PKCδ during EGF stimulation was also observed, confirming net translocation of PKCδ as opposed to de novo synthesis. Although activated PKCδ-SaaX increased at the membrane during EGF stimulation as expected, these data also indicate that activated PKCδ-CaaX was increased in membrane fractions even prior to EGF treatment. This localization prior to EGF stimulation was intended and partially obviated the need for stimulation by EGF.

In addition, this increase in phosphorylated PKCδ localization to the membrane was further tested in specific cells through a 'cell footprint' assay. Similarly, activated PKCδ localized to the membrane prior to EGF stimulation (Fig. 8b). After EGF stimulation, the activated PKCδ was found mainly to be membrane-targeted in comparison with a decrease in

non-membrane-targeted fractions. These data suggest that membrane targeting increases PKCδ localization to the membrane for activation in response to EGF and membrane targeting in itself partially acts as a stimulus.

Localization of PKCδ and its impact on force transduction was further investigated by visualizing PKCδ through tagging the membrane targeted PKCδ with GFP. Cells transfected with this construct were analyzed by cell traction force microscopy. Cells that expressed PKCδ-CaaX increased cortical tension close to the peripheries of the cell whereas the non-membrane targeted PKCδ localized throughout the cytoplasm with little effect in morphology (Fig. 8c). We furthermore found that PKCδ localization correlated with specific force being exerted onto the substratum prior and during PKCδ localization. These forces were exerted primarily behind the leading edge, along with some random specific non-peripheral force transduction. These data suggest PKCδ localization is directly associated to the distribution of force to the cells.


A
Super
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B
Cell Footprint Assay
PKCδ-SaaX
PKCδ-CaaX
PKCδ-SaaX
PKCδ-CaaX
+EGF 10 nM 60'
Membrane-stain Deep-Red
Phospho-PKCδ -Alexa-594
Dapi
C
GFP-PKCδ-CaaX
GFP
Constrained: Tractions
Phase
Merged: GFP/Traction

Figure 8. Membrane-targeted PKCδ at the membrane maps with force distribution

a) Stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT cells were stimulated with 10 nM of EGF in quiescent media. After hypotonic fractionation, lysates were divided either into supernatant, which contains cytoplasmic proteins or pellet which contains membrane-linked proteins. Lysates were subjected to SDS-PAGE and immunoblotted for indicated proteins. GFP was utilized as a negative control for cytoplasm contamination in membrane fractions. b) Stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT cells were stimulated with 10 nM of EGF in quiescent media for 60 minutes prior to fixation. Footprints were collected as described in methods and were immunostained for activated PKCδ. Images were then taken of footprints with confocal microscopy at 40x objective magnification. DAPI was utilized as a negative control for the presence of the nucleus, which is removed in the process of retaining the bottom membrane only attached to the substrate. The Deep-Red membrane stain was utilized as a positive control for membranes. c) DNA constructs with GFP linked to PKCδ-CaaX and PKCδ-SaaX were transfected in NR6-WT. Cells were then plated onto PAG/beads substrate as described previously in (Fig. 7) in the presence of culturing media. Images of cells were taken at 20x objective every 10 minutes as localization of PKCδ was observed and force was extrapolated from bead displacement represented in constrained traction force indicated in colorimetric graph. In colorimetric graph, red represents high traction force and blue represents low/no traction force. Merged images of constrained traction force and GFP PKCδ localization is indicated. Red represents strong force and white represent PKCδ localization.

2.3.3 Membrane targeted PKCδ displays increased contraction of collagen gel compared to non-targeted PKCδ expressing cells

Cell motility and isometric cell force both contribute to the eventual compaction of both wound bed collagen/ECM and artificial collagen ECM (Allen et al., 2002). To further investigate whether membrane targeted PKCδ causes increased force in a 3D ECM, the collective ability of cells to compact a collagen gel over time was investigated utilizing a gel compaction assay. Cells expressing PKCδ-CaaX mediated increased gel compaction compared to non-targeted PKCδ-SaaX (Fig. 9), at which became significant at longer time periods (Fig. 9b). These data suggest membrane targeted PKCδ predisposed cells to increased signaling for compaction which led to increased compaction of collagen gels compared to PKCδ-SaaX.

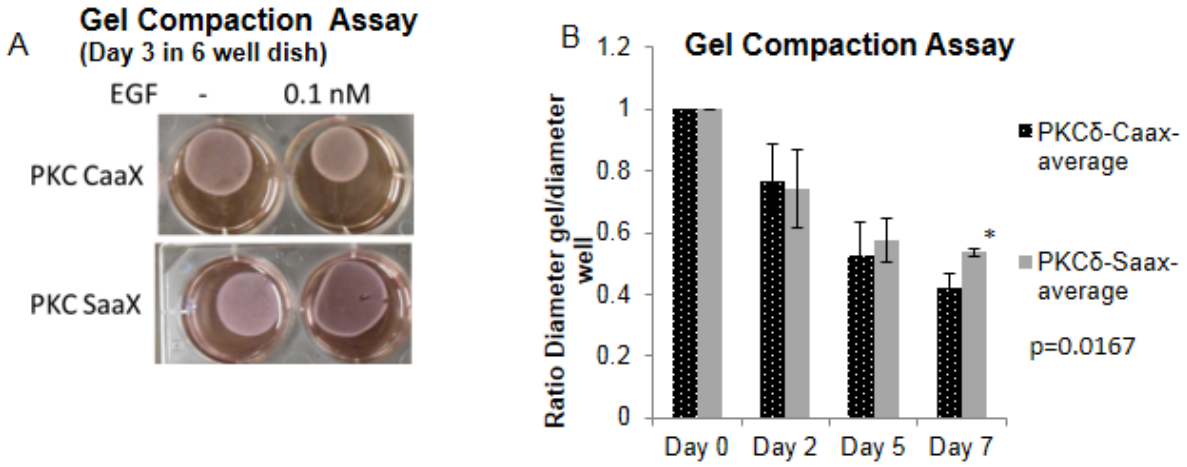


Figure 9. Gel compaction is increased in cells expressing membrane-targeted PKCδ.

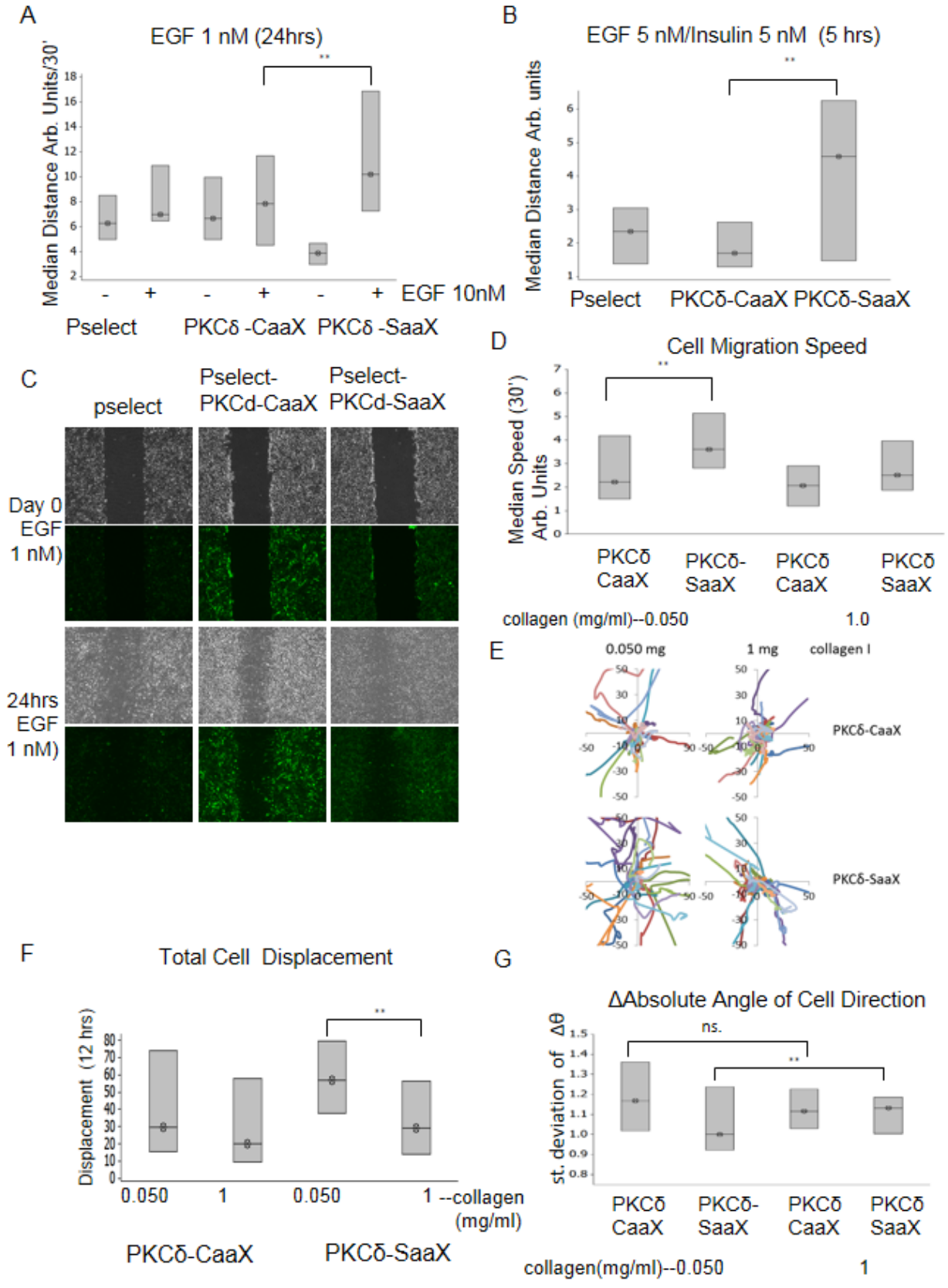
a,b) Stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT cells were incubated in 1 mg/ml of polymerized collagen. Collagen gel and cells were incubated with growth factor for indicated time points and compaction was observed by visually measuring the size of collagen gel relative to well size. a) Picture of gels were taken. b) Ratio of gel size to well was calculated by image J line scan parameter of no EGF treated sample and analysis of multiple gel compaction assays (n=3) was performed as previously described and ratios were analyzed by Student-T-test p=.006.

2.3.4 Non-membrane targeted PKCδ presents increased cell motility

Our earlier systems biology analysis of motility signaling highlighted the adhesion to contractility ratio as key to motility (Kharait et al., 2007), along with the ability to labilize or turnover adhesions. Thus, we sought to determine the effect of a lower level but tonic activation of the contractility pathway as driven by membrane-targeted PKCδ (Fig. 10). Live cell imaging of stably transfected PKCδ-C/SaaX cells were observed on a collagen coated plastic substratum with knockdown of endogenous PKCδ. Analysis of random cell motility during live cell imaging showed that cells expressing PKCδ-CaaX moved faster in an unstimulated mode. Following EGF stimulation, PKCδ-SaaX moved faster than PKCδ-CaaX (Fig. 10a). To further determine the extent of collective migration, a scratch wound healing assay was utilized. PKCδ-SaaX was found to move farther into the scratch compared to PKCδ-CaaX (Fig. 10d). These findings are consistent with increased cell adhesion leading to decreased cell motility. Our approach to utilize forced membrane targeting of PKCδ does cause increased force into adhesions unto the substratum that subsequently results in decreased cell motility.

To investigate whether membrane targeted PKCδ involves increased force exertion onto the substratum during motility, cells were challenged to migrate on an adhesive substrate. The

increased adhesion would increase PKCδ/MLC activation while causing decreased cell speed in normal fibroblasts (Kharait et al., 2007). Furthermore, increased intracellular force during active cell motility would be able to overcome the effect of an increased adhesive substrate. From our results membrane targeted PKCδ remained at the same level of persistence at low to high collagen content, with slightly decreased cell motility (Fig. 10e). Cells expressing the non-targeted PKCδ-SaaX were observed to have more persistent paths compared to PKCδ-CaaX with increased cell speed with low adhesive substratum (Fig. 10f, 10e). When challenged with an adhesive substrate, non-membrane targeted PKCδ had reduced cell speed on the adhesive substrate with decreased motility persistence compared to PKCδ-CaaX indicated in (Fig 10f, Fig 10g, Fig 10h). These data suggest in membrane targeted PKCδ expressing cells decreased cell speed may be due to increased force to the substratum at a level to overcome the effects of a very adhesive substrate.


A
EGF 1 nM (24hrs)
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- + - + - + EGF 10nM
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PKCδ -SaaX
B
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Pselect
PKCδ-CaaX
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C
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D
Cell Migration Speed
Median Speed (30') Arb. Units
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PKCδ CaaX
PKCδ-SaaX
PKCδ CaaX
PKCδ SaaX
collagen (mg/ml)--0.050
1.0
E
0.050 mg
1 mg
collagen I
PKCδ-CaaX
PKCδ-SaaX
F
Total Cell Displacement
Displacement (12 hrs)
**
0.050
1
0.050
1 --collagen (mg/ml)
PKCδ-CaaX
PKCδ-SaaX
G
ΔAbsolute Angle of Cell Direction
st. deviation of Δθ
ns.
**
PKCδ CaaX
PKCδ-SaaX
PKCδ CaaX
PKCδ SaaX
collagen(mg/ml)--0.050
1

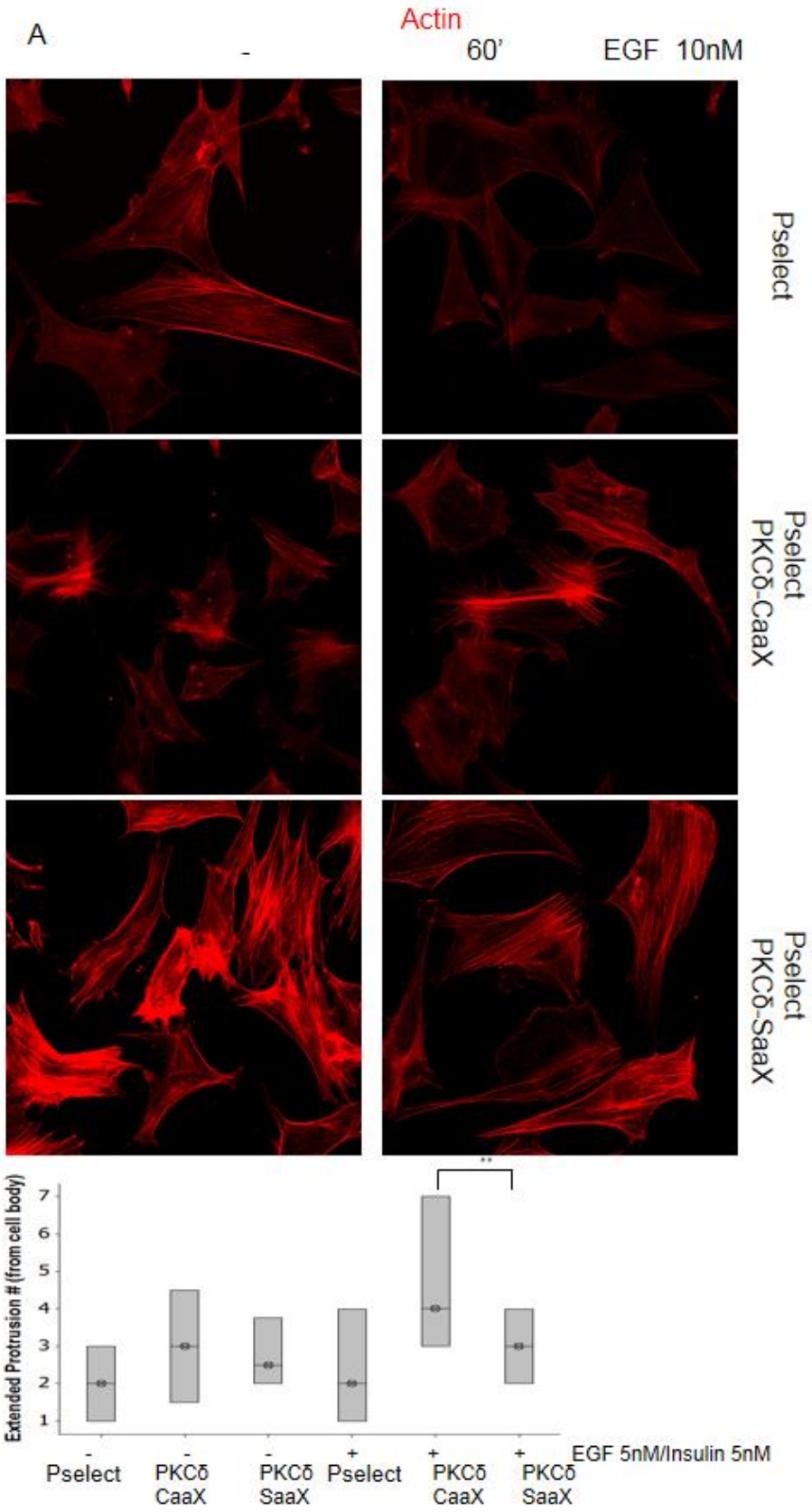
Figure 10. Cell motility is restricted by membrane targeting of PKCδ.

a). Live cell images of stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT (EGFR) cells with endogenous knockdown of PKCδ were taken every 30 minutes with and without 1 nM of EGF in quiescent media for 24 hour time period. Cell motility was analyzed by metamorph software. (Two-Sample-t-test was utilized to evaluate significance $p<.05$, $n>11$). b) Live cell images of stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT (EGFR) cells with endogenous knockdown of PKCδ were taken every 15 minutes stimulated with 5nM of EGF and 5 nM of insulin for 5 hours. Average individual cell speed/path was analyzed at 15 minute intervals for a total duration of 5 hours with metamorph software. (Two-Sample-T-test was utilized to evaluate significance ($p < 0.05$, $n >11$). c) Stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT (EGFR) were grown in a 6 well plate to 90% confluency prior to scratching with rubber policeman. Cells were treated with 1 nM of EGF in quiescent media and images of scratch were taken at 4X objective magnification. d,e,f,g) Stably transfected PKCδ-CaaX and PKCδ-SaaX NR6-WT (EGFR) *cells were grown to 90% confluency prior to plating on collagen I coated plate. Cells were then allowed to adhere overnight and then transfected with 50uM of* PKCδ specific siRNA and stimulated with 1 nM EGF. Cells were then imaged for 12 hours with 30 minute intervals and average individual cell speed (d) /path (e) /persistence (absolute displacement from origin)(f) was analyzed with metamorph software. (g) Persistence was analyzed utilizing the median of absolute angle standard deviation relative to control.

2.3.5 Membrane targeting of PKCδ activates stress fibers and leads to morphological changes independent of growth factor exposure

Cell morphology change precedes growth factor stimulated cell motility (Wells et al., 1999). To investigate how cells are affected by force signaling, stable cell lines were analyzed for stress fiber organization. To further investigate cytoskeletal tension resulting from PKCδ localization to the membrane, stress fibers of stably transfected cells were visualized by rhodamine-labeled phalloidin. Fibroblasts with PKCδ-CaaX demonstrated disorganized stress fibers, even prior to EGF stimulation. In contrast, more organized and pronounced stress fibers were observed in PKCδ-SaaX expressing cells (Fig. 11a). These results show that distribution of force to the ECM disrupts stress fibers to the cortex and cell body.

To investigate how increased membrane targeting translated to cell morphology, we induced increased kras farnesylation by adding insulin in combination with EGF. This stimulus would increase PKCδ membrane targeting. We found that increasing membrane targeting caused increased protrusions (Fig. 11b, Fig S1). Of interest, PKCδ-SaaX correlated with fewer protrusions as normal localization of activated PKCδ is cell front limited. However, this increase in protrusions only occurred with this stimulus. EGF stimulation alone did not cause these results. These data suggest decreased cortical stress fibers allow for the plasticity of membrane targeted PKCδ to exert protrusions to the ECM.


A
Actin
-
60'
EGF 10nM
Pselect
Pselect PKCδ-CaaX
Pselect PKCδ-SaaX
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Extended Protrusion # (from cell body)
1
2
3
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5
6
7
- Pselect
- PKCδ CaaX
- PKCδ SaaX
+ Pselect
+ PKCδ CaaX
+ PKCδ SaaX
EGF 5nM/Insulin 5nM

Figure 11. Membrane-targeting of PKCδ cytoskeletal structure of cells, without altering cell signaling of EGFR/ PLCγ1 / PKCδ pathway.

a) PKCδ CaaX/SaaX stably transfected cells were incubated in quiescent media prior to treatment of EGF (10 nM) for 60' and were fixed with 4% formaldehyde solution, permeabilized, and stained for phalloidin. Fluorescent images were taken utilizing confocal microscopy at mid z-stack. b) Cells were grown and transfected as previously described in (Fig 3a). Extended protrusions from cell body were manually counted after 5 hours and graphed, ($p < 0.05$, $n>11$).

2.4 DISCUSSION

We have shown that increasing PKCδ translocation by membrane tethering redistributed force signaling outward to the ECM that is partially PLCγ1 dependent (Fig 7c). In addition to isometric force exerted under the cell to the substratum, force that is exerted on the cell body is also a significant portion of cellular contraction. Force on to the cell body can be indirectly measured through live cell motility (Allen et al., 2002). Membrane targeted PKCδ caused a shift in cellular force from the cell body to the ECM. As a result, decreased cell speed was observed suggesting that the increased force 'froze' the adhesions (Fig. 10a-d). In non-membrane targeted PKCδ expressing cells, distribution of force was manifested by increased cell speed compared to membrane targeted PKCδ expressing cells. As migration involves a cycle of de-adhesion, these cells also presented a reduced net extracellular force to the ECM (Fig 7, 10). As increased restrictive forces to the cells occurred during motility, cells expressing membrane-targeted PKCδ were more resilient to the effects of an adhesive substrate as determined through

persistence measurements (Fig 10e-h). These data indicate that slightly shifting the dynamics of PKCδ localization shifts signaling of force distribution. This is a very specific effect, since cells were not manipulated with any other regulators of the cytoskeleton.

Interestingly, the difference between PKCδ-CaaX and PKCδ-SaaX were negligible in total downstream signaling to MLC-2. This implies that signaling of the proteins are the same with similar levels of expression (Fig 7e). However, localization of exerted force is the key determinant, and highlights the need to examine signaling cascades in subcellular compartments. Furthermore, from our studies only PKCδ-CaaX localizes to the membrane with increased activation of PKCδ. This furthermore correlates with cellular force distribution to the ECM (Fig 8). Considering their similarity, the differences in cellular responses are due to the intended difference in localization dynamics and resultant activation.

Force distribution to the ECM and force distribution to the cell body are both simultaneously and reciprocally being applied. As cells adhere to the ECM and actively migrate on a 2D substratum, forces emitted by these two actions are required by the cell for active motility. In a 3D-context, such as in a gel compaction assay, force exertion from the cells and forces applied to the cells are collectively systemic (Sherratt et al., 1992). Each cell integrates its force into the system with increased plasticity and synergism impacting contractions of the ECM (Schmitt-Graff et al., 1994). From this study, increased ECM compaction was observed as a result of signaling of force through membrane-targeted PKCδ (Fig 9). Signaling through growth factors and cytokines integrate cellular responses to coordinate systemic contraction of a wounded matrix. This study primarily focused on EGF signaling, since it is an essential growth factor for motility during wound healing. Downstream of EGFR signaling, EGFR stimulation of

PIP_2 hydrolysis impacts divergent regulation of motility and contraction. Although, PKCδ regulation is downstream of PIP_2 hydrolysis, it has also been found to activate m-Calpain through direct binding (Leloup et al.). As PIP_2 is being hydrolyzed at the leading edge, the rear of the cell retains PIP_2 levels where it aids in activation of m-Calpain to cleave rear adhesions (Leloup et al.; Wells et al., 2005). This further supports the subcellular directionality of EGFR mediated PIP2 hydrolysis, and reinforcing the concept that spatial localizations of signaling nexus are important for productive motility (Wells et al., 1999). Among context-specific functions of fibroblasts in wound healing, the mechanics for ECM remodeling (Allen et al., 2002) is regulated by both motility and isometric contraction critical for remodeling of the compacting ECM (Dickinson and Tranquillo, 1993; Felsenfeld et al., 1996; Plotnikov et al., 2012). These factors in combination with the transient release of both growth factor and cytokine antagonist regulate the dynamic and synchronous relationship of how fibroblasts mediate this motility and contraction of the wound. If not properly regulated, this fine-tuned system that is mediated by durotaxis and chemotaxis, may shift to exacerbate the healing tissue into fibrosis or fibroplasia (Marinkovic et al., 2012; Yates et al., 2007).

2.5 **MATERIALS AND METHODS**

Cell Culture - NR6-WT and NR6-991 cell lines were established previously from parental Swiss mouse 3T3 variant fibroblasts that lack endogenous EGFR as an original gift from Dr. Harvey Herschman (Pruss and Herschman, 1977). These cells were cultured in minimal

essential media (MEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM, nonessential amino acids, penicillin (100 units/ml), streptomycin (100 µg/ml), and G418 (350 µg/ml) for continual selection of EGFR. Subconfluent (< 75%) cultures were split every 4 days using 0.25% trypsin/0.25 mM EDTA in MEM to dislodge cells from culture and washed further with MEM before seeding.

Plasmid Construction and transfection - PKCδ constructs were established from human-derived Hs68 fibroblast cell line, in which the Kras *mouse* sequence (CaaX for farnesylation; SAAX for control) was spliced to the carboxyl-terminus of PKCδ in 3 rounds of PCR amplification. Recombinant PKCδ was then ligated into the p-select-GFP-zeocin plasmid (Invitrogen) using the *Bam*H1 and *Nhe*1 sites. Stable transfection was performed with 4µg of each plasmid (PKCδ-CaaX or PKCδ-SaaX) and lipofectamine according to the manufacturers' protocol. Following transfection, cells were grown and split in MEM containing 300µg/mL zeocin (Invitrogen).

In addition to PKCδ transfection, 50 µM mouse siRNA targeting the PKCδ 3' UTR was also transfected into transient and stably transfected cells. The primers used for siRNA synthesis were 5'-AACACAUCACCAGUCUCCUACAUGCUU-3' and 3'-TTGUGUAGUGGUCAGAGGAUGUACG-5' respectively. This sequence was designed using the online software from Integrated DNA Technologies.

Cell Traction Force Measurements - Cell Traction Force Microscopy protocol was performed as previously described (Wang and Lin, 2007). Briefly, 6 well glass bottom plates (Mattek) were first activated by treatment with 0.1M sodium hydroxide for 1 day and allowing to

air-dry overnight. The next day approximately 2 drops of 3-aminopropyltrimethoxysilane was added to each well followed by washing with de-ionized water, incubation in 0.5% glutaraledehyde for 1 hr and finally air dry. After activation, the first layer of gel was made with 11µL polyacrylamide (5% acrylamide and 0.1% bisacrylamide), 20 µL of 10% ammonium persulfate and 2µL TEMED and poured on the activated glass bottom plates. A circular glass coverslip was then placed on top of the solution. After polymerization, the glass coverslip was removed and a second layer of gel as described previously but with 0.5 µM fluorescently labeled beads was poured on top of the first layer and the gel was incubated overnight in water with a glass coverslip placed on top. Collagen was then crosslinked to the gel by adding sulfo-SANPAH on the top followed by exposure to UV-radiation. After washing 4 times with PBS, collagen I (150 µg/ml, BD Bioscience) was added on the gel and allowed to crosslink with sulfo-SANPAH overnight. Prior to plating fibroblasts onto the polyacrylamide gel, cells were transfected with 50 µM PKCδ siRNA and incubated overnight in MEM. Transfected cells were then detached by trypsinization and added to polyacrylamide gels in quiescent media and allowed to adhere for at least 5 hrs. Live cell images were taken at indicated time points with a 20X objective, and bead displacements and force were computed using the MatLab programming software as previously described (Wang and Lin, 2007).

Primary antibodies and reagents - For immunoblotting and immunostaining the following antibodies were used at a dilution of 1:1000: Anti-PKCδ antibody (BD Biosciences), anti-phospho-PKCδ (S643 PKC delta/S676 PKC-theta), anti-ppMLC-2 (S18/19), and anti-MLC-2 (Cell Signaling), and anti-GFP-FL (SantuCruz). For actin staining, phallodin-conjugated to Alexa-568 (Invitrogen) was added at 1:40 dilution.

Gel compaction assay—Stably transfected NR6-WT fibroblasts were grown in polymerized collagen I using previously described methods with modifications (Iwabu et al., 2004). Stably transfected PKCδ-CaaX and PKCδ-SaaX expressing cells were cultured to subconfluence and harvested using 0.25% trypsin/EDTA. Cells were then resuspended in MEM, diluted to 1 x 10^6 cells/mL and centrifuged at 1000 rpm for 5 min. Fibroblasts were then resuspended in quiescent media containing 1mg/mL bovine serum albumin and EGF at various concentrations. Neutralized collagen solution (1mg/ml collagen/media-pH-7) was immediately mixed with fibroblast solution and allowed to polymerize for 1 hr at 37°C. After polymerization, collagen was released from the sides of the wells by a small pipette tip. Compaction was determined by decrease in the size of the collagen gel which was documented as images. Quantification of the images was performed by line scan procedure using the image J software. From this analysis we obtained the ratio of the diameter of standardized well to the diameter of collagen gel, as diameter was preferred over area of compaction due to little difference in experimental outcome and significance.

Motility Measurements--Stably transfected cells were grown as previously described and plated onto collagen coated plates. Afterwards, cells were transfected with 50uM siRNA for 24 hours prior to imaging. Cells were then incubated in quiescent media overnight and treated with 1 nM of EGF for 24 hours in In live imaging chamber at (5% CO_2, 4% O_2). Cells treated with insulin were incubated at 5nM EGF and 5 nM insulin to increase membrane targeting at 5 hour time period. Cell speed was tracked utilizing metamorph software utilizing the track object

function. Individual cells were highlighted and the software computationally tracked cell movement as cell displacement in each frame along xy coordinates.

Hypotonic Subcellular Fractionation--Subcellular fractionation was described previously (Jiang et al., 2002). Briefly, stably transfected cells were grown to subconfluency prior to quiescence media incubation overnight. Cells were then treated with 10 nM of EGF for 60 minutes. Cells were then scraped at 4°C with rubber policeman and lysed with hypotonic buffer (10mM HEPES pH 7.4, 1.5mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). Cell lysates at 0.5 mL were then homogenized on ice further with dounce at the rate of 40 strokes. Unhomogenized cellular debris was removed by centrifugation at 1,000 x g for 5 minutes. Supernatant was then subjected to ultra-centrifugation at 100,000 x g for 1 hour at 4°C. Supernatant and pellet were then separated and extracted with 1X

sodium dodecyl sulfate (SDS) sample loading buffer.

Cell Footprinting--The dorsal part of the cell was removed as described previously (Leloup et al.; Shao et al., 2006). Stably transfected cells were plated onto collagen coated (50ug/mL) glass coverslip prior to incubation of quiescent media. Cells were then stimulated with 10 nM of EGF for 60 minutes prior to cell footprint isolation. In addition, a.ll isolation solutions were incubated at 4°C prior to use. Fibroblasts were washed with morpholineethanesulfonic acid-buffered saline (MBS; 20 mM morpholineethanesulfonic acid [pH 5.5], 135 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$). Cells were then coated with a 1% solution of cationic colloidal silica (silica prepared as a 30% stock colloid). (Cationic colloidal silica was obtained by written request from Donna Beer Stolz, University of Pittsburgh,

Pittsburgh, PA.) Repeat of wash with MBS was done prior to coating cells with 1% polyacrylic acid (Sigma Aldrich) in MBS. Polyacrylic coat was removed with another wash of MBS. Cells were then swelled for 10 minutes with hypotonic lysis buffer (2.5 mM imidazole. pH 7.0) supplemented with protease inhibitors (1:100, protease inhibitor cocktail; Sigma Aldrich). Cells were unroofed by mild application of lysis buffer through a 5-ml syringe fitted with a blunted, flattened 18-gauge needle. Periodically, the state of unroofing was observed in cells by inverted phase-contrast microscopy. Footprints were then fixed in 2% formaldehyde in PBS for 5 minutes and permeabilized with 0.1% TritonX-100 in PBS (wash buffer). After wash, cells were immunostained with phospho-PKCδ (S643 PKCδ/S676 PKC) at 1:50 dilution in 30 mg of BSA. Secondary antibody conjugated to Alexa-594 was utilized to immunostain for 1 hour. Nuclei were stained with DAPI in PBS.

2.6 SUPPORTING INFORMATION

Movie S1: Mapping of PKCδ to force exertion. GFP-linked PKCδ CaaX stably transfected cells were induced with FBS and force exerted on the substratum was calculated and false-colored red, whereas the PKC is false-colored white. Shown is a representative cell at 10 minute intervals for 80 minutes.

Movie S2: Membrane-targeted PKCδ exert increased protrusion. (2)PKCδ CaaX/ (3) SaaX stably transfected cells were induced with 5 nM EGF and 5 nM insulin as described in (Figures 11a, 12b). Images were taken at 20X objective magnification with resolution 0.35um/pixel. Movie frames were at 15 minute intervals for 5 hours.

Movie S3: Membrane-targeted PKCδ exert increased protrusion.(2)PKCδ CaaX/ (3)SaaX stably transfected cells were induced with 5 nM EGF and 5 nM insulin as described in (Figures 11a, 12b). Images were taken at 20X objective magnification with resolution 0.35um/pixel. Movie frames were at 15 minute intervals for 5 hours.

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3.0 PKCδ REGULATES FORCE SIGNALING DURING VEGF/PF4 INDUCED DISSOCIATION OF ENDOTHELIAL TUBES

Joshua Jamison, James H-C. Wang, Alan Wells

3.1 **ABSTRACT**

Wound healing requires the vasculature to re-establish itself from the severed ends. Endothelial cells within capillaries must detach from neighboring cells before they can migrate to the wound and initiate angiogenesis. The dissociation of these endothelial capillaries is driven, at least in part, by platelets' release of growth factors and cytokines, particularly the chemokine CXCL4/platelet factor-4 (PF4). As this retraction is partly mediated by increased transcellular contractility, the protein kinase c-δ/myosin light chain-2 (PKCδ/MLC-2) signaling axis becomes a candidate for this mechanism. Phospholipase C (PLC) hydrolysis of phosphoinositide bisphosphate (PIP_2), which ensues upon CXCR3 binding by CXCL4, could potentially activate PKCδ for regulation of MLC. We hypothesize that PKCδ activation promotes dissociation of endothelial cords after exposure to platelet-released CXCL4 and VEGF. To investigate this mechanism of contractility, endothelial cells were allowed to form cords with subsequent dissociation secondary to the addition of CXCL4. In this study, CXCL4-induced dissociation was reduced by a VEGFR inhibitor (sunitinib malate) and/or PKCδ inhibition. Increased contractility through MLC expression increased contractility in a PKCδ-dependent manner during combined CXCL4+VEGF treatment. As force was translated to focal adhesions, focal adhesion regulation of both individual cells and endothelial cords indicated that mechano-transduction responsive zyxin expression was upregulated after PKCδ inhibition. This study suggests that growth factor regulation of PKCδ may be involved in CXCL4-mediated dissociation of endothelial cords.

3.2 INTRODUCTION

The barrier function of the skin must be quickly re-established if compromised upon wounding. This repair requires a vascular system for tissue maintenance. However, during wounding the vessels are destroyed and thus there is a need for angiogenesis from the tips of severed vessels. This is driven by the pro-angiogenic growth factors released first by platelets and then by macrophages in the wound. Endothelial cells that are involved in angiogenesis require initial signals to 'dedifferentiate' and separate from the existing severed vessels prior to the subsequent inductive signals to migrate into the wound bed. Among these earliest signals are those released by the platelets during clotting, including the chemokine CXCL4 and growth factors VEGF, PDGF, HB-EGF, and TGFβ(Li et al., 2003).

Many of the intracellular signaling pathways that drive fibroblasts and endothelial cells to migrate are known. Downstream of growth factor receptor activation, PLC signaling triggers PKCδ to regulate cell motility via increasing transcellular contractility in fibroblasts and endothelial cells (Chen et al., 1994a; Chen et al., 1994b; Iwabu et al., 2004; Jamison et al., 2013; Joyce and Meklir, 1992; Shizukuda et al., 1999b; Yamamura et al., 1996). Growth factor and matrikine signaling through the epidermal growth factor receptor (EGFR) initiates motility via phosphorylation and activation of PLCγ1 at the membrane (Chen et al., 1994b). Activated PLCγ1 then catalyzes the hydrolysis of PIP_2 primarily at the leading edge and generates diacylglycerol (DAG) and IP3 (Insall and Weiner, 2001; Wells et al., 1999). Increased levels of DAG at the leading edge (Shao et al., 2006) synergizes the effect of PKCδ localization to the membrane (Ron and Kazanietz, 1999). DAG subsequently stabilizes the activation of PKCδ through direct binding of its N-terminal C1 domain (Kikkawa et al., 2002; Seki et al., 2005; Stahelin et al., 2005). Furthermore, PKCδ localization behind the leading edge allows it to

propel the cell body towards the extended lamellipodium and also mediate isometric force concomitant with motility (Andujar et al., 1992). EGFR-induced activation of PKCδ modulation of force has been previously shown through an intermediate kinase, myosin light chain kinase (MLCK). MLCK can directly phosphorylate (myosin-light-chain) MLC to induce regulation of contractions (Iwabu et al., 2004). Furthermore, reduced activation of PLCγ1 delayed subsequent activation of PKCδ and downstream MLC2. These data have shown that EGFR triggers contractile responses efficiently and quickly through PLCγ1/PKCδ pathway.

Dermal functions of the skin are essential for barrier function that is quickly re-established if compromised upon wounding. Dermal tissue repair involves intense anabolic processes that require vascular system for tissue maintenance. However, during wounding the vessels are destroyed and thus there is a need for angiogenesis from the tips of severed vessels. This is driven by the pro-angiogenic growth factors released by platelets and then macrophages in the wound. Endothelial cells that are involved in angiogenesis require initial signals to 'dedifferentiate' and separate from the existing severed vessels and subsequent inductive signals to migrate into the wound bed. Among these signals are the chemokine PF4 and growth factors VEGF/PDGF/HB-EGF/TGF (Li et al., 2003). These cells are also influenced by the regulation of provisional ECM by fibroblasts. Serving as a reservoir of growth factors/cytokines, this provisional ECM mediates motility/regression. It also exist as a dynamic scaffold for vessels to graft into the wound bed (Dvorak et al., 1987; Folkman, 1997; Herman, 1993; Li et al., 2003).

Therefore, we hypothesize that PKCδ regulates tension of endothelial cords for cell retraction during neo-angiogenesis as its activation is mediated by increased VEGFR signaling. Through VEGFR regulation of PKCδ, endothelial cells can mediate efficient dissociation required for neo-angiogenesis. As wound healing and angiogenesis are time- and context-specific

dependent, proper signaling of force through VEGFR signaling is required to induce normal angiogenesis.

3.3 RESULTS

3.3.1 PF4 induced dissociation is VEGFR-dependent

To investigate endothelial PF4-mediated dissociation, we utilized human microvascular endothelial cells (HMEC-1) plated on Matrigel. Cords were formed for 24 hours and then subsequently induced to dissociate with PF4 and VEGF, two factors released by platelets during the hemostatic plug of wounding. Inhibition of VEGFR/PDGFR signaling using sunitinib (2.5 uM) inhibited dissociation as noted by increased cord length (Fig. 13). In addition, PF4 had an increased effect over VEGF mediating dissociation (Fig 13).

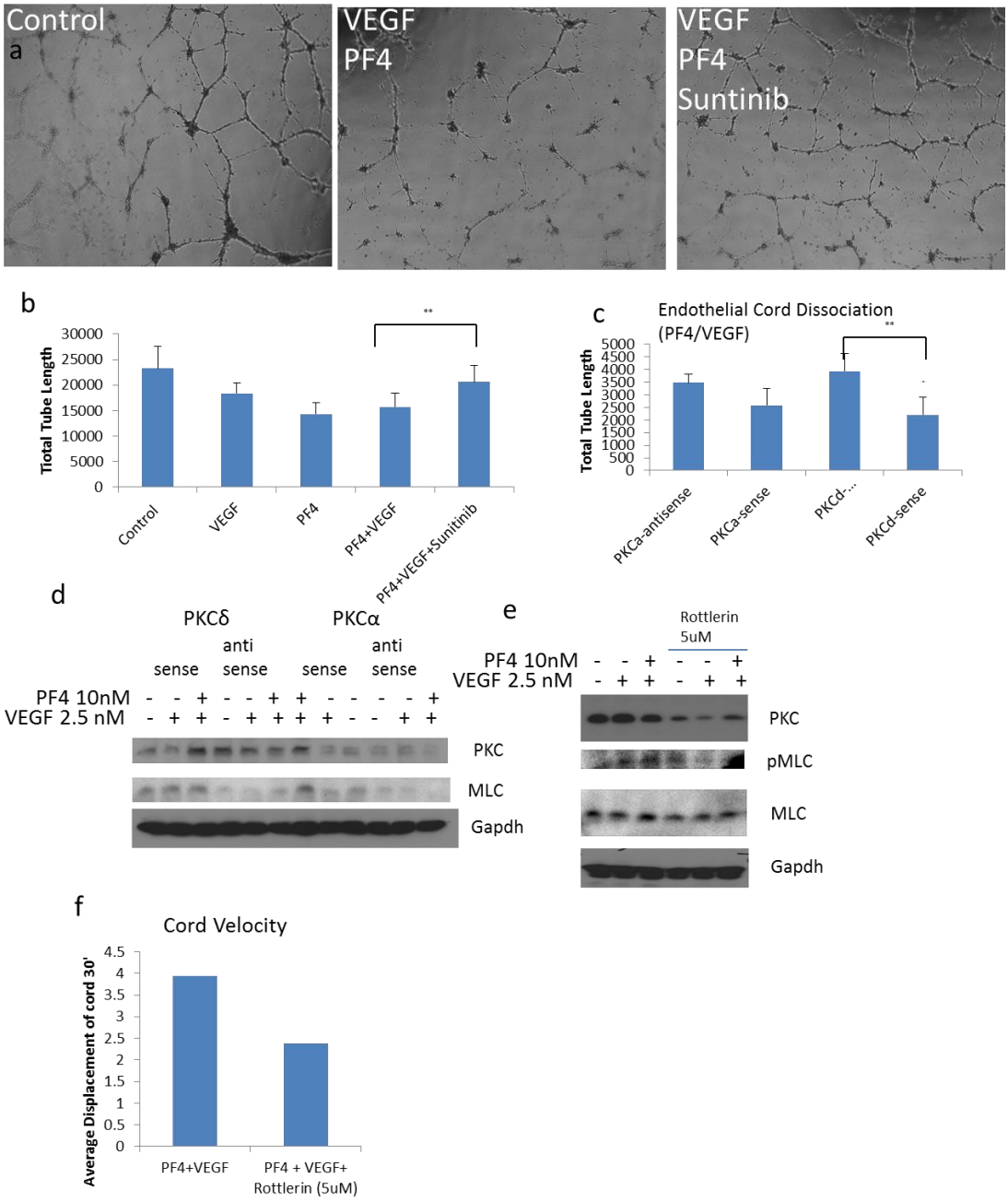

Figure 12. VEGFR/PKCδ inhibition decreases PF4 induced cord dissociation.

a) Representative phase contrast images are shown of HMEC cells treated with indicated treatments. Images were taken of live cords after 24 hours. Disruption in webbed patterning of cells indicates increased dissociation. b) Images described in (Fig 13a) were quantified as described in methods utilizing Metamorph analysis software. N = 3;

mean ± s.d. c) Quantification of cords that were induced to dissociate were quantified utilizing Metamorph as in (Fig. 13b). HMEC cells were allowed to form cords and 24 hours afterwards, 20 uM of antisense/sense oligonucleotides were added to cords as described in methods and allowed dissociate with inhibition of PKCδ for 24 hours. Cords and cord length were measured (N = 3; mean ± s.d. ** $P < 0.05$). d) Immunoblot analysis of HMEC monolayer lysates was observed during 24 hour PF4 (10 nM) and VEGF (2.5 nM) addition in the presence of antisense inhibition. GAPDH was utilized as loading control as representative blot is shown e) HMEC monolayer of cell lysates were analyzed through immunoblot analysis after PF4 (10 nM) and VEGF (2.5 nM) in the presence and absence of PKCδ inhibitor (Rottlerin 5 uM) for 24 hours. GAPDH was utilized as loading control in which representative blot is shown. f) Quantification of cord motion in supplemental movies 1 and 2. Live cell imaging of cords as they move were quantified with metamorph analysis (Sup. Movie 1/2), as each cord excluding branches were tracked and quantified as average distance displacement for 30'. N =14 cords measured; mean ± s.d. **$P < 0.01$.

3.3.2 VEGFR/PF4 induced dissociation is partially PKCδ-dependent

As tube dissociation involves the separation of cell-cell contacts, we investigated whether transcellular contractility was involved. This was blunted by downregulation of the key regulator of this tension PKCδ. Formed cords were stimulated to dissociate with PF4 and VEGF in the presence or absence of antisense towards PKCδ or PKC-alpha (as a control). After stimulation with VEGF/PF4 for cords to dissociate, endothelial cord length was partially rescued by antisense against PKCδ (Fig.12c). PKC-alpha downregulation attenuated cord dissociation to a

lesser degree. In addition, the antisense decreased PKCδ and MLC-2 levels/ppMLC-2 levels that are downstream of PKCδ regulation (Fig. 12d). PKCδ inhibition was also achieved pharmacologically utilizing the selective agent rottlerin, which limited MLC and ppMLC regulation through downregulation of PKCδ (Fig 12e). These data indicate that PKCδ affects MLC-2 actions, which in turn regulate stress fibers.

3.3.3 Dynamics of endothelial cords/capillaries tension require PKCδ-dependent motility of cords and individual cells

Cell motility is critical for vessel formation and vessel regression (Stokes et al., 1991). To further investigate the role of activating PKCδ during VEGF/PF4 mediated dissociation, endothelial cords were induced to dissociate in the presence of the PKCδ inhibitor rottlerin. This decreased random cord motion (Fig 12f, Sup. Movie 1, 2). Cord motion, a process of vessel maturation, was also observed, but to a lesser extent, in controls not being induced to dissociate. From these data, dissociation was mediated by motility of the cells and possibly isometric contractions that are PKCδ dependent.

We further investigated cord dissociation/mobility and found increased mobility and contractions in cords that were treated with PF4/VEGF (Sup. Movies 3, 4). In addition, increased force was exerted onto the Matrigel by the endothelial cells seen by the deformation of the substratum as noted with phase contrast microscopy. Individual endothelial cells at junctions were compressed and spheroid, in which its morphology appeared to integrate with movements for cord collapse (Supplemental Movie 4).

3.3.4 Activation of PKCδ is increased during PF4/VEGF induced dissociation of endothelial cords

To further investigate whether PKCδ was activated during dissociation, dissociated cords were examined for their levels of PKCδ and activated PKCδ. In PF4+VEGF induced cells, this ratio was increased at junctions with increased phosphorylated PKCδ at junction edges and decreased PKCδ in the inner part of the junction (Fig. 13). In addition, in PF4 only and PF4+VEGF mediated dissociation, endothelial cells with a spheroid morphology with increased activated PKCδ were also observed. Although, PKCδ has been shown in previous literature to mediate apoptosis (Geraldes et al., 2009; Shizukuda et al., 1999a), it seems that these cells may compress themselves to incorporate in the cords and to actively mediate tension at junctions (Fig 13, Supplemental Movie 4). This would be consistent with our findings of PKCδ directing cell contractility (Iwabu et al., 2004), Jamison et al}. These data infer that activation of PKCδ is associated with force generation and possibly force mechanics in endothelial cord dissociation.

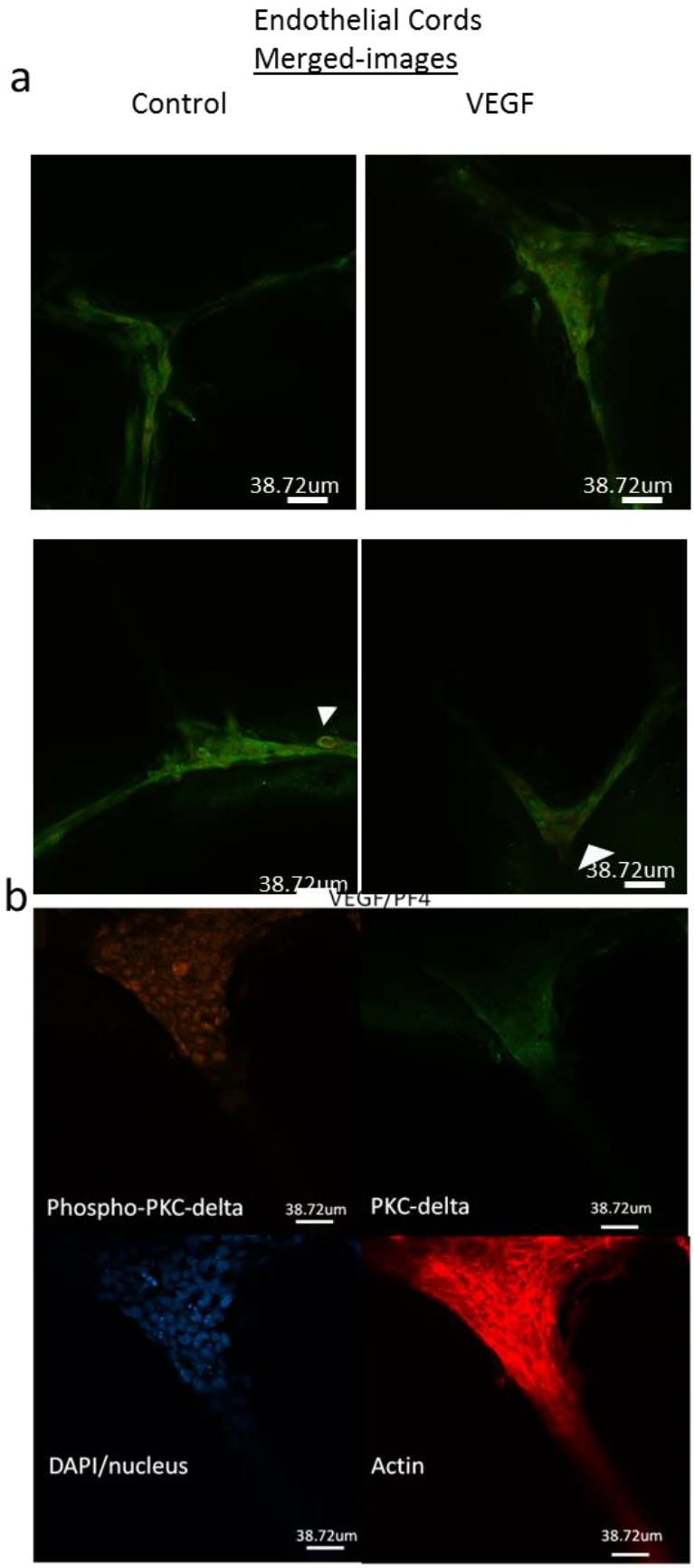
Endothelial Cords
Merged-images
a
Control
VEGF
38.72um
38.72um
38.72um
38.72um
b
VEGF/PF4
Phospho-PKC-delta
38.72um
PKC-delta
38.72um
DAPI/nucleus
Actin
38.72um
38.72um

Figure 13. Figure 13. PKCδ is activated at cord junctions during dissociation

Immunostain of HMEC endothelial cells formed into cords onto Matrigel treated with indicated treatments for 24 hours after cords are formed. a) Representative images through confocal microcopy were selected where immunostained PKCδ and phospho-PKCδ are indicated by green and orange respectively. Increased orange/red staining indicates that phospho-PKCδ has increased activation. b) Representative image of PF4 100nM/VEGF 2.5 nM treated cord that was immunostained with PKCδ and phospho-PKCδ indicated in green and orange separate images. Cells were also stained for nuclei with DAPI staining represented as blue and actin with phallodin (Alexa-633) represented as red. Representative image of a larger sized cord indicates increased effects of force translated in the ratio of activated PKCδ to PKCδ. Arrows of increased phosphorylated PKCδ expressing cells indicates positioning of possibly active cells at branch points of cords.

3.3.5 Mechanotransduction of VEGF/PF4 induced cord dissociation is PKCδ dependent

Cord dissociation involves the coordination of intercellular forces in order for capillaries to collapse. Zyxin, a focal adhesion protein that has been previously found to be induced during stretch induced response, was utilized to investigate mechanosensory input into the cords as they dissociate. Stably transfected zyxin and PKCδ was increased in Matrigel induced endothelial cords in comparison to tissue culture (data not shown). This finding may indicate endothelial cords upregulate force signaling compared to 2D monolayer of cells. Further investigation of zyxin showed that its expression is linked to PKCδ activity, as inhibition of PKCδ caused increased zyxin expression in 2D monolayer (Fig 14a). Furthermore, downregulation of PKCδ

with antisense oligonucleotides in formed endothelial cords induced to dissociate through PF4/VEGF led to increased zyxin expression (Fig 14b). These data suggest focal adhesions are dynamically regulated when force is induced and when inhibited focal adhesions are stabilized and contributing to inhibition of cord dissociation.

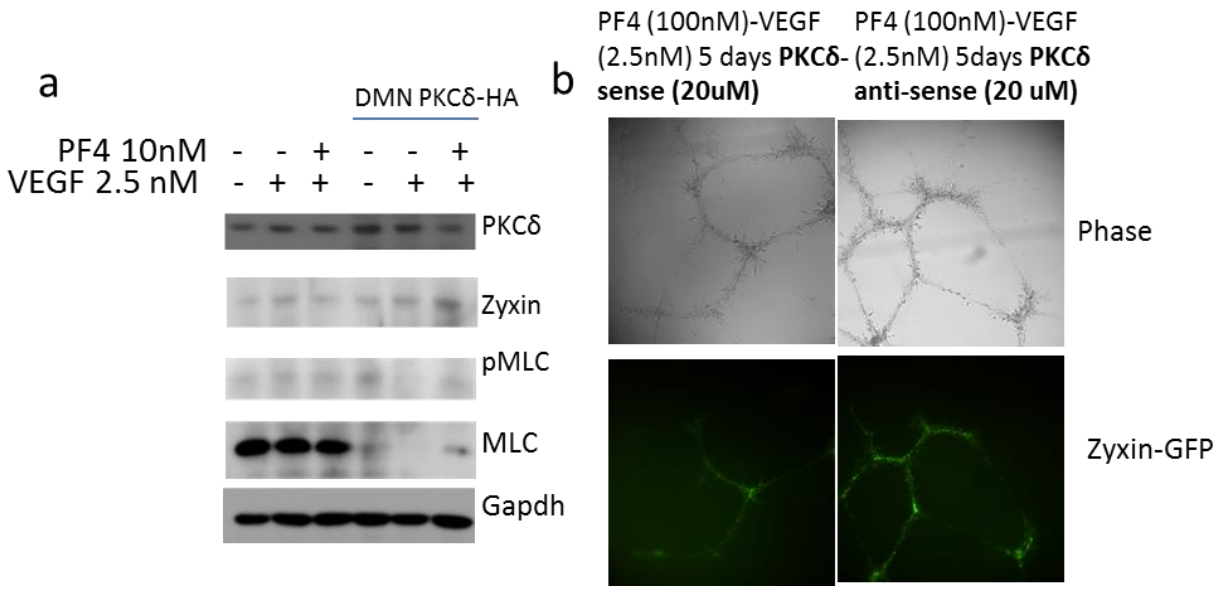

Figure 14. PKCδ inhibition stabilized focal adhesions during PF4-VEGF mediated dissociation

Immunoblot of HMEC monolayer transfected with Dominant negative PKCδ (4ug/lipofectamine) prior to treatment PF4/VEGF treatment in quiescence media for 24 hours.) Live cell fluorescent images of Zyxin expressing HMEC-1 cells that have formed cords. After cords were formed cells were incubated for 5 days with 20 uM PKCδ-sense or PKCδ-anti-sense in VEGF 2.5 nM/PF4 100 nM.

To further investigate how cords regulate force, PKCδ localization was also investigated during live cell imaging. PKCδ expression localizes to actively motile areas of the active cord, (Fig. 15, Sup. Movie 6). These data suggest force signaling through VEGFR is being applied during dissociation through motility signaling via PKCδ. By individual endothelial cells

inducing dissociation, PKCδ regulation of force is directly involved in cord stability and formation. By its regulation in cord movement, signaling through VEGFR causes PKCδ to elicit both static and dissociative regulation of force respectively for endothelial capillary stability and dissociation.

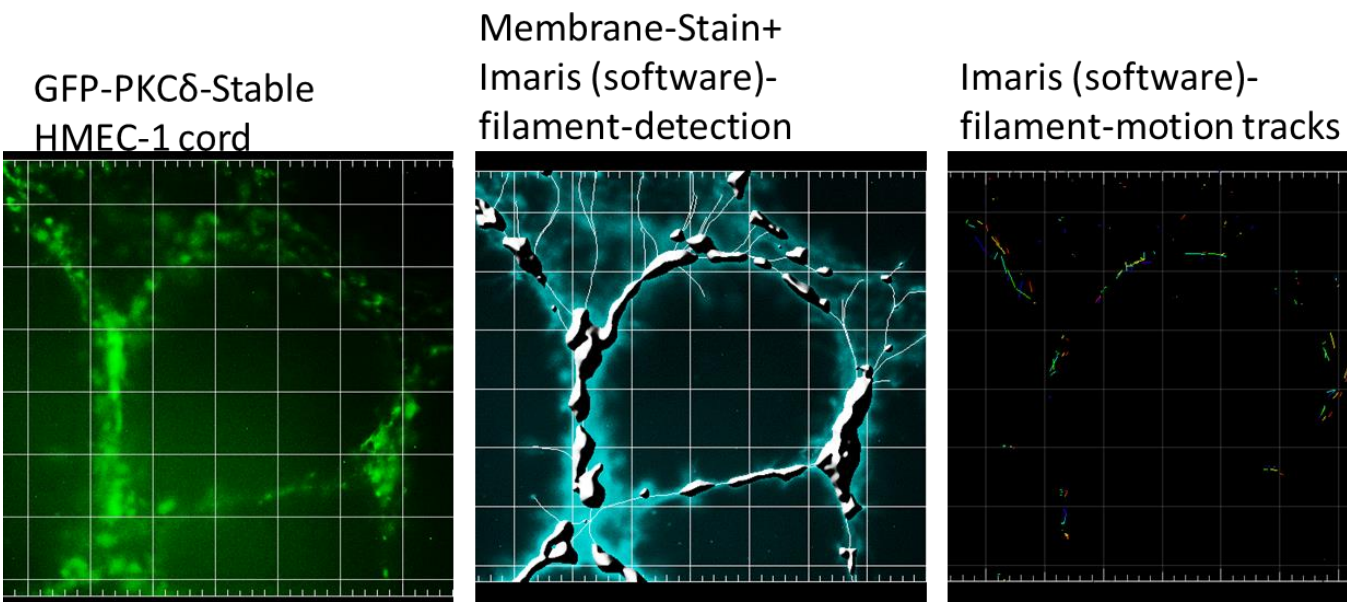

Figure 15. PF4-VEGF force induction that influences PKCδ expression in cords

GFP-PKCδ /membrane stain (light blue) fluorescence, phase were examined on live cords expression on cords after 1 day of incubation in quiescent media as images were taken during live cell imaging at 10x objective magnification. Sup Movie 5 was analyzed by imaris software utilizing filament tracking program, multi-colored lines represent different stages of motion color coded by the time interval.

These data suggest force signaling through VEGFR is being applied during dissociation through motility signaling via PKCδ. By PKCδ driven regulation in cord movement, signaling through VEGFR causes PKCδ to increase dissociative regulation of force for endothelial capillary stability and dissociation.

3.4 DISCUSSION

PF4 receptor, CXCR3, primarily regulates endothelial capillary dissociation through calcium regulation. Through upregulated calcium levels, this mechanism causes u-calpain activation which results in cleavage of focal adhesions for deadherence and increased calcium levels also modulates various proteins for cytoskeleton structure (Bodnar et al., 2006). This study investigated VEGFR signaling to PKCδ as a non-direct molecular mediator of PF4 induced cord/capillary dissociation. From this study, PKCδ that is regulated by VEGFR\tyrosine kinase signaling plays a role in dissociation in PF4 (Fig 12). Attenuation of cord dissociation through inhibition of VEGFR and PKCδ indicates that full dissociation of cords on Matrigel requires VEGFR signaling and PKCδ signaling (Fig 12a, Fig. 12b). VEGFR regulation also mediates increased regulation of PKCδ seen in our data with upregulation of PKCδ levels (Fig. 12d). PKCδ regulation of contractility through MLC was observed through data in which antisense of PKCδ and PKCα antisense caused MLC downregulation after PF4-VEGF treatment (Fig 12d). However the effect of PKCα antisense was less and may partially affect PKCδ activity indirectly through an unknown mechanism. We furthermore decreased dissociation response through inhibition of rottlerin by causing the cords to become static and non-motile (Supplemental Movie 1, 2, Fig 12f). As motility is essential for cord formation, our data also suggest importance in cord integrity (Supplemental Movie 3), that translates to cord retraction and dissociation (Supplemental Movie 4). Activation of PKCδ was also investigated during dissociation in cords. The ratio of activated PKCδ to PKCδ was observed increased at junctions in PF4-VEGF treated cells (Fig 13). Moreover, some cells that are activated with PKCδ are circular and are centrally located at junctions (Fig 13). It is interesting to postulate whether these cells are functionally important in integrating cord force as the cords dissociate or move; further experiments are

needed to fully test this hypothesis. If future data supports this idea, it offers new insight into how PKCδ regulates motility and force in the microvasculature. In this situation, motility and force would be essential if these cells act dynamically to integrate with cord motion.

We furthermore show that cord motility and dissociation is directly linked to PKCδ regulation. PKCδ contribution to dissociation seems to effect plasticity of active movement more so than disrupting intracellular junctions mediated by calpains (Fig 12f). PKCδ have been previously found to regulate focal adhesions through its kinase domain and down-regulate focal adhesions (Fordjour and Harrington, 2009; Grinnell and Harrington; Harrington et al., 2005)). Although this mechanism is not novel for PF4 induced dissociation, previous findings fail to attribute contractility as a function of this mechanism to down regulate focal adhesions. Although direction interaction of PKCδ to focal adhesion complexes have been found, it may also serve to mediate multiple functions in suppressing focal adhesions while up regulating contractility through its kinase activity. However, there is direct regulation of MLC activity through PKCδ in which MLC localizes throughout the cytoplasm along stress fibers, as focal adhesion and stress fibers are directly linked in the cytoskeleton (Wang et al., 2001a; Wang et al., 2001b). From this investigation we show that PKCδ regulates dissociation through VEGFR to PLCy1 signaling. However, it remains unknown of whether force signaling is directing the dissociation or recursively responding to force regulation. Both situations require PKCδ but in a different context for full dissociation. From this investigation, it is also realized that motility mechanisms seen in 2d migration play an important role in multi-cellular tissue. Increased calcium signaling perpetuates increased contraction and deadhesion (Bodnar et al., 2009), as VEGFR signaling is also mediating and directing the precise contractions through PKCδ signaling. Furthermore, these data indicate chemotactic signaling for sprouting and motility

regulates this dissociative response through PKCδ, as its capabilities to translocate to cellular compartments help mediate this directionality (Jamison, et al.).

Furthermore, after dissociation has occurred, stabilization of PKCδ may still mediate further contraction of cords to form stabilized vessels (Fordjour and Harrington, 2009; Grinnell and Harrington; Harrington et al., 2005). These events fine tune the normal vascular structure and provide stabilized signaling for autocrine signaling, as downregulated chemotaxis drives vasculature stabilization and formation. VEGF induced chemotaxis upregulation of PKCδ drives destabilization of the vasculature that is responsive to motile signaling. This is combined with extracellular signaling of other molecular mediators and regulation by the stroma. Furthermore, pericytes also regulate the microvascular with force exertion (Lee et al., 2010; Murphy and Wagner, 1994) and the study of how pericytes regulate cord stability/dissociation would further elucidate how force signaling is modulated *in vivo*.

3.5 MATERIALS AND METHODS

HMEC-1 cell culture--HMEC-1 (human dermal microvascular endothelial cells) were obtained from CDC, Atlanta, Georgia. Endothelial cells were grown in MCDB 131 (Gibco) media with 10 mM L-glutamine supplemented and 10% fetal bovine serum. In addition, stable cell lines of HMEC-1 cells were also established with neomycin selection (350 ng/mL) of Zyxin-gfp plasmid obtained commercially from (Origene). Stable selection of focal adhesion marker

Matrigel Tube formation assay--Cells were grown to 50% confluency prior to seeding onto Matrigel. Matrigel (BD biosciences) was seeded onto u-chamber slide at 10 ul per well or

120 uL per well for 24 well plate and incubated for 30 minutes at 37℃ for polymerization. Cells were then re-plated onto matrigel at 75,000 cells per well (24 well), 15,000 cells per well (u-chamber slide, Ibidi) for 24 hours. Cords were grown overnight in quiescent media 0.5% FBS. Afterwards endothelial cords were allowed to dissociate with VEGF-BB growth factor and Recominbnat PF4 (PeproTech) that was added at 2.5 nM and 100 nM respectively for 24 hours or indicated time points in figure/movie. Some groups cells were quantified for cord length by adding 2.5 uM Cell Tracker Green for better quantification of cords.

Antisense inhibition: DNA oligonucleotides sequence targeting PKCδ and PKCα were obtained from the literature () and ordered from IDT adding phopho-thioate bonds at the end nucleotides. Oligonucleotides sense and anti-sense of human PKCδ are GTGGCATGATGGAGCCTTTT and 5'TTTTCCGAGGTAGTACCGTG-3' respectively. Oligonucleotide sense and antisense PKCα are 5'-CGGGCAACGACTCCACGGCG-3' respectively. DNA oligonucleotides at 20 uM were added after HMEC cords were formed in quiescence media, and additional antisense was added to the cells via lipofectamine according manufacturer protocol.

Live Cell Imaging—HMEC-1 cells were grown and plated in a 6 well glass bottom plate on matrigel as previously described and then put into live cell chamber with (5% O_2, 5% CO_2, 90% N_2) at 37℃. HMEC cells were imaged for either 7 hours at 30 minute intervals (Movie 1, 2) or for 1 hour and 20 minutes at 5 minute intervals (Move 3 and Movie 4). Size bars are indicated in image. HMEC cells in movie 5 were taken for 4 hours at 15 minute intervals in 6 well glass bottom well, cords were induced to dissociate after 3 days of quiescent media, then induced to dissociate with PF4 100 nM and VEGF2.5 nM for 4 hours where pictures were taken every 15

minutes.

Immunostaining/Confocal Microscopy—HMEC-1 cells cultured and plated onto 15 well u-chamber slides (Ibidi) as previously described. After selected treatments, cells were fixed in 2% formaldehyde in PBS for 10 minutes. Cells were then permeabilize with 0.1% Triton-X-100 in PBS (wash buffer) for 10 minutes. Afterwards, cells were washed for 30 minutes (3x washes) prior to incubation with antibody. Antibodies were diluted (1:50) for anti-phospho-PKC-theta/delta (S643/S676)(Cell Signaling) polyclonal rabbit and (1:100) anti-PKCδ (polyclonal anti-mouse) in wash buffer with 30 mg of bovine serum albumin and cells were immunostained overnight at 4°C. Cells were then washed for 15 minutes (3x) and incubated with secondary antibody at (1:100) (Alexa-488-antimouse and Alexa-594-antirabbit- LifeTechnologies) in wash buffer/30mg of BSA/5% goat serum, as secondary antibodies were raised in goat. Antibody was incubated onto cells for 1 hour at room temperature. Cells were then washed (3x). Alexafluor-633 phalloidin (1:40) in PBS and DAPI (1:10000) was added to cell cells. Afterwards, cells were desiccated for 60' at RT to decrease matrigel depth (z). PBS was added to cells for 30 minutes prior to imaging.

4.0 DISCUSSION

Individual cells regulate force throughout various tissues to provide proper structure and function for various physiological and molecular processes. This study suggests PKCδ as an important regulator of motile cellular force through EGFR signaling. As specific localization of PKCδ directs force spatially, regulation of force signaling exerted by the cell is primarily directed by growth factor signaling. By creating constructs of PKCδ that increased localization to the membrane, we increased specific signaling of EGFR activation to PKCδ through PLCy1 activity. We found that targeting PKCδ to the membrane increased force to the ECM through cell traction force microscopy (Fig 7). Through this directed cellular localization, it also caused a measurable increase in cellular force to the 3D ECM as well (Fig 9). However, regulation of MLC was relatively the same when comparing negative control that is not targeted to the membrane (data not shown), as there is slight increase in PKCδ activation. From these data, EGFR signaling to PLCγ1 may actually position rather than initiate activation. Although, EGFR signaling to PI3 kinase may alternatively mediate a more direct role in regulating PKCδ molecular activation through PDK1. These data also suggested that force signaling through PKCδ was PLCγ1 dependent by utilizing EGFR-mutant cell lines that is PLCγ1 activation deficient which exhibited response to EGF with reduced force signaling (Fig 7). These results were similar to a previous study demonstrating that these cell lines were deficient of force signaling at early time points but also activated force through PKCδ and MLC at later time

points with sustained contractility (Iwabu et al., 2004). This also infers that EGFR signaling to PKCδ to regulate force signaling is modulated under more direct cytoskeletal regulators for force possibly mediated byPLCγ1 independent pathway such as Rho-GTPASE during sustained contractility (Totsukawa et al., 2004).

4.1 FORCE SIGNALING IS SPATIALLY LOCALIZED

Through the technique of membrane targeting, PKCδ localization was directed spatially closer towards PLCγ1 signaling. Force signaling that is normally mediated by PKCδ remains intact for cell motility. Membrane targeting PKCδ caused increased force towards the ECM but restricting maximum speed due to increased adhesiveness. This is suggested from our data in which membrane targeting caused attenuation of cell speed for cell motility (Fig 10). PKCδ localization to the membrane directs the signaling of cell force through RTK signaling as this translocation to mediate signaling is relevant to our understanding of the coordination of cell motility. While lamellipodia and filopodia extending forward during cell motility, the signaling for contraction of the cell body is needed to connect towards the lamellipod. Activation and translocation of PKCδ partially mediates this contraction of the cell body towards the lamellipodia during 2D cell motility. Moreover, concomitantly with this process, it is also likely that in order for contraction to occur sufficiently, cell force must be initiated from the ECM that is connected to the cell membrane. The membrane may also be utilized in anchoring a molecular scaffolds involving PKCδ to direct the location of force signaling. At the cytoplasm with a less diverse milieu of PKC activators, regulation of kinase signaling possibly occurs but is delayed as it is not coordinated from the extended membrane of the lamellipod. Alternatively, increased

contractility can occur from the membrane through Rho Gtpase signaling. In both cases, PKCδ kinase activity can signal downstream at which retrograde flow of activated pools would then direct cell body contraction perinculear where PKCδ accumulates (Fan et al., 2006). All of this process is initiated by EGFR/VEGFR kinase activity.

This process is dynamic where the leading edge is driven by adherence and persistence based on focal contacts (Weiger et al., 2009). PKCδ signaling was shown to drive protrusions but may biophysically orient the cell to the direction of lamellipodia extensions. However, increased membrane targeting may conversely cause increased sensitivity to lamellipodium protrusion, at which would decrease persistence due to increased ambiguity of directional signaling (Fig. 10). Instead of directional migration,ambiguous remodeling of the provisional matrix for proper wound healing may also occur (Allen et al., 2002). In this context, these lamellipodia and filopodia are not only needed for active 3D motility, but also mediate compaction of the ECM. As the lamellipodia and filopodia connect to collagen through focal adhesions and integrins, both motile and isometric force are acting simultaneously for compaction (Allen et al., 2002).

4.2 PROJECTED REGULATION OF PKCδ IN 3D ENVIRONMENT FOR FIBROBLASTS

Our experiments to capture PKCδ regulation of force signaling may be modest compared to the 3d environment. Previous findings indicate that cell protrusions in 2D are more relevant predictors of how cells migrate in 3D (Meyer et al., 2012). Furthermore, increased regulation of force due to cell extensions/protrusions in a 3D ECM may occur while the cell is supported and suspended via these connections to the ECM. In contrast, the cell is suspended only by

gravity, impacting its weight and force signaling on a 2D flat substrate. The force directed at 2D protrusions is projected to be minimal since it is not supported. In addition, durotaxis signaling that correlates with 3D environment could also be investigated through challenging the cells to migrate on an adhesive substrate. When membrane targeted PKCδ expressing cells were challenged to migrate on an increased collagen substrate, they were more resilient to increased adhesiveness by less deviation in their persistence when challenged (Fig. 10). This indicates that although membrane targeting causes slower cell speed, it has increased adhesiveness to the substrate.

4.3 FORCED MEMBRANE TARGETING OF PKCδ

To explore the role of PKCδ regulation through PLCy1, the increase of membrane targeting was achieved by genetically adding a k-ras farnesylation motif at the c-terminus to PKCδ. To isolate the engineered product, the endogenous PKCδ was knocked-down while the human PKCδ was transiently and stably transfected into NR6-WT mouse fibroblast cell lines. With transient transfections, many cells would over-express the PKCδ construct with the high levels of proteins possibly accumulating and mislocalizing to different cellular compartments. Furthermore, overexpression of PKCδ also saturates total levels but not activated levels making it difficult for experimental analysis.

A second confounding variable is that adding a farnesylation moiety increases the hydrophobicity of the protein. This modification may be independent of the regulatory region of PKCδ, at which the c-terminus interacts with neither the regulatory region nor the kinase domain. However, this modification may cause changes in PKCδ leading to alternative protein-

protein interactions to cause the altered phenotype observed. As previously discussed, PKCδ has a number of permissive interactions. These nonspecific interactions may occur, but it is highly unlikely to confound the major hypothesis. Moreover, multiple experiments (PLC inhibition and EGFR deficiency that regulates PLC) show that membrane-targeted PKCδ activation is under less stringent regulation.

One known activator of PKCδ which may play role in EGFR induced signaling is PDK-1. By constitutively localizing PKCδ at the membrane, the membrane anchored PDK-1 may activate PKCδ in an unregulated manner. This may possibly be the cause of a lower threshold to PLCy1 noted with these constructs. This may factor into PKCδ seemingly constitutive activation.

However, the main pitfall of experimentation but conversely the greatest insight in extrapolating PKCδ mediated effects on motility is that membrane targeting does not increase molecular MLC activation. Transiently and stably expressing PKCδ does increase MLC activation with the addition of PKCδ, but increase in activation does not occur when PKCδ is targeted to the membrane. From this lack of increase compared to negative control suggests that increased membrane localization of PKCδ in itself does not cause increased MLC activation. However, membrane targeting induced increased de-alignment in stress fibers. As discussed previously, this could be an indirect effect of increasing cytoskeletal regulation through PKCδ activation, or from what I proposed in (Chapter 2) that this membrane targeting of PKCδ causes redistribution of MLC activation. Unfortunately, it would be very difficult to test causative interactions, since PKCδ interaction to MLC is not direct but through MLCK. And to further confound rationalizing this molecular pathway in motility, PKCδ interaction to MLCK is not direct. To possibly investigate this question with current research tools, is to develop a light-

activated PKCδ construct and have MLC conformational FRET construct. As positional activation PKCδ with fluorescence light the activation could be linked to MLC status. But this is even more artificial than this project, though a positive finding would further confirm that localization regulates the alignment of MLC for increased contractility.

Furthermore, there is also a possibility of testing an indirect effect, where MLC activity and distribution may be indirectly affected by PKCδ activation. To partially circumvent indirect effects, this project could further be strengthen by evaluating substrate specificity directly. Computational modeling and critical mutations to PKCδ that would alter kinase specificity could be generated to determine whether the specificity of MLC in this pathway is specific. Further studies in kinase specificity would be needed by modulating bona fide interactions and nonspecific interactions through mutations. This artificial system would be able to test specificity as experimental tools are becoming more available and engineered DNA more accessible in addition to the technology of high throughput screening PKCδ construct activity. Nevertheless, systems biology and *a priori* predictions may be an alternative and cleaner way to define loosely connected pathways for testing.

4.4 PKCδ MAY NOT BE INVOLVED IN DUROTAXIS SENSING

For PKCδ regulation of cellular contractility, durotaxis responsiveness/sensitivity may not be augmented. To some extent, this may correspond to the rationale of membrane targeted-PKCδ expressing cells migrate slower in 2D, but are better able to withstand a very adhesive substrate, necessitating force signaling to emanate from membrane localized PKCδ to the cell body. Whether PKCδ signaling is connected to contractile units that sense restrictive environments during durotaxis is largely unknown. However, increased signaling of PKCδ to

direct force signaling to the ECM may come at a cost that may override a cell's tendency to move at faster speeds/maintain constant deformation (Roca-Cusachs et al., 2013). I speculate that PKCδ signaling is working independently of this durotactic sensing, by membrane targeted PKCδ results in reduced cell motility. It is also presumed that if it is a part of durotactic sensing that it would increase persistence in a restrictive environment rather than maintaining persistence at a constant level. Regulation of PKCδ in these experiments suggests that its lack of responsiveness to adhesiveness may possibly make it independent to durotactic force sensing (Plotnikov et al., 2012; Roca-Cusachs et al., 2013). This may be relevant to the general understanding of chemotaxis.

Downstream of EGFR chemotaxis signaling, fibroblasts must direct force irrespective of substrate stiffness in order to migrate appropriately into heterogeneous tissue. In the wound milieu, fibroblasts and other cell types must migrate through ECM that has different rigidity. This raises an important experimental question that should be investigated: does chemotaxis function above durotaxis during cell migration? Rationalizing the differences and key molecular players in cell migration would fuel pursuits investigating 3D environments, as the differences in regulation during 2D migration is more obvious.

4.5 PKCδ REGULATION OF FORCE ENDOTHELIAL CORDS

To additionally investigate PKCδ dependent regulation of cellular force, another experimental model involving endothelial cells in cords was utilized during dissociation induced by CXCL-4 and VEGF. In human body microvasculature, endothelial cells experience continual cell force through either shear stress and pulsatile lamellar flow of force, by which they modulate their cytoskeleton to align with these fluid forces (Cucina et al., 1995; Galbraith et al., 1998; Imberti et al., 2000). These cells would also experience increased force during dissociation

during the initial stages of angiogenesis. From this study, PF4-VEGF induced dissociation of endothelial cords were in part mediated by PKCδ regulation as this dissociation was also mediated by VEGFR signaling (Fig. 12). Cell motility is important part of vascular sprouting, but it is also a part of vascular stability since the vascular must respond to hydrostatic force to properly respond to changes in blood flow (Fu and Tarbell, 2013). As intercellular force is mostly directed through intercellular bonds, motile force signaling would modulate permeability. Interestingly PKCδ regulates force signaling in this study through motile contractile signaling, in which we found PKCδ inhibition decreased this mobility of cords (Fig. 12, Sup Movie S2.1, S2.2). This mobility is linked to the regulation of dissociation, since VEGF signaling mediates increased vascular migration and decreases permeability in vessels. Previously, this study investigated PKCδ regulation of cell force through membrane translocation (Jamison et al., 2013). This ability to translocate, positions PKCδ to modulate force, especially in a quick and efficient fashion. Regulation in this manner may also be essential in competitive regulation of vascular sprouting that requires the modulation of VEGFR upregulation in sprouting tip cells (Hellstrom et al., 2007). PKCδ regulation intracellularly may affect these sprouting cells by mediating force towards the extended protrusions that sense the ECM as it projects the cell body away from the main endothelial cord. Furthermore, this regulation of endothelial cords as a whole, causes cords to collapse through this contraction of the cell body. This coordinated contraction causes vessels or cords to involute, shifting cell distribution in the vessel or cords at branch points as a result of retraction. Involution of the original vessel may serve as a source for increased cells at branch points, where new vessels can reorient themselves more towards chemotactic signaling. Furthermore, we found that this force signaling translate to increased focal adhesion activity, as PKCδ inhibition caused increased upregulation of Zyxin that is

mechanotransduction regulated (Fig. 15). From these data, it is concluded the cell body and membrane/focal adhesions are affected by PKCδ activity to translate force to the ECM.

4.6 DUROTAXIS REGULATION OF ENDOTHELIAL CORDS ON SUBSTRATE/STROMA

In addition, our findings also show that endothelial cords exert force onto the ECM during live cell imaging, even in non-treated cords. These data suggest the ECM is not just an important scaffold and reservoir of growth factors to modulate endothelial cords, it is also a regulator of durotactic signaling. Endothelial cords exert force onto the Matrigel ECM, which is a soft substrate that deforms considerably. Previous findings have shown that soft substrates, affect the differentiation of many cell types (Evans et al., 2009). Force that is exerted on the substrate actually may be another form of signaling through durotaxis that coordinate cells spatially in the vasculature/cords (Heo et al., 2011; Teh et al., 2011, 2013). The leading endothelial cells can also digest and secrete the ECM, guided similarly to juxtacrine signaling through ECM modulation. Thus, this facilitates the migration and capillary formation of endothelial cells that receives these signals. However, PKCδ is only contributing to force regulation and not durotactic signaling. Dissociation that is induced by VEGF would reorient this ECM tracking behavior of endothelial cells, as this is required for angiogenesis to form new sprouting capillaries that are channeled towards the chemotactic signaling. Downstream of VEGFR signaling, PKCδ regulates force in cell migration. It is postulated that dual function of PKCδ stabilizes vascular permeability by orienting ECM mediated stress fibers, which would conversely place the pathway critical for contributing to the disorganization of stress fibers in response to VEGF. These events that channel ECM deposition (Yates et al., 2011) may also

channel durotactic force on the ECM to modulate new orientations of capillary formation. Mechanisms of how force signaling drive this process would have to be elucidated.

4.7 MULTIPLE TASKS OF PKCδ MAY BE LINKED INFLUENCING DIFFERENTIATION

PKCδ has been implicated in regulating differentiation in keratinocytes (Joseloff et al., 2002), as cells may differentiate through force signaling by PKCδ. Regulation of the cytoskeleton may be a part of mediating differentiation in multiple cell types through PKCδ. Moreover, complicated involvement of PKCδ regulation of vesicular trafficking, is being attributed to mediating differentiation in many cell types.

4.7.1 PKCδ regulation of Exocytosis

It is known that PKCδ regulation directly influences trafficking, as first seen through EGFR attenuation of signaling (Wells et al., 1998). Additional studies, are correlating this regulation of exocytosis in the effects in bone metabolism, granule exocytosis, to differentiation (Cremasco et al., 2012; Ma et al., 2008). PKCδ regulation of force signaling and chemotactic motility may coordinate both cytoskeleton signaling with vesicular trafficking for exocytosis in certain cell types. Furthermore, in osteoclasts PKCδ has been known to regulate remodeling by regulating exocytosis through cathepsin K (Cremasco et al., 2012). In lymphocytes, PKCδ functions in exocytosis during lytic granule exocytosis utilized by CD8+ CTL in response to intracellular pathogens (Ma et al., 2008). Although different from the wound bed, these

functions correlate with fibroblasts functions of secreting the collagen ECM. A direct link between chemotactic signaling through growth factors and contraction of the ECM would be interesting as PKCδ may be a central player of both. Furthermore, as PKCδ mediated exocytosis may affect autocrine signaling, it may also be correlated to the release of factors necessary for appropriate differentiation of cells in the tissue. Furthermore, as new studies implicate PKCδ in additional roles, its cytoskeletal regulation may overarch other functions in the cell. These functional studies also highlight the need for analysis of signaling proteins such as PKCδ that has broad downstream effectors but converge and coordinate functions. As PKCδ was successfully investigated through a systems biology approach, it is very likely that this approach is needed to also fully characterize multi-specificity kinases.

4.8 FUTURE EXPERIMENTAL DIRECTIONS

PKCδ has been implicated in numerous pathways that possibly converge on one another in signaling and functionality. Additional studies are needed to investigate the exact consequence of PKCδ localization as it relates to receptor internalization. Furthermore, studies are also needed to investigate how vascular pruning is directed through cheomtactic signaling. Active motility signaling in the microvasculature through PKCδ may also involve pericyte regulation of vessel stability. Further investigation of how this occurs during normal and pathological angiogenesis would also be of interest to pursue. This project's investigation of cord mobility highlights that migration does not stop when the cells are coalesced in a capillary. Migration does occur in intact capillaries/cords and further study is needed in how it is implicated in vessel stability in different systems.

It is also essential that PKCδ along with its family members undergo a thorough pathway analysis, considering additional parameters of localization and asymmetric signaling in cells. But as computational software advances with more powerful informatics algorithms, it may even be more advantageous to pursue pathway analysis of how the PKC family functions converge or diverge from lower to highly evolved organisms. As this family of kinases is highly conserved in addition to multiple functionality of each of its members, investigating the evolution/progression of the family's functions may help to understand more comprehensive implication of signaling. Integrating the analysis of this type of study wound increase the knowledge of how PKC is functioning in the cell. PKC function may also be compared across multiple cellular lineages. Fibroblast utilization of PKCδ may be similar to lymphocyte utilization of PKCδ during rolling adhesions. This type of study may be helpful in getting a grasp of how PKCδ signaling translate to multiple cellular functions. Translational research would require inhibition/activation of a particular PKCδ mediated function to avert a pathological phenotype. Translational research utilizing this approach would extrapolate defined signaling from a complex multi-signaling pathway. This would become especially essential in designing therapeutics in complicated disease processes such as wound healing.

Knowledge that PKCδ regulates force in an RTK dependent manner may help in advancing strategies for therapies in various diseases such as wound healing requirements of PKCδ regulation of force.

4.9 FUTURE CLINICAL APPLICATIONS

4.9.1 Clinical Applications in Wound Healing

Wound healing is a very dynamic and complex process for regeneration in which exogenous addition of growth factors have been largely unsuccessful in correcting scarring and failure to heal (Yates et al., 2012). As a model for almost perfect wound healing early gestation in-utero wound healing leads to regeneration, whereas late utero into adulthood, wound healing leads to a scar, with increased severity of scar with gestational age (Yates et al., 2011). The difference in the wound fibroblasts in-utero vs. adult is drastically different wherein fetal fibroblasts have reduced force exertion but with increased chemotactic response to EGF. This appears superficially to be conflicting since EGF is supposed to increase the signaling of force exertion. However my study supports the hypothesis that PKCδ may be regulated at reduced levels during fetal wound healing in fibroblasts. However, due to the complexity of signaling through EGFR (which is increased in the fetus), force regulation may be reduced only to a lower threshold of EGFR mediated regulation of motile signaling. Moreover, EGF stimulation would require a lower threshold to stimulate force through a less restrictive matrix environment in the fetus (Yates et al., 2012). Unpublished data also suggest that levels of membrane-targeted PKCδ were drastically lower in cells that were in a 3D collagen matrix compared to non-membrane targeted PKCδ expressing stable cells. In one perspective, this may indicate that the feed-forward mechanism of progressive contraction does not necessitate higher PKCδ or even MLC levels. It was also previously shown that contractility is modulated in biphasic manner with lower levels of activation of MLC for efficient contractility during haptokinesis (Kharait et al., 2007). Nevertheless, PKCδ regulation in fetal fibroblasts must be investigated. Furthermore, it is

known that a stiffer matrix supports increased myofibroblasts differentiation and increased scarring (Yates et al., 2011). It would even be more interesting if wound healing was dependent on matrix stiffness and composition, since in scar-less wound healing, the fetal ECM is not mature and has a less stiff matrix (Yates et al., 2012).

With knowledge that wound healing may be affected by having a less stiff matrix, additional investigation, including epidemiological, is needed on prophylactic skin care. To date, there is no published studies on whether increased utilization of skin care products (lotions, serums, etc.) to soften and hydrate the epidermis have an effect on wound healing in humans. In addition, it is not known whether the stiffness of the dermis impacts the epidermis as far as wound healing. Knowledge of the parameters of chemotaxis and durotaxis would help in the design of ECM scaffold stiffness, to graft in the wound bed.

Translational research in wound healing treatment would possibly need to consider scaffold compliance and also its ability to be modulated by fibroblasts (especially if growth factors are added to scaffold). Experimental extrapolation of force through proposed PKCδ activity may help in bioengineering a substrate tensile stiffness which would optimize the survival and differentiation of cells in efforts to reproduce tensile stiffness of the dermal ECM. As the design would implement chemotaxis, normal cells would graft into the scaffold through migration.

Negative pressure therapy is also another aspect in wound care management that utilizes force. The appropriate tension applied through this therapy to stimulate a robust response of migration into the wound may be critical in optimizing this therapy. This study may help in extrapolating the pressure need to facilitate appropriate cell response to force as they exert force during chemotaxis.

In sum, the effects of skin stiffness on wound healing, and conversely the results of increased cell contractility on wound outcome hold promise for novel approaches to limit scarring while improving healing.

APPENDIX

A.1 ABBREVIATIONS

ACN-4 – α-actinin-4

ATP – adenosine thriphosphate

Cdc42 – cell division cycle protein 42

COL - Collagen

CXCL4 – CXC ERL+ 4 (chemokine)

CXCL10 – CXC ERL+ 10/IP-10 (chemokine)

CXCL11 – CXC ERL+ 11/IP-9 (chemokine)

E-cadherin - epithelial cadherin

ECM – extracellular matrix

EGF- epidermal growth factor

EGFR – epidermal growth factor receptor

FAK-focal adhesion kinase

FBS – fetal bovine serum

FN - Fibronectin

GAPDH – glyceraldehide-3-phosphate dehydrogenase

GF- growth factor

GFP-green fluorescent protein

HB-EGF- Heparin bound- Epidermal Growth factor

KO-knock-out

MLC – myosin light chain

MLCK – myosin light chain kinase

PDGF-Platelet Derived Growth factor

PDK1- phosphoinositide dependent kinase-1

PIP_2 - Phosphatidylinositol 4,5-bisphosphate

PIP_3 – Phosphatidylinositol 3,4,5-trisphosphate

PF4- Platelet factor 4/CXCL4

PLCγ1- Phospo-lipase- γ1

Rac – Ras-related C3 botulinum toxin substrate

Ras – Rat sarcoma oncogene

Rho – Ras homolog

ROCK – Rho associated kinase

RTK- receptor tyrosine kinase

TGF-β – tumor growth factor β

VEGF- vascular endothelial growth factor

VEGFR- Vascular endothelial growth factor receptor

BIBIOLIOGRAPHY

Allen, F.D., Asnes, C.F., Chang, P., Elson, E.L., Lauffenburger, D.A., and Wells, A. (2002). Epidermal growth factor induces acute matrix contraction and subsequent calpain-modulated relaxation. Wound Repair Regen *10*, 67-76.

Andujar, M.B., Melin, M., Guerret, S., and Grimaud, J.A. (1992). Cell migration influences collagen gel contraction. J Submicrosc Cytol Pathol *24*, 145-154.

Bai, X., Margariti, A., Hu, Y., Sato, Y., Zeng, L., Ivetic, A., Habi, O., Mason, J.C., Wang, X., and Xu, Q. (2010). Protein kinase C{delta} deficiency accelerates neointimal lesions of mouse injured artery involving delayed reendothelialization and vasohibin-1 accumulation. Arteriosclerosis, thrombosis, and vascular biology *30*, 2467-2474.

Bailly, M., Condeelis, J.S., and Segall, J.E. (1998). Chemoattractant-induced lamellipod extension. Microsc Res Tech *43*, 433-443.

Baker, B.M., and Chen, C.S. (2012). Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. J Cell Sci *125*, 3015-3024.

Bates, D.O., Hillman, N.J., Williams, B., Neal, C.R., and Pocock, T.M. (2002). Regulation of microvascular permeability by vascular endothelial growth factors. Journal of anatomy *200*, 581-597.

Bertram, A., and Ley, K. (2011). Protein kinase C isoforms in neutrophil adhesion and activation. Archivum immunologiae et therapiae experimentalis *59*, 79-87.

Bodnar, R.J., Yates, C.C., Rodgers, M.E., Du, X., and Wells, A. (2009). IP-10 induces dissociation of newly formed blood vessels. J Cell Sci *122*, 2064-2077.

Bodnar, R.J., Yates, C.C., and Wells, A. (2006). IP-10 blocks vascular endothelial growth factor-induced endothelial cell motility and tube formation via inhibition of calpain. Circ Res *98*, 617-625.

Breitkreutz, D., Braiman-Wiksman, L., Daum, N., Denning, M.F., and Tennenbaum, T. (2007). Protein kinase C family: on the crossroads of cell signaling in skin and tumor epithelium. Journal of cancer research and clinical oncology *133*, 793-808.

Chan, C.E., and Odde, D.J. (2008). Traction dynamics of filopodia on compliant substrates. Science *322*, 1687-1691.

Chaturvedi, L.S., Marsh, H.M., and Basson, M.D. (2011). Role of RhoA and its effectors ROCK and mDia1 in the modulation of deformation-induced FAK, ERK, p38, and MLC motogenic signals in human Caco-2 intestinal epithelial cells. Am J Physiol Cell Physiol *301*, C1224-1238.

Chen, C.L., and Chen, H.C. (2009). Functional suppression of E-cadherin by protein kinase Cdelta. J Cell Sci *122*, 513-523.

Chen, P., Gupta, K., and Wells, A. (1994a). Cell movement elicited by epidermal growth factor receptor requires kinase and autophosphorylation but is separable from mitogenesis. J Cell Biol *124*, 547-555.

Chen, P., Murphy-Ullrich, J.E., and Wells, A. (1996). A role for gelsolin in actuating epidermal growth factor receptor-mediated cell motility. J Cell Biol *134*, 689-698.

Chen, P., Xie, H., Sekar, M.C., Gupta, K., and Wells, A. (1994b). Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but mitogen-activated protein kinase activity is not sufficient for induced cell movement. J Cell Biol *127*, 847-857.

Choi, Y.S., and Jeong, S. (2005). PI3-kinase and PDK-1 regulate HDAC1-mediated transcriptional repression of transcription factor NF-kappaB. Molecules and cells *20*, 241-246.

Clark, R.A., Tonnesen, M.G., Gailit, J., and Cheresh, D.A. (1996). Transient functional expression of alphaVbeta 3 on vascular cells during wound repair. The American journal of pathology *148*, 1407-1421.

Cremasco, V., Decker, C.E., Stumpo, D., Blackshear, P.J., Nakayama, K.I., Nakayama, K., Lupu, T.S., Graham, D.B., Novack, D.V., and Faccio, R. (2012). Protein kinase C-delta deficiency perturbs bone homeostasis by selective uncoupling of cathepsin K secretion and ruffled border formation in osteoclasts. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *27*, 2452-2463.

Cucina, A., Sterpetti, A.V., Pupelis, G., Fragale, A., Lepidi, S., Cavallaro, A., Giustiniani, Q., and Santoro D'Angelo, L. (1995). Shear stress induces changes in the morphology and cytoskeleton organisation of arterial endothelial cells. European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery *9*, 86-92.

del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J.M., and Sheetz, M.P. (2009). Stretching single talin rod molecules activates vinculin binding. Science *323*, 638-641.

Dembo, M., and Wang, Y.L. (1999). Stresses at the cell-to-substrate interface during locomotion of fibroblasts. Biophys J *76*, 2307-2316.

Dickinson, R.B., and Tranquillo, R.T. (1993). A stochastic model for adhesion-mediated cell random motility and haptotaxis. J Math Biol *31*, 563-600.

Dvorak, H.F., Harvey, V.S., Estrella, P., Brown, L.F., McDonagh, J., and Dvorak, A.M. (1987). Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. Lab Invest *57*, 673-686.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. (2006). Matrix elasticity directs stem cell lineage specification. Cell *126*, 677-689.

Evans, N.D., Minelli, C., Gentleman, E., LaPointe, V., Patankar, S.N., Kallivretaki, M., Chen, X., Roberts, C.J., and Stevens, M.M. (2009). Substrate stiffness affects early differentiation events in embryonic stem cells. European cells & materials *18*, 1-13; discussion 13-14.

Faassen, A.E., Schrager, J.A., Klein, D.J., Oegema, T.R., Couchman, J.R., and McCarthy, J.B. (1992). A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J Cell Biol *116*, 521-531.

Fan, J., Guan, S., Cheng, C.F., Cho, M., Fields, J.W., Chen, M., Denning, M.F., Woodley, D.T., and Li, W. (2006). PKCdelta clustering at the leading edge and mediating growth factor-enhanced, but not ecm-initiated, dermal fibroblast migration. The Journal of investigative dermatology *126*, 1233-1243.

Felsenfeld, D.P., Choquet, D., and Sheetz, M.P. (1996). Ligand binding regulates the directed movement of beta1 integrins on fibroblasts. Nature *383*, 438-440.

Ferguson, K.M. (2004). Active and inactive conformations of the epidermal growth factor receptor. Biochemical Society transactions *32*, 742-745.

Folkman, J. (1997). Angiogenesis and angiogenesis inhibition: an overview. EXS *79*, 1-8.

Fordjour, A.K., and Harrington, E.O. (2009). PKCdelta influences p190 phosphorylation and activity: events independent of PKCdelta-mediated regulation of endothelial cell stress fiber and focal adhesion formation and barrier function. Biochim Biophys Acta *1790*, 1179-1190.

Fu, B.M., and Tarbell, J.M. (2013). Mechano-sensing and transduction by endothelial surface glycocalyx: composition, structure, and function. Wiley interdisciplinary reviews Systems biology and medicine *5*, 381-390.

Galbraith, C.G., Skalak, R., and Chien, S. (1998). Shear stress induces spatial reorganization of the endothelial cell cytoskeleton. Cell motility and the cytoskeleton *40*, 317-330.

Gan, L., Miocic, M., Doroudi, R., Selin-Sjogren, L., and Jern, S. (2000). Distinct regulation of vascular endothelial growth factor in intact human conduit vessels exposed to laminar fluid shear stress and pressure. Biochemical and biophysical research communications *272*, 490-496.

Geraldes, P., Hiraoka-Yamamoto, J., Matsumoto, M., Clermont, A., Leitges, M., Marette, A., Aiello, L.P., Kern, T.S., and King, G.L. (2009). Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy. Nat Med *15*, 1298-1306.

Germain, R.N., Robey, E.A., and Cahalan, M.D. (2012). A decade of imaging cellular motility and interaction dynamics in the immune system. Science *336*, 1676-1681.

Ghassemi, S., Meacci, G., Liu, S., Gondarenko, A.A., Mathur, A., Roca-Cusachs, P., Sheetz, M.P., and Hone, J. (2012). Cells test substrate rigidity by local contractions on submicrometer pillars. Proceedings of the National Academy of Sciences of the United States of America *109*, 5328-5333.

Ghosh, K., Thodeti, C.K., Dudley, A.C., Mammoto, A., Klagsbrun, M., and Ingber, D.E. (2008). Tumor-derived endothelial cells exhibit aberrant Rho-mediated mechanosensing and abnormal angiogenesis in vitro. Proceedings of the National Academy of Sciences of the United States of America *105*, 11305-11310.

Goeckeler, Z.M., and Wysolmerski, R.B. (1995). Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. J Cell Biol *130*, 613-627.

Grinnell, K.L., and Harrington, E.O. Interplay between FAK, PKCdelta, and p190RhoGAP in the regulation of endothelial barrier function. Microvasc Res *83*, 12-21.

Hall, A. (2012). Rho family GTPases. Biochemical Society transactions *40*, 1378-1382.

Harrington, E.O., Shannon, C.J., Morin, N., Rowlett, H., Murphy, C., and Lu, Q. (2005). PKCdelta regulates endothelial basal barrier function through modulation of RhoA GTPase activity. Exp Cell Res *308*, 407-421.

Hellstrom, M., Phng, L.K., and Gerhardt, H. (2007). VEGF and Notch signaling: the yin and yang of angiogenic sprouting. Cell adhesion & migration *1*, 133-136.

Heo, S.J., Nerurkar, N.L., Baker, B.M., Shin, J.W., Elliott, D.M., and Mauck, R.L. (2011). Fiber stretch and reorientation modulates mesenchymal stem cell morphology and fibrous gene expression on oriented nanofibrous microenvironments. Annals of biomedical engineering *39*, 2780-2790.

Herman, I.M. (1993). Molecular mechanisms regulating the vascular endothelial cell motile response to injury. J Cardiovasc Pharmacol *22 Suppl 4*, S25-36.

Huttenlocher, A., Sandborg, R.R., and Horwitz, A.F. (1995). Adhesion in cell migration. Current opinion in cell biology *7*, 697-706.

Imberti, B., Morigi, M., Zoja, C., Angioletti, S., Abbate, M., Remuzzi, A., and Remuzzi, G. (2000). Shear stress-induced cytoskeleton rearrangement mediates NF-kappaB-dependent endothelial expression of ICAM-1. Microvasc Res *60*, 182-188.

Insall, R.H., and Weiner, O.D. (2001). PIP3, PIP2, and cell movement--similar messages, different meanings? Dev Cell *1*, 743-747.

Iwabu, A., Smith, K., Allen, F.D., Lauffenburger, D.A., and Wells, A. (2004). Epidermal growth factor induces fibroblast contractility and motility via a protein kinase C delta-dependent pathway. The Journal of biological chemistry *279*, 14551-14560.

Jamison, J., Lauffenburger, D., Wang, J.C., and Wells, A. (2013). PKCdelta Localization at the Membrane Increases Matrix Traction Force Dependent on PLCgamma1/EGFR Signaling. PloS one *8*, e77434.

Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002). Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol Cell Biol *22*, 7158-7167.

Joseloff, E., Cataisson, C., Aamodt, H., Ocheni, H., Blumberg, P., Kraker, A.J., and Yuspa, S.H. (2002). Src family kinases phosphorylate protein kinase C delta on tyrosine residues and modify the neoplastic phenotype of skin keratinocytes. The Journal of biological chemistry *277*, 12318-12323.

Joyce, N.C., and Meklir, B. (1992). Protein kinase C activation during corneal endothelial wound repair. Invest Ophthalmol Vis Sci *33*, 1958-1973.

Katanosaka, Y., Bao, J.H., Komatsu, T., Suemori, T., Yamada, A., Mohri, S., and Naruse, K. (2008). Analysis of cyclic-stretching responses using cell-adhesion-patterned cells. Journal of biotechnology *133*, 82-89.

Kharait, S., Hautaniemi, S., Wu, S., Iwabu, A., Lauffenburger, D.A., and Wells, A. (2007). Decision tree modeling predicts effects of inhibiting contractility signaling on cell motility. BMC Syst Biol *1*, 9.

Kikkawa, U., Matsuzaki, H., and Yamamoto, T. (2002). Protein kinase C delta (PKC delta): activation mechanisms and functions. Journal of biochemistry *132*, 831-839.

Kurpinski, K., Chu, J., Hashi, C., and Li, S. (2006). Anisotropic mechanosensing by mesenchymal stem cells. Proceedings of the National Academy of Sciences of the United States of America *103*, 16095-16100.

Langrana, N.A., Alexander, H., Strauchler, I., Mehta, A., and Ricci, J. (1983). Effect of mechanical load in wound healing. Annals of plastic surgery *10*, 200-208.

Lanner, F., Sohl, M., and Farnebo, F. (2007). Functional arterial and venous fate is determined by graded VEGF signaling and notch status during embryonic stem cell differentiation. Arteriosclerosis, thrombosis, and vascular biology *27*, 487-493.

Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. Cell *84*, 359-369.

Lauritzen, C., Bagge, U., and Bjursten, L.M. (1981). Determination of wound strength for quantitation of skin damage after pressure ischemia. An experimental study in rabbits. Scandinavian journal of plastic and reconstructive surgery *15*, 93-95.

Lee, S., Zeiger, A., Maloney, J.M., Kotecki, M., Van Vliet, K.J., and Herman, I.M. (2010). Pericyte actomyosin-mediated contraction at the cell-material interface can modulate the microvascular niche. Journal of physics Condensed matter : an Institute of Physics journal *22*, 194115.

Leibovich, S.J., and Wiseman, D.M. (1988). Macrophages, wound repair and angiogenesis. Prog Clin Biol Res *266*, 131-145.

Leloup, L., Shao, H., Bae, Y.H., Deasy, B., Stolz, D., Roy, P., and Wells, A. m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate. The Journal of biological chemistry *285*, 33549-33566.

Li, J., Zhang, Y.P., and Kirsner, R.S. (2003). Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. Microsc Res Tech *60*, 107-114.

Lizotte, F., Pare, M., Denhez, B., Leitges, M., Guay, A., and Geraldes, P. (2013). PKCdelta impaired vessel formation and angiogenic factor expression in diabetic ischemic limbs. Diabetes *62*, 2948-2957.

Llado, A., Tebar, F., Calvo, M., Moreto, J., Sorkin, A., and Enrich, C. (2004). Protein kinaseCdelta-calmodulin crosstalk regulates epidermal growth factor receptor exit from early endosomes. Mol Biol Cell *15*, 4877-4891.

Ma, J.S., Haydar, T.F., and Radoja, S. (2008). Protein kinase C delta localizes to secretory lysosomes in CD8+ CTL and directly mediates TCR signals leading to granule exocytosis-mediated cytotoxicity. Journal of immunology *181*, 4716-4722.

Madri, J.A., Graesser, D., and Haas, T. (1996). The roles of adhesion molecules and proteinases in lymphocyte transendothelial migration. Biochemistry and cell biology = Biochimie et biologie cellulaire *74*, 749-757.

Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D.R., Zilberstein, A., Ullrich, A., Pawson, T., and Schlessinger, J. (1990). The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. EMBO J *9*, 4375-4380.

Marinkovic, A., Mih, J.D., Park, J.A., Liu, F., and Tschumperlin, D.J. (2012). Improved throughput traction microscopy reveals pivotal role for matrix stiffness in fibroblast contractility and TGF-beta responsiveness. Am J Physiol Lung Cell Mol Physiol *303*, L169-180.

Markova, A., and Mostow, E.N. (2012). US skin disease assessment: ulcer and wound care. Dermatologic clinics *30*, 107-111, ix.

Masumura, T., Yamamoto, K., Shimizu, N., Obi, S., and Ando, J. (2009). Shear stress increases expression of the arterial endothelial marker ephrinB2 in murine ES cells via the VEGF-Notch signaling pathways. Arteriosclerosis, thrombosis, and vascular biology *29*, 2125-2131.

McClain, S.A., Simon, M., Jones, E., Nandi, A., Gailit, J.O., Tonnesen, M.G., Newman, D., and Clark, R.A. (1996). Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. The American journal of pathology *149*, 1257-1270.

Mellor, H., and Parker, P.J. (1998). The extended protein kinase C superfamily. The Biochemical journal *332 (Pt 2)*, 281-292.

Meyer, A.S., Hughes-Alford, S.K., Kay, J.E., Castillo, A., Wells, A., Gertler, F.B., and Lauffenburger, D.A. (2012). 2D protrusion but not motility predicts growth factor-induced cancer cell migration in 3D collagen. J Cell Biol *197*, 721-729.

Mima, A., Kitada, M., Geraldes, P., Li, Q., Matsumoto, M., Mizutani, K., Qi, W., Li, C., Leitges, M., Rask-Madsen, C., *et al.* (2012). Glomerular VEGF resistance induced by PKCdelta/SHP-1 activation and contribution to diabetic nephropathy. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *26*, 2963-2974.

Miyamoto, A., Nakayama, K., Imaki, H., Hirose, S., Jiang, Y., Abe, M., Tsukiyama, T., Nagahama, H., Ohno, S., Hatakeyama, S., *et al.* (2002). Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta. Nature *416*, 865-869.

Murphy, D.D., and Wagner, R.C. (1994). Differential contractile response of cultured microvascular pericytes to vasoactive agents. Microcirculation *1*, 121-128.

Negishi, M., and Katoh, H. (2002). Rho family GTPases as key regulators for neuronal network formation. Journal of biochemistry *132*, 157-166.

Newton, A.C. (2003). Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. The Biochemical journal *370*, 361-371.

Oancea, E., and Meyer, T. (1998). Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell *95*, 307-318.

Paradis, S., Ailion, M., Toker, A., Thomas, J.H., and Ruvkun, G. (1999). A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. Genes & development *13*, 1438-1452.

Pasapera, A.M., Schneider, I.C., Rericha, E., Schlaepfer, D.D., and Waterman, C.M. (2010). Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. J Cell Biol *188*, 877-890.

Paszek, M.J., Zahir, N., Johnson, K.R., Lakins, J.N., Rozenberg, G.I., Gefen, A., Reinhart-King, C.A., Margulies, S.S., Dembo, M., Boettiger, D., *et al.* (2005). Tensional homeostasis and the malignant phenotype. Cancer cell *8*, 241-254.

Pathak, A., and Kumar, S. (2011). Biophysical regulation of tumor cell invasion: moving beyond matrix stiffness. Integrative biology : quantitative biosciences from nano to macro *3*, 267-278.

Plotnikov, S.V., Pasapera, A.M., Sabass, B., and Waterman, C.M. (2012). Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. Cell *151*, 1513-1527.

Pollard, T.D., and Borisy, G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453-465.

Pruss, R.M., and Herschman, H.R. (1977). Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. Proc Natl Acad Sci U S A *74*, 3918-3921.

Puceat, M., and Vassort, G. (1996). Signalling by protein kinase C isoforms in the heart. Molecular and cellular biochemistry *157*, 65-72.

Rahman, A., Anwar, K.N., Uddin, S., Xu, N., Ye, R.D., Platanias, L.C., and Malik, A.B. (2001). Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. Molecular and cellular biology *21*, 5554-5565.

Ridley, A.J. (2013). RhoA, RhoB and RhoC have different roles in cancer cell migration. Journal of microscopy *251*, 242-249.

Roca-Cusachs, P., Iskratsch, T., and Sheetz, M.P. (2012). Finding the weakest link: exploring integrin-mediated mechanical molecular pathways. J Cell Sci *125*, 3025-3038.

Roca-Cusachs, P., Sunyer, R., and Trepat, X. (2013). Mechanical guidance of cell migration: lessons from chemotaxis. Current opinion in cell biology *25*, 543-549.

Ron, D., and Kazanietz, M.G. (1999). New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *13*, 1658-1676.

Rosse, C., Linch, M., Kermorgant, S., Cameron, A.J., Boeckeler, K., and Parker, P.J. (2010). PKC and the control of localized signal dynamics. Nature reviews Molecular cell biology *11*, 103-112.

Saito, N. (1995). [Differential involvement of PKC subspecies in neuronal function]. Nihon yakurigaku zasshi Folia pharmacologica Japonica *105*, 127-136.

Satish, L., Blair, H.C., Glading, A., and Wells, A. (2005). Interferon-inducible protein 9 (CXCL11)-induced cell motility in keratinocytes requires calcium flux-dependent activation of mu-calpain. Molecular and cellular biology *25*, 1922-1941.

Schmitt-Graff, A., Desmouliere, A., and Gabbiani, G. (1994). Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. Virchows Arch *425*, 3-24.

Seki, T., Matsubayashi, H., Amano, T., Shirai, Y., Saito, N., and Sakai, N. (2005). Phosphorylation of PKC activation loop plays an important role in receptor-mediated translocation of PKC. Genes Cells *10*, 225-239.

Shao, H., Chou, J., Baty, C.J., Burke, N.A., Watkins, S.C., Stolz, D.B., and Wells, A. (2006). Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation. Molecular and cellular biology *26*, 5481-5496.

Sheldon, R., Moy, A., Lindsley, K., Shasby, S., and Shasby, D.M. (1993). Role of myosin light-chain phosphorylation in endothelial cell retraction. Am J Physiol *265*, L606-612.

Sherratt, J.A., Martin, P., Murray, J.D., and Lewis, J. (1992). Mathematical models of wound healing in embryonic and adult epidermis. IMA J Math Appl Med Biol *9*, 177-196.

Shi, Y., Zhang, J., Mullin, M., Dong, B., Alberts, A.S., and Siminovitch, K.A. (2009). The mDial formin is required for neutrophil polarization, migration, and activation of the LARG/RhoA/ROCK signaling axis during chemotaxis. Journal of immunology *182*, 3837-3845.

Shizukuda, Y., Helisch, A., Yokota, R., and Ware, J.A. (1999a). Downregulation of protein kinase cdelta activity enhances endothelial cell adaptation to hypoxia. Circulation *100*, 1909-1916.

Shizukuda, Y., Tang, S., Yokota, R., and Ware, J.A. (1999b). Vascular endothelial growth factor-induced endothelial cell migration and proliferation depend on a nitric oxide-mediated decrease in protein kinase Cdelta activity. Circ Res *85*, 247-256.

Singer, A.J., and Clark, R.A. (1999). Cutaneous wound healing. The New England journal of medicine *341*, 738-746.

Soltoff, S.P., Grubman, S.A., and Jefferson, D.M. (1998). Development of salivary gland cell lines for studies of signaling and physiology. Annals of the New York Academy of Sciences *842*, 100-107.

Stahelin, R.V., Digman, M.A., Medkova, M., Ananthanarayanan, B., Melowic, H.R., Rafter, J.D., and Cho, W. (2005). Diacylglycerol-induced membrane targeting and activation of protein kinase Cepsilon: mechanistic differences between protein kinases Cdelta and Cepsilon. The Journal of biological chemistry *280*, 19784-19793.

Stahelin, R.V., Digman, M.A., Medkova, M., Ananthanarayanan, B., Rafter, J.D., Melowic, H.R., and Cho, W. (2004). Mechanism of diacylglycerol-induced membrane targeting and activation of protein kinase Cdelta. The Journal of biological chemistry *279*, 29501-29512.

Stokes, C.L., Lauffenburger, D.A., and Williams, S.K. (1991). Migration of individual microvessel endothelial cells: stochastic model and parameter measurement. J Cell Sci *99 (Pt 2)*, 419-430.

Stossel, T.P. (1993). On the crawling of animal cells. Science *260*, 1086-1094.

Tan, J.L., Tien, J., Pirone, D.M., Gray, D.S., Bhadriraju, K., and Chen, C.S. (2003). Cells lying on a bed of microneedles: an approach to isolate mechanical force. Proceedings of the National Academy of Sciences of the United States of America *100*, 1484-1489.

Teh, T.K., Toh, S.L., and Goh, J.C. (2011). Aligned hybrid silk scaffold for enhanced differentiation of mesenchymal stem cells into ligament fibroblasts. Tissue engineering Part C, Methods *17*, 687-703.

Teh, T.K., Toh, S.L., and Goh, J.C. (2013). Aligned fibrous scaffolds for enhanced mechanoresponse and tenogenesis of mesenchymal stem cells. Tissue engineering Part A *19*, 1360-1372.

Timmenga, E.J., Andreassen, T.T., Houthoff, H.J., and Klopper, P.J. (1991). The effect of mechanical stress on healing skin wounds: an experimental study in rabbits using tissue expansion. British journal of plastic surgery *44*, 514-519.

Tinsley, J.H., Teasdale, N.R., and Yuan, S.Y. (2004). Involvement of PKCdelta and PKD in pulmonary microvascular endothelial cell hyperpermeability. Am J Physiol Cell Physiol *286*, C105-111.

Toker, A. (2003). PDK-1 and protein kinase C phosphorylation. Methods in molecular biology *233*, 171-189.

Tonnesen, M.G., Feng, X., and Clark, R.A. (2000). Angiogenesis in wound healing. The journal of investigative dermatology Symposium proceedings / the Society for Investigative Dermatology, Inc [and] European Society for Dermatological Research *5*, 40-46.

Totsukawa, G., Wu, Y., Sasaki, Y., Hartshorne, D.J., Yamakita, Y., Yamashiro, S., and Matsumura, F. (2004). Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. J Cell Biol *164*, 427-439.

Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D.J., Sasaki, Y., and Matsumura, F. (2000). Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J Cell Biol *150*, 797-806.

Tu, X., Joeng, K.S., Nakayama, K.I., Nakayama, K., Rajagopal, J., Carroll, T.J., McMahon, A.P., and Long, F. (2007). Noncanonical Wnt signaling through G protein-linked PKCdelta activation promotes bone formation. Dev Cell *12*, 113-127.

Turner, T., Chen, P., Goodly, L.J., and Wells, A. (1996). EGF receptor signaling enhances in vivo invasiveness of DU-145 human prostate carcinoma cells. Clin Exp Metastasis *14*, 409-418.

Upton, M.L., Gilchrist, C.L., Guilak, F., and Setton, L.A. (2008). Transfer of macroscale tissue strain to microscale cell regions in the deformed meniscus. Biophys J *95*, 2116-2124.

Urbich, C., Stein, M., Reisinger, K., Kaufmann, R., Dimmeler, S., and Gille, J. (2003). Fluid shear stress-induced transcriptional activation of the vascular endothelial growth factor receptor-2 gene requires Sp1-dependent DNA binding. FEBS letters *535*, 87-93.

Vardouli, L., Moustakas, A., and Stournaras, C. (2005). LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta. The Journal of biological chemistry *280*, 11448-11457.

Vega, F.M., Fruhwirth, G., Ng, T., and Ridley, A.J. (2011). RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. J Cell Biol *193*, 655-665.

Wadsworth, S.J., and Goldfine, H. (2002). Mobilization of protein kinase C in macrophages induced by Listeria monocytogenes affects its internalization and escape from the phagosome. Infection and immunity *70*, 4650-4660.

Wang, H., Riha, G.M., Yan, S., Li, M., Chai, H., Yang, H., Yao, Q., and Chen, C. (2005). Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line. Arteriosclerosis, thrombosis, and vascular biology *25*, 1817-1823.

Wang, H.B., Dembo, M., Hanks, S.K., and Wang, Y. (2001a). Focal adhesion kinase is involved in mechanosensing during fibroblast migration. Proceedings of the National Academy of Sciences of the United States of America *98*, 11295-11300.

Wang, J.G., Miyazu, M., Matsushita, E., Sokabe, M., and Naruse, K. (2001b). Uniaxial cyclic stretch induces focal adhesion kinase (FAK) tyrosine phosphorylation followed by mitogen-activated protein kinase (MAPK) activation. Biochemical and biophysical research communications *288*, 356-361.

Wang, J.H. Cell Traction Forces (CTFs) and CTF Microscopy Applications in Musculoskeletal Research. Oper Tech Orthop *20*, 106-109.

Wang, J.H., and Li, B. (2009). Application of cell traction force microscopy for cell biology research. Methods in molecular biology *586*, 301-313.

Wang, J.H., and Lin, J.S. (2007). Cell traction force and measurement methods. Biomech Model Mechanobiol *6*, 361-371.

Wang, N., Ostuni, E., Whitesides, G.M., and Ingber, D.E. (2002). Micropatterning tractional forces in living cells. Cell motility and the cytoskeleton *52*, 97-106.

Wang, Y.L. (2009). Traction forces and rigidity sensing of adherent cells. Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference *2009*, 3339-3340.

Weiger, M.C., Wang, C.C., Krajcovic, M., Melvin, A.T., Rhoden, J.J., and Haugh, J.M. (2009). Spontaneous phosphoinositide 3-kinase signaling dynamics drive spreading and random migration of fibroblasts. J Cell Sci *122*, 313-323.

Weinstein, I.B. (1991). The roles of specific isoforms of protein kinase C in growth control and human colon cancer. Princess Takamatsu symposia *22*, 277-283.

Wells, A., Gupta, K., Chang, P., Swindle, S., Glading, A., and Shiraha, H. (1998). Epidermal growth factor receptor-mediated motility in fibroblasts. Microsc Res Tech *43*, 395-411.

Wells, A., Huttenlocher, A., and Lauffenburger, D.A. (2005). Calpain proteases in cell adhesion and motility. Int Rev Cytol *245*, 1-16.
Wells, A., Ware, M.F., Allen, F.D., and Lauffenburger, D.A. (1999). Shaping up for shipping out: PLCgamma signaling of morphology changes in EGF-stimulated fibroblast migration. Cell motility and the cytoskeleton *44*, 227-233.
Wu, Q., Dhir, R., and Wells, A. Altered CXCR3 isoform expression regulates prostate cancer cell migration and invasion. Mol Cancer *11*, 3.
Yamamura, S., Nelson, P.R., and Kent, K.C. (1996). Role of protein kinase C in attachment, spreading, and migration of human endothelial cells. J Surg Res *63*, 349-354.
Yang, M.T., Reich, D.H., and Chen, C.S. (2011). Measurement and analysis of traction force dynamics in response to vasoactive agonists. Integrative biology : quantitative biosciences from nano to macro *3*, 663-674.
Yates, C.C., Bodnar, R., and Wells, A. (2011). Matrix control of scarring. Cellular and molecular life sciences : CMLS *68*, 1871-1881.
Yates, C.C., Hebda, P., and Wells, A. (2012). Skin wound healing and scarring: fetal wounds and regenerative restitution. Birth defects research Part C, Embryo today : reviews *96*, 325-333.
Yates, C.C., Whaley, D., Kulasekeran, P., Hancock, W.W., Lu, B., Bodnar, R., Newsome, J., Hebda, P.A., and Wells, A. (2007). Delayed and deficient dermal maturation in mice lacking the CXCR3 ELR-negative CXC chemokine receptor. The American journal of pathology *171*, 484-495.
Yuan, S.Y. (2002). Protein kinase signaling in the modulation of microvascular permeability. Vascul Pharmacol *39*, 213-223.