PROFILIN-1 IN CAPILLARY MORPHOGENESIS OF VASCULAR ENDOTHELIAL CELLS

by

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Vascular endothelial cells (VEC) assemble into capillary-like structures during angiogenesis, and this neovascularization process plays an important role in a wide range of physiological and pathological scenarios. Based on significant upregulation of its expression in VEC during capillary morphogenesis, profilin-1 (Pfn1 - a ubiquitously expressed actin-binding protein) was previously implicated in capillary morphogenesis of VEC. The overall objective of the present study was to investigate whether and how loss of Pfn1 function affects a) the various cellular functions that are important for capillary morphogenesis such as VEC migration, invasion and proliferation, and b) the overall capillary forming ability of VEC. Loss of Pfn1 function in VEC was achieved either by suppressing the overall expression of Pfn1 by RNA interference method or selectively abrogating specific ligand-interactions (actin, proline-rich ligands) of Pfn1 by expressing various point-mutants of Pfn1 in a near-null endogenous Pfn1 background (knockdown and knock-in approach). Loss of Pfn1 expression causes a major change in actin cytoskeleton in VEC. Particularly, there is a significant depletion of actin filaments and focal adhesions in VEC when Pfn1 expression was silenced. Silencing of Pfn1 expression also significantly impairs the migratory ability of VEC. Analyses of leading edge dynamics revealed that Pfn1 depletion results in decreased velocity and frequency of lamellipodial protrusion. Further experiments with point-mutants of Pfn1 showed that both actin and polyproline interactions of Pfn1 are required for efficient lamellipodial protrusion and overall migration of

VEC. Loss of Pfn1 expression is associated with reduced dynamics of VE-cadherin dependent cell-cell adhesion, which was also found to be correlated with increased nuclear accumulation of $p27^{Kip1}$ (a major cell-cycle inhibitor) and reduced VEC proliferation. Finally, we found that loss of overall expression of Pfn1 significantly impairs collagen gel invasion and three-dimensional (3-D) capillary morphogenesis of VEC. Abolishing either of actin or polyproline interactions of Pfn1 also leads to a dramatic inhibition of capillary mophogenesis of VEC. Taken together, these results demonstrate that Pfn1 plays a critical role in capillary morphogenesis of VEC through its interactions with both actin and polyproline ligands. This study may further imply that Pfn1 could be a novel angiogenesis target.

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PREFACE

First and foremost, I would like to thank my mentor, Dr. Partha Roy. I could not have imagined a better mentor for my graduate study. Without his guidance, patience, support, and encouragement, it would be impossible for me to finish my PhD study and write down this paragraph.

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NOMENCLATURE

3-D, three-dimensional

ABP, actin-binding protein

ADP, adenosine diphosphate

ATP, adenosine triphosphate

bFGF, basic fibroblast growth factor

C, non-targeting control siRNA construct

CDKI, cyclin-dependent kinase inhibitor

ECM, extracellular matrix

FA, focal adhesion

F-actin, filamentous actin

FAK, focal adhesion kinase

FBS, fetal bovine serum

G-actin, globular actin

GEF, guanine-nucleotide exchange factor

GFP, green fluorescent protein

HmVEC-1, a dermal human microvascular endothelial cell line

HUVEC, human umbilical vein endothelial cell

MMP, matrix-metalloprotease

N-WASP, Neural Wiscott Aldrich syndrome protein

P, profilin-1-specific siRNA

Pfn1, profilin-1

PI(3,4)P_2, phosphatidylinositol-3,4-bisphosphate

PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate

PI(3,4,5)P_3, phosphatidylinositol-3,4,5-triphosphate

PIP2, phosphatidylinositol biphosphate

PIP3, phosphatidylinositol triphosphate

VASP, vasodilator stimulated phosphoprotein

VEC, vascular endothelial cell

VEGF, vascular endothelial growth factor

WAVE, WASP family verprolin-homologous protein

1.0 INTRODUCTION

1.1 SIGNIFICANCE

Angiogenesis (capillary morphogenesis) is a process of new vessels formation from the pre-existing ones. It is a fundamental process that plays an important role in both physiological adaption and pathological conditions. While angiogenesis is critical for wound healing (Li et al., 2003) and may have therapeutic potential in certain cardiovascular diseases such as myocardial infarction (Goncalves, 2000), aberrant angiogenesis can also exacerbate several other disease conditions. For example, growth and metastatic spreading of tumor cells is critically dependent on angiogenesis (Carmeliet and Jain, 2000). Excessive angiogenesis has also been correlated with atherosclerosis, diabetic retinopathy and arthritis (Quesada et al., 2006). Therefore, depending on the disease, there is a clinical need for identifying either pro- or anti-angiogenetic therapeutic targets. Although anti-angiogenetic therapy using angiogenesis inhibitors have shown promise in certain clinical settings, such as in cancer treatment, the benefit is still very modest (Burger, 2007; Srinivasan et al., 2007). Present therapeutic interventions that are targeted to the action of either a single angiogenic mediator [example: vascular endothelial growth factor (VEGF) or VEGF receptor] or a specific signaling pathway in angiogenesis have limited efficacy since usually multiple agonists and signaling pathways act in concert in disease states. Furthermore, inhibiting one pathway often leads to compensatory up-regulation of other

pathways. Thus, for better anti-angiogenic therapy, one need to consider alternative strategies that could target cellular processes essential for angiogenesis at a more fundamental level, such as vascular endothelial cell (VEC) migration and proliferation.

During angiogenesis, quiescent VECs from the parent vasculature undergo a series of events that are marked by: 1) cell mobilization, 2) matrix-metalloproteases (MMPs) secretion and subsequent degradation of underlying basement membrane matrix, 3) cell migration and invasion through extracellular matrix (ECM) to the target sites, 4) cell proliferation, 5) re-establishment of cell-cell adhesions, 6) lumen formation via vesicular fusion of neighboring cells, and finally 7) recruitment of pericytes that stabilizes the newly generated blood vessels (Figure 1) (Bauer et al., 2005; Carmeliet, 2000; Yancopoulos et al., 2000). Although angiogenesis is an incredibly complex process involving interactions of a multi-cellular environment, several lines of experimental evidence in the literature demonstrate that VEC migration/invasion and proliferation are absolutely crucial for capillary morphogenesis of VECs. For example, molecular perturbations or agents that suppress VEC migration/invasion and proliferation exhibit anti-angiogenic effect (Browne et al., 2006; Ho et al., 2004; Liu and Senger, 2004) and conversely, known angiogenesis inhibitors suppress these fundamental processes (O'Reilly et al., 1997; O'Reilly et al., 1994). Consistent with these observations, proangiogenic factors, such as VEGF and bFGF up-regulate VEC migration/invasion and proliferation (Yancopoulos et al., 2000). Finally, highly vascularized atherosclerotic plaques show a dramatic increase in the number of proliferating VECs (O'Brien et al., 1994), thus further scoring the importance of VEC proliferation in angiogenesis.

Since actin cytoskeleton lies at the heart of either of these two cellular processes of angiogenesis, i.e. cell migration and proliferation. Perturbing an important actin binding protein

(ABP) to inhibit capillary morphogenesis may have a fundamental advantage over the current choices of targeting a specific receptor/ agonist or a single pathway.

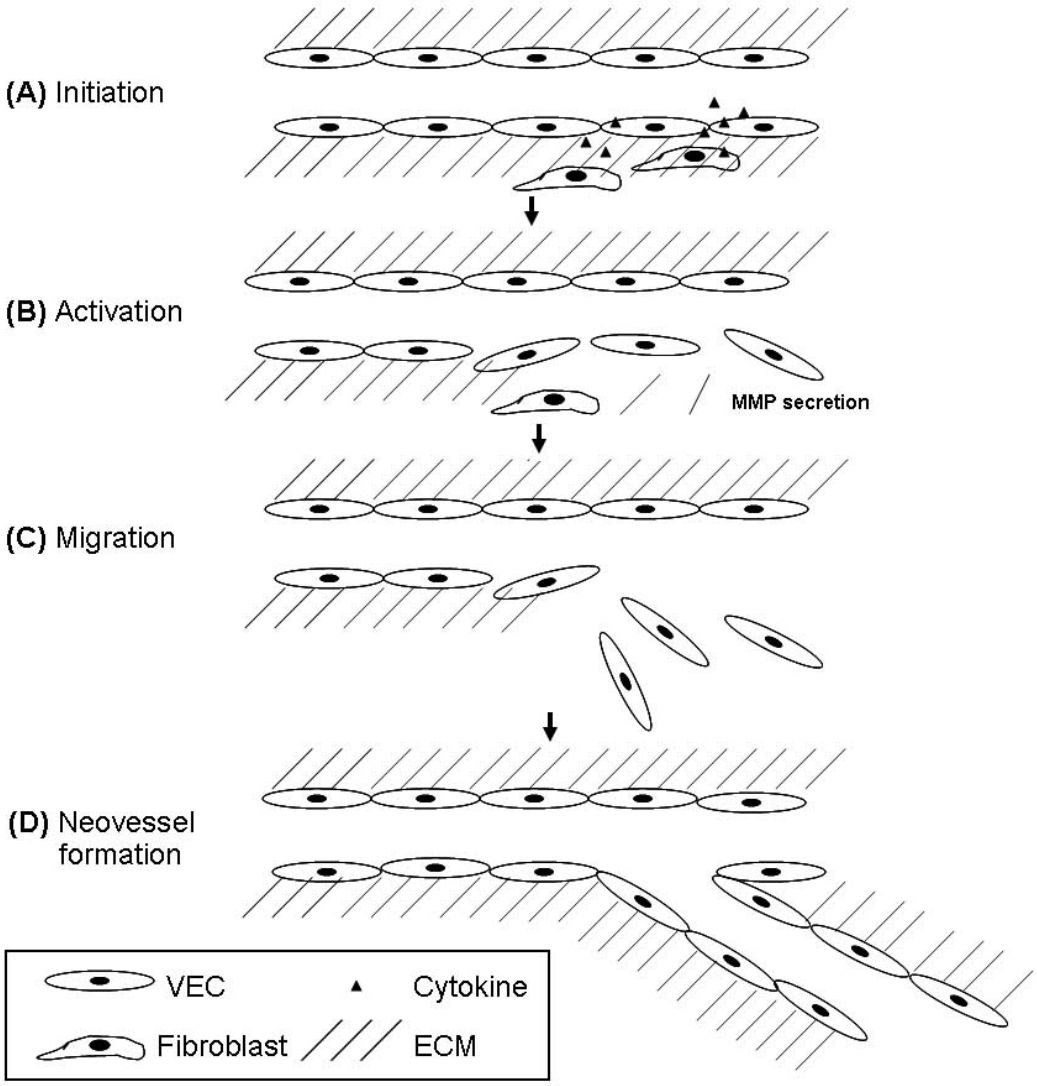

Figure 1. *A Schematic Model of Angiogenesis*

(A) Angiogenesis is initiated by cytokines secreted by fibroblast, monocytes, and platelets. **(B)** Activated VECs disrupt cell-cell adhesion with neighboring VECs, and digest ECM by secreting MMPs. **(C)** VECs migrate and invade through ECM, and proliferate. **(D)** VECs re-establish cell-cell adhesion, and form new vessels. [adapted from (Bauer, 2005)]

1.2 PROFILIN-1 AND CAPILLARY MORPHOGENESIS

1.2.1 Role of Actin Cytoskeleton and Actin Binding Proteins in Capillary Morphogenesis

Although the exact molecular details of how VECs assemble into capillary structures are not completely understood, in the past couple of years, *in vitro* models where VECs plated either on reconstituted basement membrane or embedded in 3-D ECM form polygonal network of pre-capillary cords (akin to the capillary structures *in vivo*) have been used to identify the key molecular players in this process (Davis et al., 2002). Those studies suggest ECM-integrin interaction and signaling to actin cytoskeleton that regulates cell migration and shape change, drive the process of capillary morphogenesis (Davis et al., 2002; Davis et al., 2000; Davis and Camarillo, 1995). Marked shape change and migration of VECs clearly implies active reorganization of actin cytoskeleton during capillary morphogenesis. Actin cytoskeleton is regulated by various ABPs including those involved in G(globular)-actin sequestering, nucleating, severing, depolymerizing, and capping activities (Davis et al., 2002; Pollard and Borisy, 2003). However, only very few studies have been reported to date that examined the roles of different ABPs in capillary morphogenesis. Thymosin β4 (a G-actin sequestering protein) was first reported to be up-regulated in VECs when plated on matrigel (Grant et al., 1995), which was later shown to be a potent stimulator of angiogenesis (Grant et al., 1999). Similarly, increased expression of three other ABPs including profilin-1 (Pfn1, a ubiquitously expressed ABP encoded by the Pfn family of genes), VASP (vasodilator stimulated phosphoprotein), and gelsolin was observed in VECs undergoing capillary morphogenesis, the functional significance of which was, however, not known (Salazar et al., 1999). Based on the significant up-regulation

of Pfn1 during capillary morphogenesis, I proposed the following hypothesis: ***Pfn1 plays an important role in capillary morphogenesis of VEC*** **(HYPOTHESIS).**

1.2.2 Pfn1 As a Regulator of Actin Cytoskeleton

Although Pfn1 was initially considered to be a molecule that sequesters monomeric actin (Carlsson et al., 1977), later study showed that Pfn1 actually promotes actin polymerization primarily at the barbed end by accelerating ATP-ADP nucleotide exchange on G-actin and shuttling the Pfn1-actin complex directly to the free barbed end of actin filaments (Figure 2) (Goldschmidt-Clermont et al., 1991; Kang et al., 1999; Pantaloni and Carlier, 1993; Perelroizen et al., 1996; Pollard and Cooper, 1984; Pring et al., 1992). *In vitro* study also demonstrated that with capping protein blocking the barbed end of actin filaments Pfn1's role was limited to actin sequestering (Perelroizen et al., 1996). While Pfn1 may act as an actin sequesting protein in prokaryotic or lower eukaryotic cells, Pfn1 in higher eukaryotes promotes actin polymerization (Schluter et al., 1997). This may be partly due to that in higher eukaryotes Pfn1's concentration does not appear to be sufficient to account for high G-actin concentration in those cells. (Balasubramanian et al., 1994; Ding et al., 2006; Magdolen et al., 1993; Zou et al., 2007).

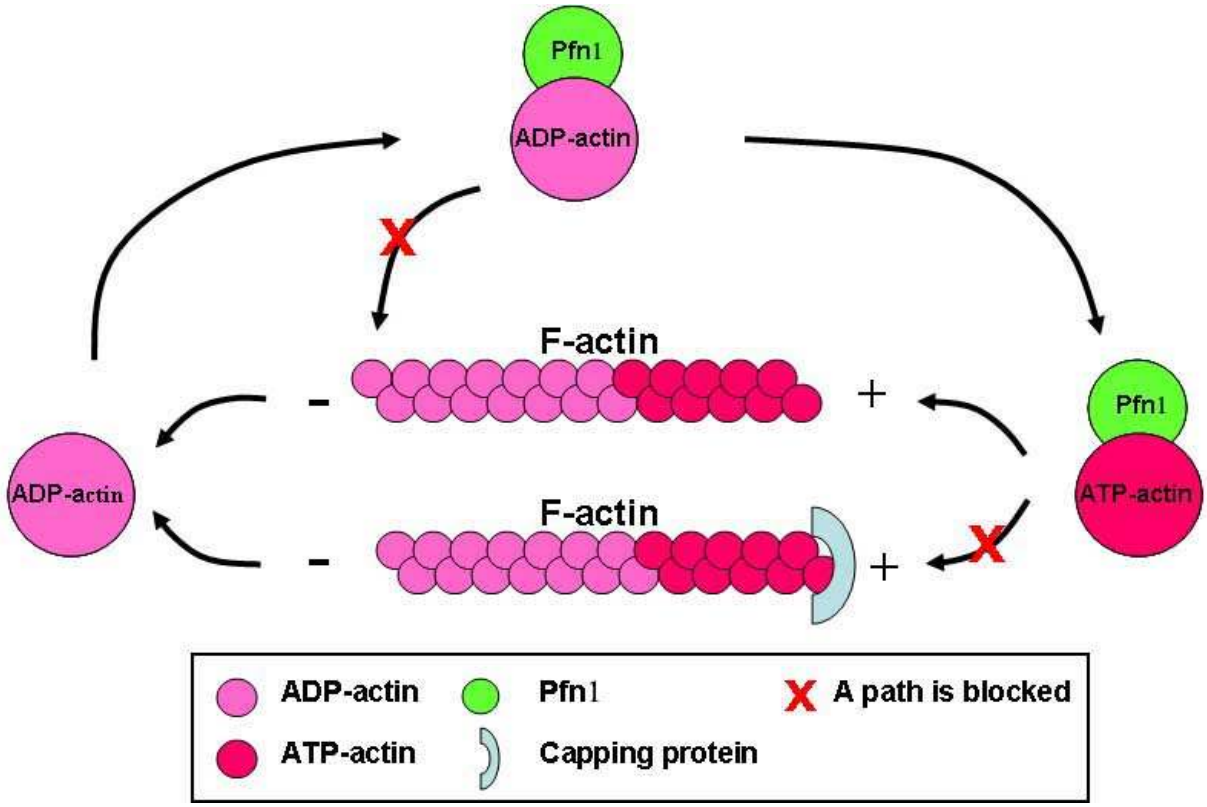

Figure 2. *Pfn1 in Actin Polymerization*

Pfn1 facilitates ADP-ATP nucleotide exchange on G-actin and shuttles the Pfn1-actin complex directly to the free barbed (plus) ends, but not to the pointed (minus) ends of actin filaments. When capping protein occupies the plus end of actin filaments, Pfn1's role as an actin promoter is limited to actin sequestering [modified from (Schluter et al., 1997)].

1.2.3 Pfn1 and Its Binding Ligands

Four different genes of mammalian Pfns have been identified thus far: Pfn1 (the focus of the study) is ubiquitously expressed in all cell types; Pfn2 is mainly expressed in brain; Pfn3 is expressed in testis and kidney; and Pfn4 is expressed in testis only (Hu et al., 2001; Kwiatkowski and Bruns, 1988; Lambrechts et al., 2000; Obermann et al., 2005).

In addition to G-actin, Pfn1 physically binds to phosphoinositides including phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$], phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], and phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] (Lu et al., 1996, Machesky and Poland, 1993). *In vitro* study showing that Pfn1 binds to $PI(4,5)P_2$ and inhibits its hydrolysis by phospholipase C-gamma 1 (Goldschmidt-Clermont et al., 1990) suggests Pfn1 might regulate phosphoinositide metabolism. In a very recent study by our group, overexpression of Pfn1 in breast cancer cells was found to suppress growth factor induced generation of $PI(3,4,5)P_3$ (Das et al., 2009). Given the fact that membrane phosphoinositides are important regulators of actin cytoskeleton (Yamamoto et al., 2001), Pfn1 could play a role as a potential mediator between cell membrane and cytoskeleton.

With its ability to bind to polyproline sequences Pfn1 interacts with almost all major actin nucleation/elongation proteins, including those important for cytoskeletal control such as vasodilator stimulated phosphoprotein (VASP), Neural Wiscott Aldrich syndrome protein (N-WASP), WASP family verprolin-homologous protein (WAVE), and Diaphanous (Miki et al., 1998; Reinhard et al., 1995; Suetsugu et al., 1998; Watanabe et al., 1997). Since some of these proteins are activated at the downstream of Rho-GTPase signaling, Pfn1 could be an important nexus between signaling and cytoskeletal control.

1.2.4 Pfn1's Role in Cell Migration

Gene deletion of Pfn1 and Pfn2 resulting in impaired motility of *Dictyostelium* amoebae directly showed for the first time Pfn's role in cell migration (Haugwitz et al., 1994). In *Drosophila*, gene deletion of *chickadee* (a Pfn1 homolog) also resultes in impaired cell migration and developmental defects (Verheyen and Cooley, 1994). Pfn1 is required for embryogenesis in *C.*

elegans (Velarde et al., 2007). In *Xenopus*, Pfn1 has been shown to be a downstream effector of Daam1 (a formin homolog) in non-canonical Wnt signaling and is required for gastrulation (Sato et al., 2006). Similarly in zebrafish, simultaneous knocking down Pfn1 and diaphanous-related formin 2 results in inhibited gastrulation (Lai et al., 2008). Since Pfn1 deletion results in early embryonic lethality for mammalian development, how Pfn1 might affect higher eukaryotic cell migration has not been explored in detail. Model studies have shown that immuno-depletion of Pfn1 inhibits intracellular movement of bacterial pathogens (mimics actin polymerization at the leading edge of migrating cells) thus suggesting Pfn1 may play a role in actin-based protrusion during cell migration (Grenklo et al., 2003; Laurent et al., 1999; Sanger et al., 1995; Theriot et al., 1994). This is further supported by indirect immunofluorescence studies demonstrating preferential localization of Pfn1 at the leading edge of migrating cells (Buss et al., 1992; Mayboroda et al., 1997; Neely and Macaluso, 1997). While, based on these studies, Pfn1 could be conceived as a positive regulator of cell migration, there are other findings that appear to be contradictory to this notion. For example, many different types of highly invasive adenocarcinomas have very low levels of Pfn1 expression compared to their normal counterparts (Belot et al., 2002; Gronborg et al., 2006; Janke et al., 2000; Wittenmayer et al., 2004). Also, our laboratory previously showed that a very moderate over-expression of Pfn1 dramatically inhibits the migration of breast cancer cells (Roy and Jacobson, 2004). Conversely, silencing Pfn1 expression in breast cancer cells significantly increases their motility (Bae et al., 2009; Zou et al., 2007). Thus, it appears that Pfn1's role in cell migration can be complex and cell-type specific.

1.2.5 Pfn1's Role in Cell Proliferation

Actin cytoskeleton exerts its influence on cell-division process in at least several different ways. First, contact inhibition is a hallmark of most normal adherent cells including VECs, where engagement of cell-cell adhesion receptors prevents G1-to-S phase entry of cell cycle (St Croix et al., 1998). Since tethering of adhesion receptors to cortical actin cytoskeleton determines the integrity of cell-cell adhesion, the state of actin cytoskeleton can have an indirect influence on cell cycle via regulation of adhesion receptors. Second, cytokinesis, the physical act of cell division, requires acto-myosin based contraction and is thus clearly dependent on actin cytoskeleton (Glotzer, 2005). Because of involvement of actin cytoskeleton in cell division, it is natural that ABPs, the main regulators of actin cytoskeleton, should also play important roles in this process. Indeed, there are a few studies in the literature that point to Pfn1's importance in cell proliferation. For example, Pfn1,2-null *Dictyostelium* amoebae exhibit defects in cytokinesis (Haugwitz et al., 1994). Gene deletion of Pfn homolog in *Drosophila* leads to defects in proliferation (Verheyen and Cooley, 1994). Pfn1-null mouse embryo is lethal at two-cell stage, which suggests Pfn1 may play an important role also in mammalian cell proliferation and/or survival (Witke et al., 2001). A recent study showed that in mouse chondrocytes disruption of Pfn1 gene causes defects in abscission during late cytokinesis (Bottcher et al., 2009), which partly explains Pfn1's role in mammalian cell proliferation.

1.2.6 Relevance of Pfn1 in Disease

Recently Pfn1's role has queried in the context of cardiovascular diseases and cancer.

Overexpression of Pfn1 in mouse blood vessels causes vascular hypertrophy and hypertension (Moustafa-Bayoumi et al., 2007). In another study, up-regulated Pfn1 was found to be associated with pulmonary hypertension (Dai et al., 2006). Expression level of Pfn1 was found to be dramatically up-regulated in atherosclerotic plaques, and consistent with this a further study showed heterozygous mutated mice with 50% reduction in Pfn1 have reduced probability to form atherosclerotic lesions (Romeo et al., 2004; Romeo et al., 2007).

Although Pfn1 is required for normal cell proliferation and migration, surprisingly many different types of highly invasive adenocarcinomas have very low levels of Pfn1 expression compared to their normal counterparts (Belot et al., 2002; Gronborg et al., 2006; Janke et al., 2000; Wittenmayer et al., 2004). A recent study from our laboratory showed at least in breast cancer cells, loss of Pfn1 expression enhances cell migration through Ena/VASP (Bae et al., 2009), which might partly explain why tumor cells have less Pfn1 amount compared to their normal counterparts. Conversely, over-expression of Pfn1 level in these breast cancer cells inhibits their motility (Roy and Jacobson, 2004; Zou et al., 2007).

1.3 HYPOTHESIS AND SPECIFIC AIMS

Based on significant up-regulation of Pfn1 expression in human umbilical vein endothelial cells (HUVECs) during capillary morphogenesis (Salazar et al., 1999), one envisions that Pfn1 might play an instrumental role during endothelial morphogenesis. Related to this, we specifically hypothesized that **Pfn1 plays an important role in capillary morphogenesis of VECs (HYPOTHESIS).** Related to this overall hypothesis, I will determine:

Specific Aim 1: The role of Pfn1 in migration of VEC. Specifically, I will determine whether loss of Pfn1 function alters:

a) VEC migration.

b) The kinetics of cell protrusion.

Specific Aim 2: The role of Pfn1 in capillary morphogenesis of VEC. Specifically, I will determine whether loss of Pfn1 function impairs:

a) Ability of VEC to form capillary-like structures *in vitro*.

b) ECM invasion of VEC.

c) VEC proliferation.

2.0 MATERIAL AND METHODS

2.1 CELL CULTURE

Human umbilical vein endothelial cells (HUVECs) and an immortalized human microvascular endothelial cell line (HmVEC-1) were used in the experiments. HUVECs (source: Cambrex Biosciences, Walkersville, MD) were cultured in the complete EBM2 growth media (also commercially available from the same source). HmVEC-1 were cultured in MCDB 131 media (Gibco, Gaithersburg, MD) supplemented with 10% FBS, 10 mM L-Glutamine (Gibco, Gaithersburg, MD), 1 ng/mL EGF (BD Biosciences, Bedford, MA), and 1 μg/ml hydrocortisone (Sigma, St. Louis, MO).

2.2 GENE SILENCING OF PROFILIN-1

To silence Pfn1 expression, a custom-designed siRNA (sense strand: 5'-AGA AGG UGU CCA CGG UGG U UU-3'; antisense-strand: 5'-ACC ACC GUG GAC ACC UUC U UU-3') was synthesized by Dharmacon (Lafayette, CO) and was transfected into VECs using a proprietary reagent according to the manufacturer's protocol. Either a control siRNA (sense strand 5'-UAG CGA CUA AAC ACA UCA A UU-3'; antisense strand: 5’-UUG AUG UGU UUA GUC GCU A UU-3’) that bears no significant homology with any known mouse or human gene and

commercially available from the same source or Smart-pool of non-targeting control siRNA from the same source was used for the control experiments. Briefly, 100 nM of either of the siRNA constructs was transfected into cells for 24 hours. The transfection media was then replaced with the regular growth media and cells were cultured for another 24-72 hours before performing the experiments.

2.3 GENERATION OF PROFILIN-1 CONSTRUCTS

Generation of our original plasmids encoding GFP-Pfn1 and its point mutants (GFP-Pfn1-H119E and GFP-Pfn1-H133S) has been previously described and confirmed (Zou et al., 2007). These constructs were further modified by introducing a two base-pair silent mutation (does not change the peptide encoding) in the Pfn1-siRNA targeting region before subcloning into pQCXIP retroviral vector (Clontech, Mountainview, CA) at Not1 and BamH1 restriction sites. Retrovirus packaging and subsequent infection of HmVEC-1 cells were carried out according to the manufacturer's instructions. Infected cells were selected for puromycin resistance (250 ng/ml) and finally, stable cells were sorted based on their GFP-fluorescence before experimental use.

2.4 ANTIBODIES AND REAGENTS

Polyclonal antibodies specific for Pfn1 and Pfn2 were generous gifts of Drs Sally Zigmond (University of Pennsylvania) and Walter Witke (European Molecular Biology Laboratory, Italy), respectively. Polyclonal antibody for N-WASP was kindly provided by Dr Hideki Yamaguchi

(Albert Einstein College of Medicine). Monoclonal antibodies for VASP, p27 ^{Kip1} and ZO-1 (zonula occludens-1) were obtained from Pharmingen (San Diego, CA). Monoclonal antibodies for GAPDH and actin are products of Chemicon (Temecula, CA). Monoclonal antibody for vinculin is a product of Sigma (St Louis, MO). Polyclonal antibody for mDia1 was obtained from Abcam (Cambridge, MA). Monoclonal antibody for VE-cadherin (vascular endothelial cadherin) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For immunoblotting, the antibodies were used at the following concentrations: Pfn1 (1:500), Pfn2 (1:1000), VASP (1:500), p27 ^{Kip1} (1:2000), GAPDH (1:200), actin (1:1000), ZO-1 (1:500), VE-cadherin (1:1000), N-WASP (1:1000), and mDia1 (1:2500). Rhodamine-phalloidin and DAPI were purchased from Molecular Probes (Carlsbad, CA). Collagen type I and growth-factor-reduced matrigel are products of BD Biosciences (Bedford, MA).

2.5 PROTEIN EXTRACTION AND IMMUNOBLOTTING

For protein extraction, cells were washed twice with cold PBS and lysed on ice for 30 min in modified RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1mM sodium pervanadate, and protease inhibitors (10 µg/ml of leupeptin, aprotinin, pepstatin and 1 mM phenylmethylsulfonyl fluoride). The lysates were clarified at 13,000 rpm for 15 min at 4 ºC and the protein concentration was determined using a coomassie-based protein assay kit (Pierce; Rockford, IL). For protein electrophoresis, equal amounts of protein samples were loaded on a gel and transferred onto a nitrocellulose membrane. After blocking the membrane with 5% non-fat dry milk in TBST for 1 hour at room temperature, immunoblotting was performed overnight with the

appropriate antibodies. After extensive washing with TBST, the blot was incubated with the appropriate secondary antibody (1:1000 dilution, Pharmingen, San Diego, CA) and washed 3 times with TBST before performing chemiluminescence for the visualization of protein bands.

2.6 IMMUNOSTAINING

Cells cultured on collagen or collagen-coated cover-slips were washed 3 times with PBS fixed with 3.7% formaldehyde for 15 minutes, permeablized with 0.5% Triton X-100 for 5 minutes and then blocked with 10% goat-serum for 30 minutes. After incubating with either vinculin (1:100 dilution), VASP (1: 100 dilution), and p27 ^{Kip1} (1:200 dilution) antibody for 1 hour at room temperature, cells were washed 4 times (first twice with PBS containing 0.02% tween and then twice with PBS), each of duration 3 minutes, before incubating with a FITC-conjugated anti-mouse secondary antibody (1:100 dilution). The stained cells were washed 4 times using similar procedures and then mounted on slides for fluorescence microscopy on an IX-71 Olympus inverted microscope. For F-actin visualization, cells were stained with rhodamine-phalloidin. For VE-cadherin and ZO-1 staining, cells were fixed and permeabilized using cold methanol at -20°C for 20 minutes and then blocked with 5% BSA (containing 15% glycine) for 45 minutes at room temperature. After incubating with either VE-cadherin (1:200 dilution) or ZO-1 (1:250 dilution) antibody for 1 hour at room temperature, cells were washed five times with 5% BSA (containing 15% glycine) followed by washing five times with PBS before incubating with the appropriate secondary antibody. Stained cells were then washed five times using similar procedures. All fluorescence images were acquired using the Metamorph imaging software.

2.7 QUANTIFICATION OF PHALLOIDIN FLUORESCENCE

For each experiment, we acquired images of phalloidin-stained cells at 6-12 random fields of observation using a 20X objective. After performing background subtraction of the images, the average fluorescence intensity per cell was calculated for each field of observation. These values were then normalized with respect to the average fluorescence value calculated for the control cells for a given experiment. Normalized fluorescence data of control and Pfn1-siRNA treated cells were pooled from 2 experiments, the average values of which were then statistically compared using a Student's T-test.

2.8 WOUND HEALING ASSAY

Confluent monolayers of HUVECs cultured in the wells of a 24-well plate was mechanically scratched using a pipet-tip. Cell debris was removed by washing with PBS before adding complete growth media to the cells. Images of the wound edges were acquired at three random locations first immediately after wounding and then at the same locations after 12 hours to assess the wound closure by migrating HUVECs. Wound closure was quantified by the percentage change in the wound area per unit time and averaged for three locations per well from a triplicate set of samples for each experimental condition. This assay was performed 48, 72, and 96 hours after siRNA transfection.

2.9 SINGLE-CELL MIGRATION ASSAY

HUVECs or HmVEC-1, of different treatments, were sparsely plated on a 35 mm tissue-culture dish and after an overnight incubation, time-lapse videomicroscopy of 3 random fields were simultaneously performed at an interval of 2.5-3 minutes for a total duration of 90 minutes. The acquired images were analyzed using the NIH ImageJ software.

2.10 CELL SPREADING ASSAY

HUVECs were plated on the wells of a 24-well plate that were pre-coated with different concentrations of matrigel (25 and 100 μg/ml) and the percentages of cells that showed spreading morphology (appears phase-dense) were determined at different time-points (30 and 60 minutes) after cell seeding. Data for three random fields of observation from a duplicate set of samples for each experimental condition were averaged for statistical comparison using a Student's T-test.

For HmVEC-1 spreading on matrigel, two-hundred microliters of matrigel was polymerized in the wells of a 48-well plate at 37 ºC for 30 minutes prior to seeding 25,000 VECs on the top of matrigel. Phase-contrast images of cells acquired 0.5, 1, 2, and 4 hours after plating them onto matrigel.

2.11 KYMOGRAPH ANALYSIS OF MEMBRANE PROTRUSION

Time-lapse imaging of sparsely seeded VECs, of different treatments, was performed at an interval of 5 seconds for a total duration of 10 minutes. Around 3~5 kymographs were generated for each cell by the Metamorph software (Downingtown, PA), from which the average values of the protrusion velocity, protrusion distance (magnitude) and number of protrusion within the ten-minute duration (frequency) were calculated. These values were averaged for a total of 10-15 cells of each group pooled from 2-3 independent experiments for statistical comparison.

2.12 CORD FORMATION ASSAY

Two-hundred microliters of matrigel was polymerized in the wells of a 48-well plate at 37 ºC for 30 minutes prior to seeding 25,000 VECs on the top of matrigel. Phase-contrast images of cells acquired 8 hours after plating revealed matrigel-induced cord formation by VECs. Cord-formation data was quantified by counting the number of nodes that have at least three branches or total length of cords in a given field of observation, which was then averaged for three fields per well from a duplicate set of samples of each experimental condition.

2.13 3-D MORPHOGENESIS ASSAY

HUVECs were initially transfected with either control or Pfn1-siRNA. One day after transfection, 150 μl of neutralized collagen-I solution will be premixed with the transfected HUVECs and

plated in duplicate in the wells of a 8-well Lab-Tek chamber slide (Nunc, Rochester, NY) at the final concentrations of collagen and cells equal to 2.5 mg/ml and 2E6 /ml, respectively. The collagen solution will be allowed to polymerize for 30 minutes, and then overlaid with the complete growth medium with 50 ng/ml bFGF, 50 ng/ml VEGF, and 50 ng/ml PMA. As experimental readouts, capillary length per 10X field was scored at multiple random locations (3/well) at the end of 96 hours of incubation. The average number of capillary length per field between the two groups was compared using a Student's T-test.

2.14 INVASION ASSAY

HUVECs were first transduced with GFP-encoded adenovirus, and then transfected with either control or Pfn1-siRNA. One day after transfection, 400 μl of neutralized collagen-I solution will be premixed with the transfected HUVECs and plated in duplicate in the wells of a 24-well plate at the final concentrations of collagen and cells equal to 2.5 mg/ml and 5E6 /ml, respectively. The collagen solution was allowed to polymerize for 30 minutes, and then overlaid with the complete growth medium with 50 ng/ml bFGF, 50 ng/ml VEGF, and 50 ng/ml PMA. Time-lapse videomicroscopy of two independent experiments with a duplicate set of samples for each experimental condition was taken at an interval of 10 minutes for a total duration of 72 hours. Cell tracking was carried out in Image J software. The average invasion speed between the two groups was compared using a Student's T-test.

2.15 CELL-PROLIFERATION ASSAY

HUVECs were plated in triplicate at a density of 20,000 cells per well of a 24-well plate 24 hours after transfection with the appropriate siRNAs. At different time-points after transfection (48-96 hours), cells were washed with PBS and stained with 0.5% crystal violet for 15 minutes. After washing cells three times with PBS, dye was eluted from cells by adding 300 μl of 100% ethanol in each well and its absorbance was measured using a plate-reader. Absorbance data based on triplicate set of samples for each experimental condition from a total of three independent experiments were then averaged for statistical comparison using a Student's T-test.

2.16 DATA REPRESENTATION

In most cases, experimental data were represented as box and whisker plots where dot represents the mean, middle line of box indicates median, top of the box indicates 75th percentile, bottom of the box measures 25th percentile and the two whiskers indicate the 10th and 90th percentiles, respectively.

3.0 THE ROLE OF PROFILIN-1 IN MIGRATION OF VASCULAR ENDOTHELIAL CELLS

This specific aim tests an overall working postulate that **loss of Pfn1 function impairs membrane protrusion and migration of VEC (Working postulate #1).** Specifically, I will determine whether loss of Pfn1 function alters:

a) VEC migration.

b) The kinetics of cell protrusion.

Some of the contents in this chapter have been or will be published in the following publications:

Ding, Z., Lambrechts, A., Parepally, M. and Roy, P. (2006). Silencing profilin-1 inhibits endothelial cell proliferation, migration and cord morphogenesis. *J Cell Sci* **119**, 4127-37.

Bae, Y. H., Ding, Z., Zou, L., Wells, A., Gertler, F. and Roy, P. (2009). Loss of profilin-1 expression enhances breast cancer cell motility by Ena/VASP proteins. *J Cell Physiol* **219**, 354-64.

Ding, Z., Gau, D., Deasy, B., Wells, A. and Roy, P. (2009) Both actin and polyproline interactions of profilin-1 are required for vascular endothelial cell migration and capillary morphogenesis. *(under review)*

3.1 RESULTS

3.1.1 Suppression of Pfn1 Expression in VEC by RNA Interference

To suppress Pfn1 expression, we adopted transient transfection of VEC with either a non-targeting control (C) or Pfn1-specific (P) siRNA construct. Initially, to determine the specificity of Pfn1-siRNA, we transiently transfected the siRNA constructs into MDA-MB-231 breast carcinoma cells, which were genetically engineered by us to provide stable expression of different point-mutants of GFP-Pfn1. These mutants involved a 2 base-pair alteration either within (mutant-1) or outside (mutant-2) the region targeted by Pfn1-siRNA. As judged by the decrease of intensity in immunoblots, our Pfn1-siRNA down-regulated the expression of mutant-2 as expected, but was ineffective in suppressing the expression of mutant-1. In negative control experiments, Pfn1-siRNA treatment did not non-specifically decrease the intensity of immunoblot of GFP-expressing MDA-MB-231 cells (Figure 3A). Taken together, these data thus demonstrate the specificity of action of our Pfn1-siRNA. The bar graph in Figure 3B displays the silencing efficiency of Pfn1-siRNA in HUVECs as a function of time (48-96 hours), which shows a time-dependent progressive loss of Pfn1 expression with ~97% gene-silencing achieved 96 hours after transfection. The representative Pfn1 immunoblots at different time points after transfection are shown in the inset of Figure 3B (the GAPDH blot serves as the loading control). We have also confirmed that the polyclonal Pfn1 antibody used in this study is specific and does not cross-react with Pfn2. Since Pfn2's function is similar to Pfn1, we checked the expression level of Pfn2 in HUVECs. We could not detect Pfn2 expression by immunoblotting under either of the experimental conditions (inset of Figure 3B) thus suggesting that there is no compensatory up-regulation of Pfn2 when Pfn1 expression is suppressed in VEC.

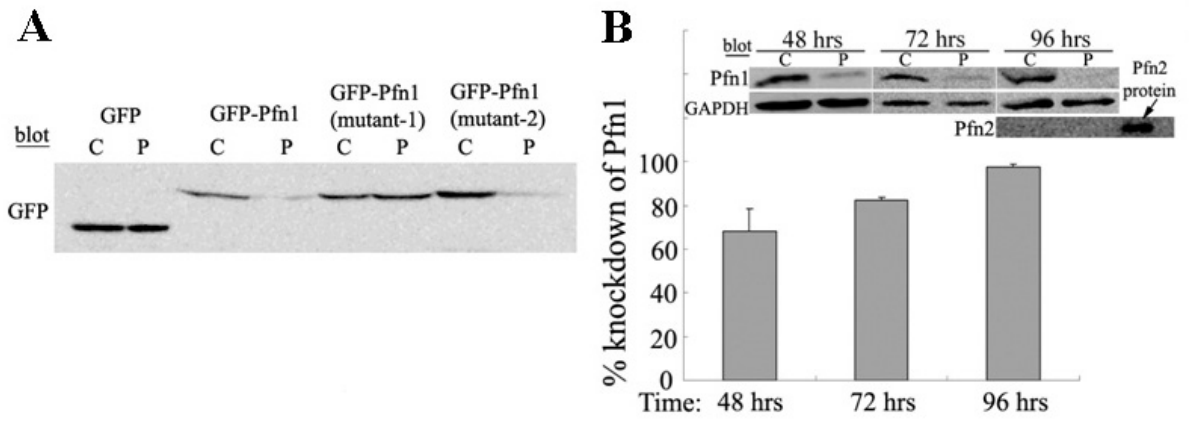

Figure 3. *Suppression of Pfn1 Expression in VEC by RNA Interference*

(A) Immunoblots of GFP shows that Pfn1 siRNA (denoted by "P") was able to knockdown the expression of GFP-Pfn1 and GFP-Pfn1 (mutant-2) but not that of GFP-Pfn1 (mutant-1) and GFP, thus confirming the specificity of Pfn1 siRNA. **(B)** A bar graph shows time-dependent progressive loss of Pfn1 expression with nearly 97% suppression of Pfn1 expression 96 hours after transfection. The insets show representative immunoblots of Pfn1 48, 72, and 96 hours after transfection of either control siRNA (denoted by "C") or Pfn1 siRNA (denoted by "P"). No compensatory up-regulation of Pfn 2 after suppressing Pfn1 (purified Pfn2 protein serves as a positive control for the blot).

3.1.2 Perturbing Ligand Interactions of Pfn1 in VEC

We stably expressed Pfn1 and its mutant constructs (GFP-Pfn1, GFP-Pfn1-H133S, and GFP-Pfn1-H119E) in an immortalized human dermal microvascular VEC line (HmVEC-1) by retroviral transduction. Figure 4A shows the strategy to design these Pfn1 constructs. As a control, HmVEC-1 was transduced with GFP-encoding retrovirus. We suppressed endogenous Pfn1 expression by Pfn1 siRNA treatment. In order to first demonstrate the efficacy of our "knockdown and knock-in" system, we transfected all of our stable HmVEC-1 sublines with Pfn1-siRNA. As a control group for all of the experiments, GFP expressers were also transfected with control siRNA (this treatment condition will be referred to as "control GFP group" from here on). Figure 4B shows a representative Pfn1-immunoblot confirming the expression of exogenous GFP-Pfn1 or its mutants in various HmVEC-1 sublines against a strongly suppressed endogenous Pfn1 background. The average expression levels of GFP-Pfn1 and its mutants are comparable between the different cell lines, and were estimated to be equal to ~70% of the endogenous Pfn1 level.

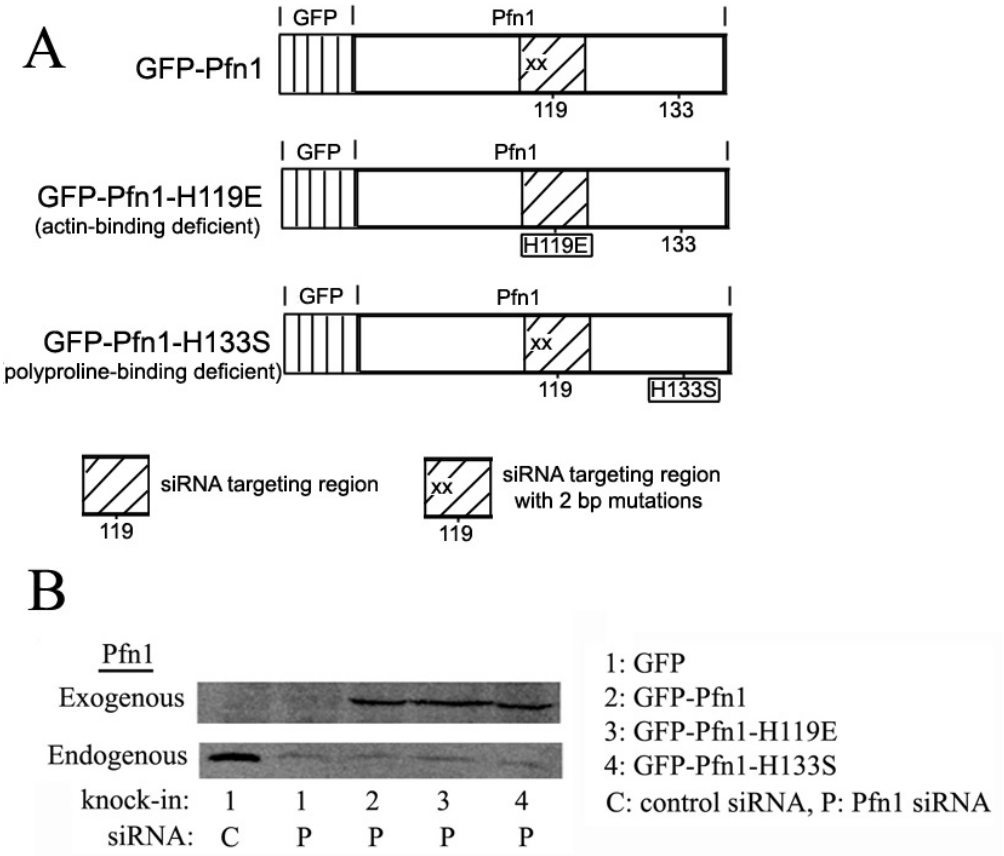

Figure 4. *"Knockdown and Knock-in" of Pfn1 and Its Mutants in VEC*

(A) A schematic drawing of "knockdown and knock-in" constructs of GFP-Pfn1. **(B)** A representative Pfn1 immunoblot shows stable expression of exogenous GFP-Pfn1 or its mutants (GFP-Pfn1-H119E, GFP-Pfn1-H133S) in HmVEC-1 in the silenced endogenous Pfn1 background.

3.1.3 Silencing Pfn1 Reduces Polymerized Actin Filament

The effect of silencing Pfn1 on endothelial actin cytoskeleton was evaluated by rhodamine-phalloidin staining of HUVECs 96 hours after transfection, which showed that cells bearing Pfn1-siRNA have significantly less actin filaments, particularly those comprising stress-fibers, compared with the control-siRNA transfected cells (Figure 5A). Quantification of phalloidin-fluorescence showed approximately 29% reduction in the overall F-actin content of HUVECs due to loss of Pfn1 expression (Figure 5B). Since Pfn1 has been recently implicated in gene transcription (Lederer et al., 2005), we next asked whether expression levels of actin and some of the ABPs that are important for actin assembly in response to growth factor signaling such as VASP, N-WASP and mDia1 are altered as a result of silencing Pfn1. Immunoblots of whole cell lysates showed no appreciable change in the expression levels of actin and the indicated ABPs at any time-point after transfection (Figure 5C). Consistent with the observation in HUVECs, phalloidin staining revealed that F-actin content of GFP expressing HmVEC-1 is significantly reduced when endogenous Pfn1 expression is silenced (Figure 5D). Taken together, these data suggest that loss of Pfn1 expression alters actin cytoskeleton via direct modulation of actin polymerization and/or bundling of actin filaments in VEC.

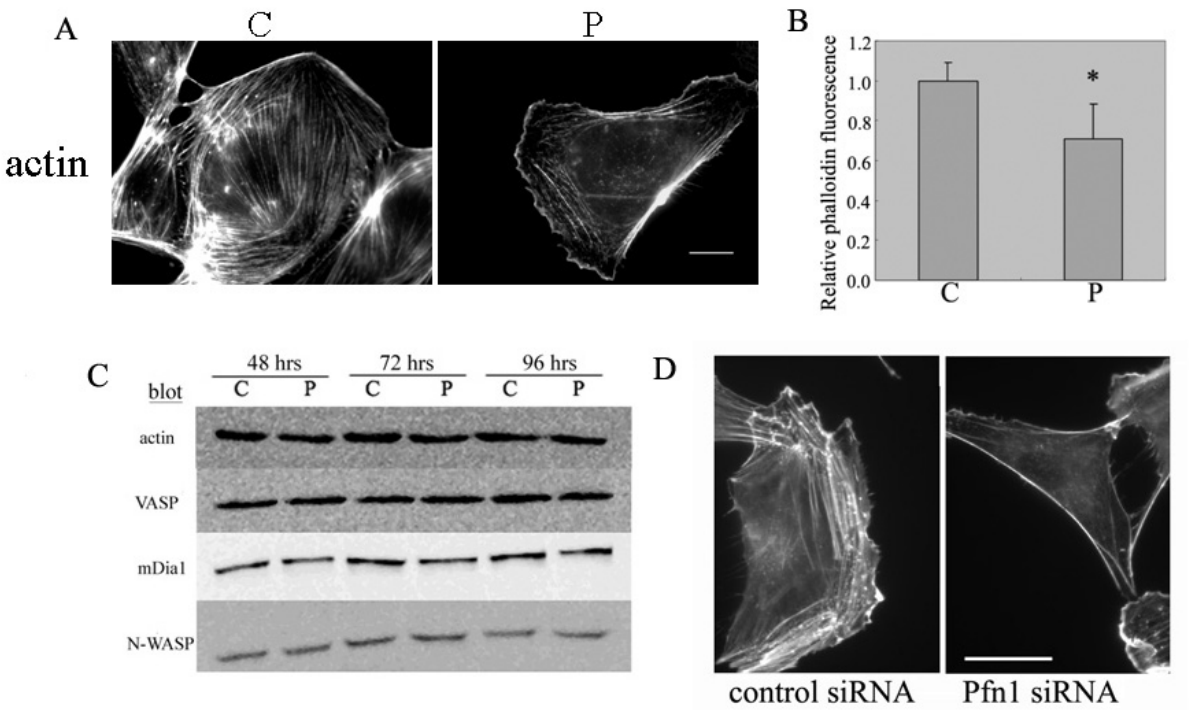

Figure 5. *Silencing Pfn1 Reduces Polymerized Actin Filament*

(A) Rhodamine-phalloidin staining of HUVECs shows that silencing Pfn1 dramatically inhibits the formation of actin stress-fibers. Bar, 20 μm. **(B)** A bar graph displaying the relative (normalized with respect to the control cells) fluorescence intensity of phalloidin shows a 29% decrease in the average level of F-actin in Pfn1-deficient cells. These data were obtained from analyses of 640 control and 584 Pfn1-deficient cells from two independent experiments, the difference of which was found to be statistically significant (the asterisk indicates $P<0.0001$). **(C)** Immunoblots show comparable expression levels of actin and several ABPs such as VASP, mDia1, and N-WASP at 48, 72 and 96 hours after transfection. **(D)** Phalloidin staining of GFP expressing HmVEC-1 treated with either control or Pfn1-siRNA. Bar, 30 μm.

3.1.4 Loss of Pfn1 Expression Decreases Focal Adhesion Formation in HUVEC

Since cell-matrix adhesion complexes physically associate with actin cytoskeleton, we next asked whether loss of Pfn1 expression has any impact on these adhesive structures in VECs. Previous data showed that Pfn1-overexpression causes human aortic VECs to form more focal contacts and display increased adhesion on fibronectin-substrate (Moldovan et al., 1997), thus suggesting a possible role of Pfn1 in regulating cell-matrix adhesion. To further this line of inquiry, we performed vinculin [a marker for focal adhesion (FA)] immunostaining of HUVECs, which showed a marked inhibition of FA assembly when Pfn1 expression was silenced (Figure 6A). A bar graph in Figure 6B summarizes the results from quantitative analyses of vinculin-staining data, which showed significantly lower FA density (defined as the number of FA plaques per 100 μm^2 of cell area) in Pfn1-deficient cells (2.7±1.9) when compared with control cells (6.1±2.1). The FAs observed for the control cells also appeared to be larger in size compared with those in Pfn1-deficient cells. Overall, these data demonstrate that Pfn1 plays an important role in regulating cell-matrix adhesions in VECs.

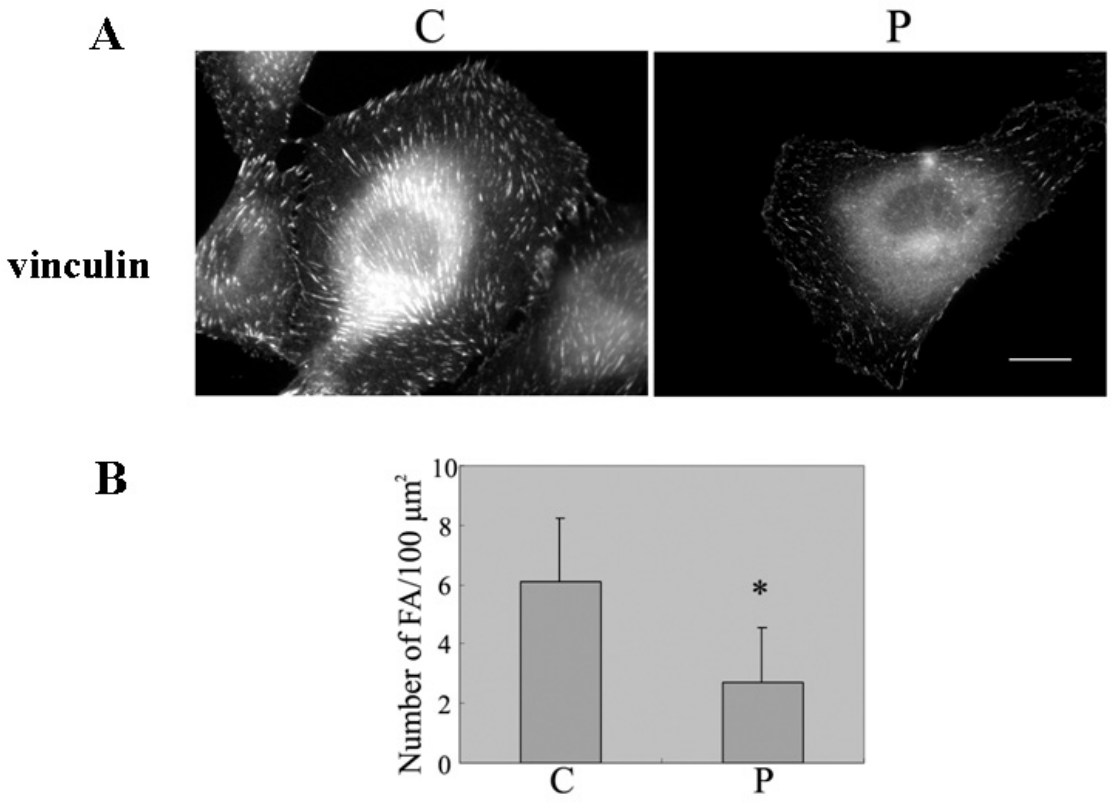

Figure 6. *Loss of Pfn1 Expression Decreases Focal Adhesion Formation in HUVEC*

(A) Vinculin-immunostaining shows a dramatic reduction in FA formation when Pfn1 expression is silenced (C, control siRNA; P, Pfn1-siRNA). Bar, 20 μm. **(B)** A bar graph shows a significantly (P<0.001) higher FA density (number of FA/100 μm^2 of cell area) in control cells (6.1±2.1) than in Pfn1-deficient cells (2.7±1.9). These data are based on analyses of 56 control and 69 Pfn1-deficient cells that were randomly selected from two independent experiments.

3.1.5 Loss of Pfn1 Expression Inhibits HUVEC Migration

We next examined whether directed migration of VECs is affected by loss of Pfn1 expression using a standard wound-healing assay. Figure 7A depicts the results of a typical wound-healing experiment performed 96 hours after siRNA transfection, where Pfn1-deficient cells clearly showed significant impairment in wound closure when compared with the control cells. Proliferation is not likely to play a role here simply because the wound-healing experiment only lasted 12 hours. A bar graph in Figure 7B summarizes the wound-healing data as a function of time where Pfn1-siRNA treatment inhibited wound-closure by 25%, 36%, and 47% at 48, 72 and 96 hours after transfection, respectively. These data thus demonstrate increasing inhibition of VEC migration with progressive loss of Pfn1 expression.

Cell migration in a monolayer set-up, as in a wound-healing assay, can be affected by the strength of cell-cell adhesion. We additionally performed time-lapse imaging of individual HUVECs to determine whether intrinsic VEC migration is affected by loss of Pfn1 expression. Figure 7C shows the results of a typical set of time-lapse experiments where HUVECs bearing control-siRNA displayed directed migration involving significant translocation of their cell bodies during the course of the experiment (upper panel). Consistent with our findings from wound-healing experiments, a marked inhibition in random cell migration was observed in the case of Pfn1-deficient cells (lower panel). The single-cell migration data are summarized in Figure 7D, which depicts the trajectories of individual cell obtained by frame-by-frame analyses of the centroid positions of cell-nuclei (assumed to be the representations of cell-bodies in this case). The average net velocity of control and Pfn1-deficient cells were 29.1±17.3 μm/h (n=27 cells) and 7.5±6.4 μm/h (n=21 cells), respectively, the difference of which was found to be statistically significant (P<0.001). Overall, the results from wound-healing and time-lapse

imaging experiments clearly demonstrate that Pfn1 plays an important role in VEC migration as hypothesized.

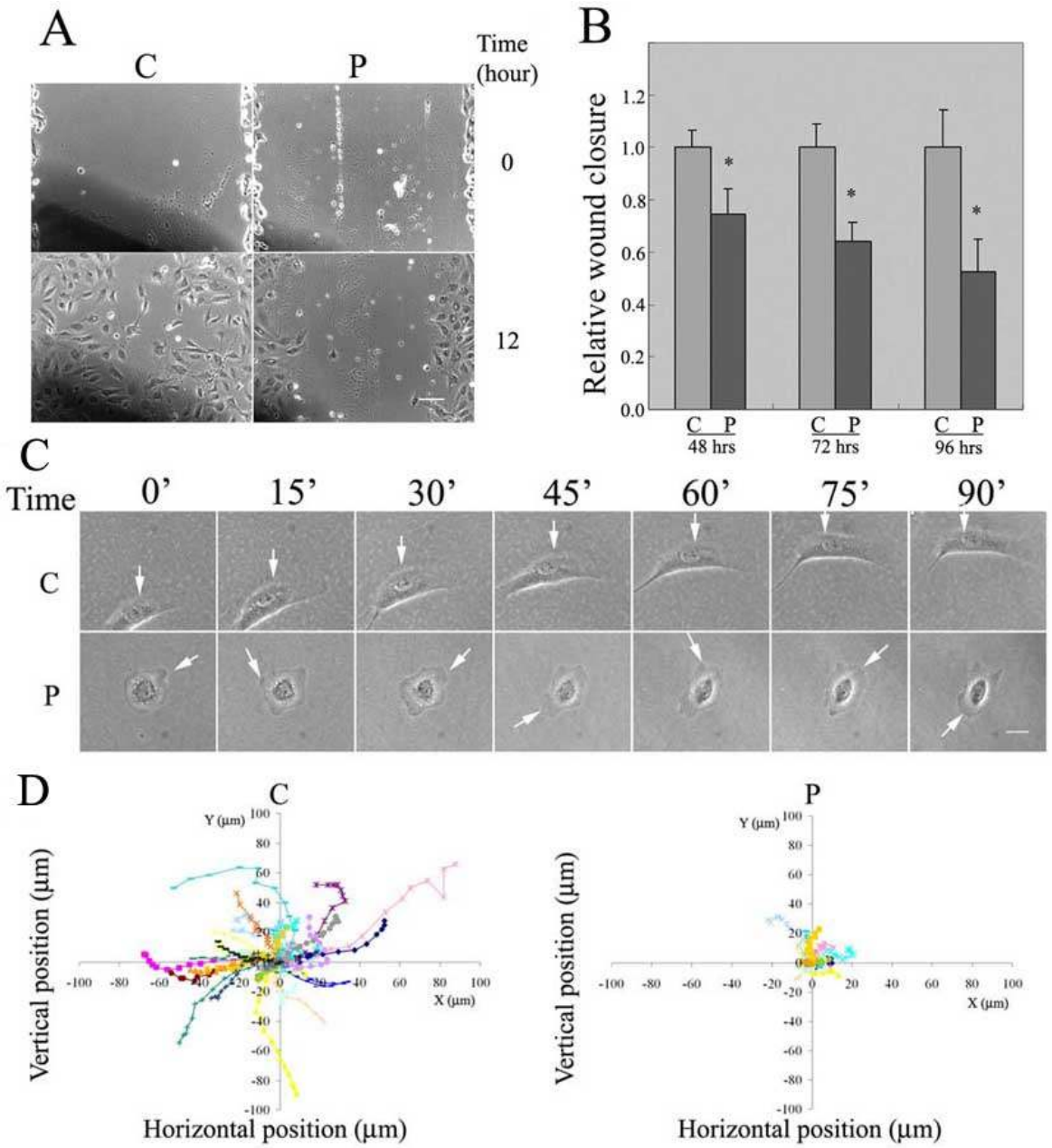

Figure 7. *Loss of Pfn1 Expression Inhibits HUVEC Migration*

(A) Representative images of the wound margins immediately and 12 hours after wounding show significant impairment in wound closure by HUVECs due to loss of Pfn1

expression (C, control siRNA; P, Pfn1-siRNA). Bar, 100 μm. **(B)** A bar graph plotting the relative efficiency of wound closure shows Pfn1-siRNA treatment inhibited wound-closure by 25%, 36% and 47% when evaluated at 48, 72 and 96 hours after transfection, respectively. (These data are summarized from three independent experiments and the asterisk indicates $P<0.002$) **(C)** A typical time-lapse imaging experiment shows directed migration of control cells (denoted by "C") involving directed protrusion and significant net cell translocation. By contrast, Pfn1-deficient cells (denoted by "P") produce small, randomly directed protrusion with much less net cell translocation (the direction of protrusion is indicated by the arrow). Bar, 30 μm. **(D)** Trajectories of individual cells from the frame-by-frame analyses of the centroid of cell nuclei show a significantly ($P<0.001$) larger net velocity of control cells (29.1±17.3 μm/h) compared with the Pfn1-deficient cells (7.5±6.4 μm/h) during the 90-minute observation period (migration data of 27 control and 21 Pfn1-deficient cells from a total of three independent experiments were pooled for the analysis).

3.1.6 Both Actin and Polyproline Interactions of Pfn1 Are Required in Regulating HmVEC-1 Migration

To determine whether Pfn1's interaction with both actin and polyproline ligands contribute to overall VEC migration, we performed time-lapse motility experiments of various HmVEC-1 sublines and compared the average speed of migration between the different cell lines. Because of certain degree of variation in the expression of GFP-tagged proteins in the polyclonal culture of our stable cell lines, cells which were fairly bright for GFP-fluorescence were only chosen for time-lapse measurements. Also, between the different experimental groups, cells with relatively similar levels of GFP-fluorescence were selected for final data analyses. A box and whisker plot comparing the relative speed of migration between the different groups of cells demonstrates that silencing Pfn1 expression inhibits the average speed of migration of GFP-expressing HmVEC-1 (n=96) by nearly 37% (Figure 8). Re-expression of GFP-Pfn1 in a silenced endogenous Pfn1 background (n=80) resulted in an average migration speed close to 91% of that of control GFP group of cells (n=106). In fact, we did not find any statistically significant difference in the average speed of migration between the control GFP group and GFP-Pfn1 re-expressers suggesting that expression of GFP-Pfn1 is able to fully rescue the inhibition of migration resulting from endogenous Pfn1 depletion. However, re-expression of neither of the Pfn1 mutants was able to rescue the motility defect induced by silencing Pfn1 expression since the average speed of migration of GFP-Pfn1-H119E (n=71) and GFP-Pfn1-H133S (n=82) expressers were found to be 40% and 37% less than that of control cells, respectively, and these differences were statistically significant ($P<0.01$). Overall, these results demonstrate that both actin and polyproline interactions of Pfn1 are indispensable for efficient VEC migration.

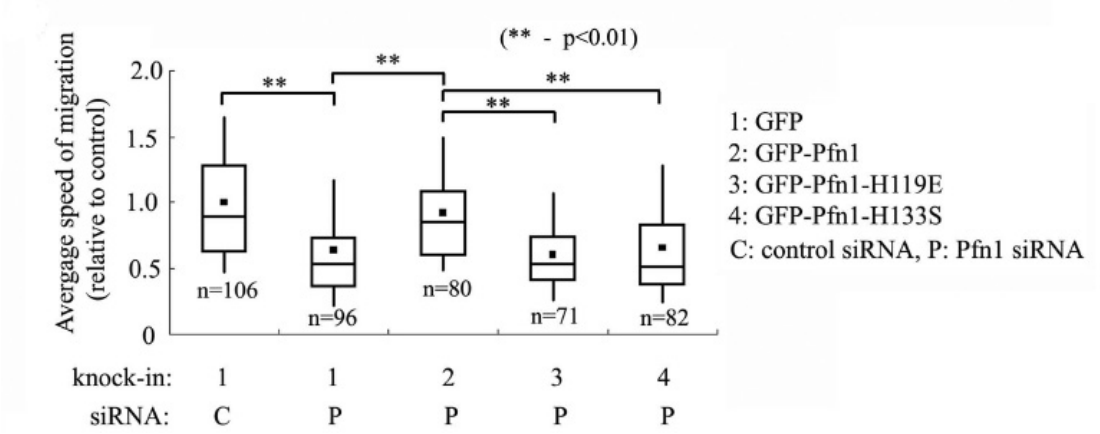

Figure 8. *Both Actin and Polyproline Interactions of Pfn1 Are Required in Regulating HmVEC-1 Migration*

A box and whisker plot showing the average speed of migration of different HmVEC-1 sublines relative to that of control cells. (n: number of cells analyzed from a total of 4 independent experiments; ** indicates $P<0.01$).

3.1.7 Loss of Pfn1 Expression Reduces Velocity and Frequency of Membrane Protrusion of HUVEC

Membrane protrusion is considered as the first step of cell migration, and it is effective actin polymerization at the cell membrane that translates to effective membrane protrusion. Pfn1 has been extensively studied in actin polymerization in biochemical experiments and pathogen-based model systems that mimic actin polymerization at the leading edge of migrating cells. To determine whether Pfn1 plays a role in membrane protrusion in VEC, we performed kymograph analyses of membrane protrusion of control or Pfn1-deficient HUVECs. Each kymograph was generated from time-lapse images with a five-second interval and ten-minute duration. Figure 9A shows a typical kymograph from either control (left) or Pfn1-deficient (right) HUVECs. We found the number of protrusions (sawtooth shapes) during the course of experiments (protrusion frequency), and the velocity of protrusion (slope of ascending part of each sawtooth waveform) were both smaller in Pfn1-deficient HUVECs. Box and whisker plots in Figure 9B and C show the quantitative results of protrusion velocity and frequency, both of which are important parameters to characterize protrusions. There is a 1.8-fold reduction in protrusion velocity, and the protrusion frequency decreases dramatically when Pfn1 expression was silenced. These data therefore demonstrate that Pfn1 plays an important role in regulating velocity and frequency of membrane protrusion of VEC.

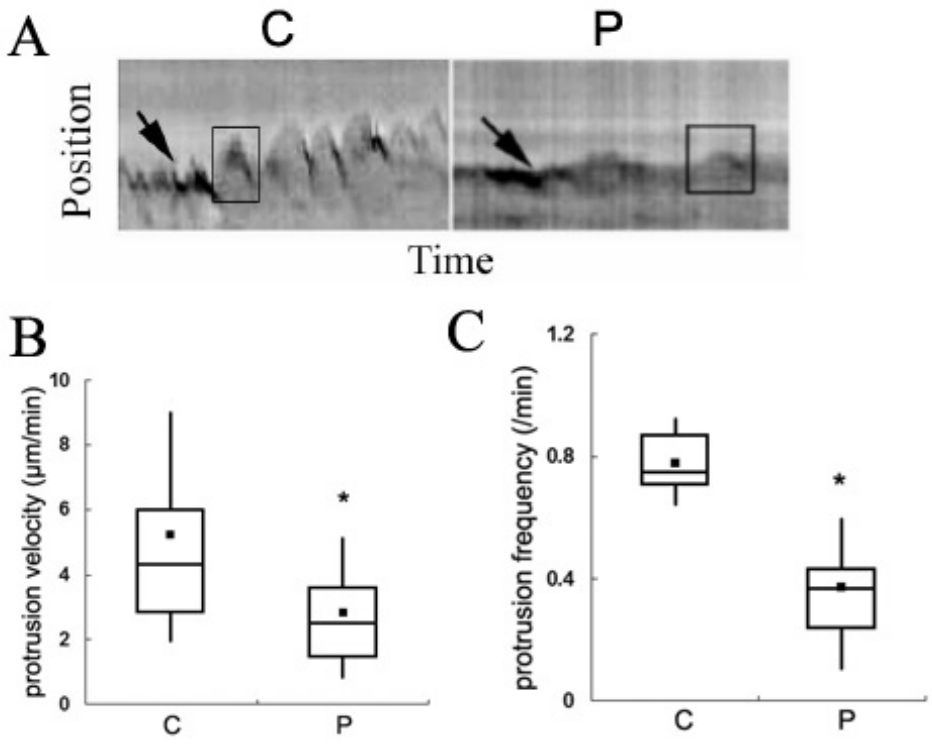

Figure 9. *Loss of Pfn1 Expression Reduces Velocity and Frequency of Membrane Protrusion of HUVEC*

(A) Representative kymographs of control (denoted by "C") and Pfn1-deficient HUVECs (denoted by "P") were generated from time-lapse images with a five-second interval and ten-minute duration. Black arrows indicate the leading edge of cell membrane, and the boxes outline the "sawtooth" shape of a single protrusion event. **(B)** A box and whisker plot depicting the protrusion velocity shows loss of Pfn1 expression lead to a 1.8-fold reduction in protrusion velocity of VEC. These data are based on analyses of 678 single protrusion event from control cells and 297 from Pfn1-deficient ones from three independent experiments. (* indicates $P<0.05$). **(C)** A box and whisker plot comparing the protrusion frequency shows a dramatic decrease in frequency when Pfn1 expression was suppressed (* indicates $P<0.05$).

3.1.8 Both Actin and Polyproline Interactions of Pfn1 Are Required For Efficient Membrane Protrusion of HmVEC-1

We further asked whether perturbing actin or polyproline interactions of Pfn1 alters the lamellipodial dynamics of HmVEC-1. To address this question, we analyzed the leading edge movement of the different groups of HmVEC-1 from kymographs of 1-pixel (0.3 μm) wide lines that were drawn normal to the leading edge and in the direction of protrusion. Each kymograph was generated from time-lapse images with a five-second interval and ten-minute duration. Figure 10A depicts a set of representative kymographs of the protrusion events of HmVEC-1 under different experimental conditions where leading edge traces (marked by the arrows) reveal cycles of typical lamellipodial protrusion and withdrawal (resemble sawtooth waveforms). It is evident from Figure 10A that either silencing the overall expression of Pfn1 or expression of Pfn1-mutants leads to a much flatter kymograph trace suggesting that membrane dynamics is suppressed by inhibition of Pfn1 function. We performed quantitative analyses of the actual protrusion velocity (equal to the slope of the ascending portion of a sawtooth waveform) of the different experimental groups and these data are summarized in the form of a box and whisker plot in Figure 10B. The average protrusion velocity of Pfn1-depleted HmVEC-1 (= 2.3 μm/min) was found to be nearly 40% less than that of control siRNA-treated cells (= 4 μm/min), and this data is consistent with our earlier finding with HUVEC (Bae et al., 2009). Re-expression of GFP-Pfn1 in the silenced endogenous Pfn1 background increased the average protrusion velocity to 3.6 μm/min, and this value was not statistically different from the velocity scored for control cells. The average velocity of protrusion of both GFP-Pfn1-H119E (= 2.7 μm/min) and GFP-Pfn1-H133S (=2.5 μm/ min) expressers were found to be less than that of GFP-Pfn1 expressing cell line with statistical significance. We also noted that both Pfn1-depleted and the mutant cells

display reduced frequency of protrusion (data not shown). Overall, these data demonstrate that both actin and polyproline interactions of Pfn1 are required for efficient lamellipodial protrusion.

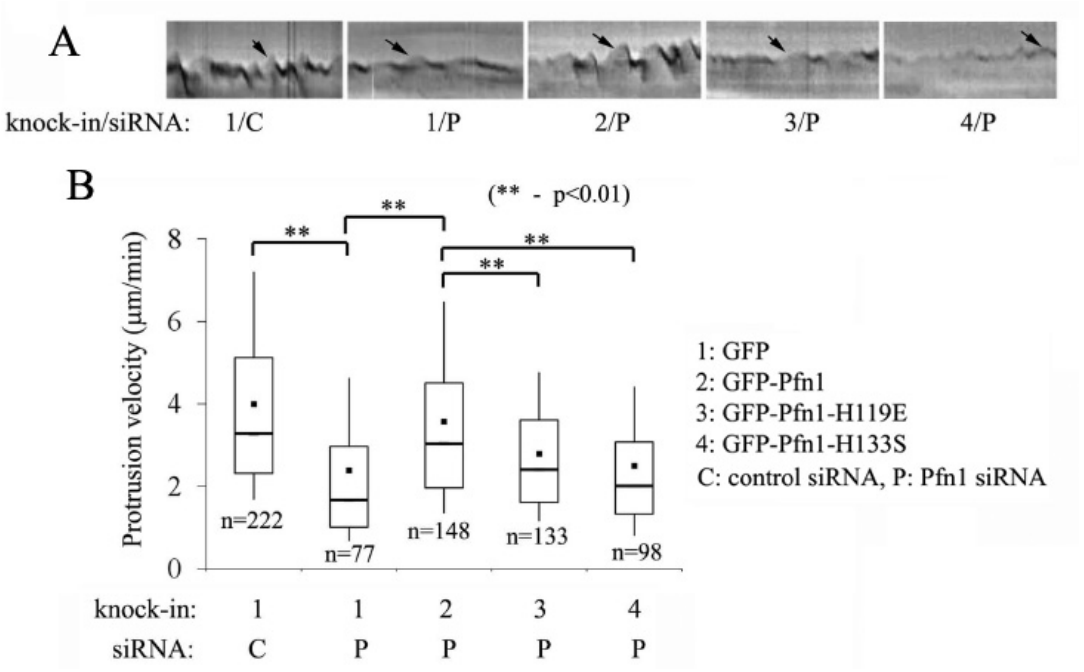

Figure 10. *Both Actin and Polyproline Interactions of Pfn1 Are Required For Efficient Membrane Protrusion of HmVEC-1*

(A) Representative kymographs of different groups of cells (black arrow marking the ascending portion of a sawtooth waveform indicates membrane protrusion; construct and siRNA annotations are same as in panel (B). **(B)** A box and whisker plot comparing the average protrusion velocity between the different groups (n: number of protrusion events analyzed from a total of 4-5 experiments).

3.1.9 Pfn1 Is Important for HUVEC Spreading

I next evaluated whether early cell spreading (an event that involves active cell protrusion), on ECM-coated substrate is affected by loss of Pfn1 expression. Figure 11A shows the morphology of HUVECs on matrigel (100 μg/ml)-coated substrate within 1 hour after plating. From phase contrast images where spreading cells appear darker (phase-dense), it is evident that Pfn1-deficient cells are much less efficient in spreading when compared to the control cells. Figure 11B summarizes these data in the form of a bar graph plotting the percentage of spreading cells on substrates coated with different concentrations of matrigel. Although a higher concentration of matrigel generally facilitated cell spreading, a 3-fold decrease in the spreading efficiency was observed when Pfn1 expression was silenced, thus confirming that Pfn1 plays a key role in regulating VEC protrusion. To further determine whether loss of Pfn1 actually inhibits or only delays cell spreading, we compared HUVEC-morphology on matrigel-coated substrates at later time-points. Even at 22 hours after cell-seeding, the extent of spreading of Pn1-deficient cells was clearly much less compared with that of control cells (Figure 11C), therefore meaning that silencing Pfn1 actually inhibits VEC spreading.

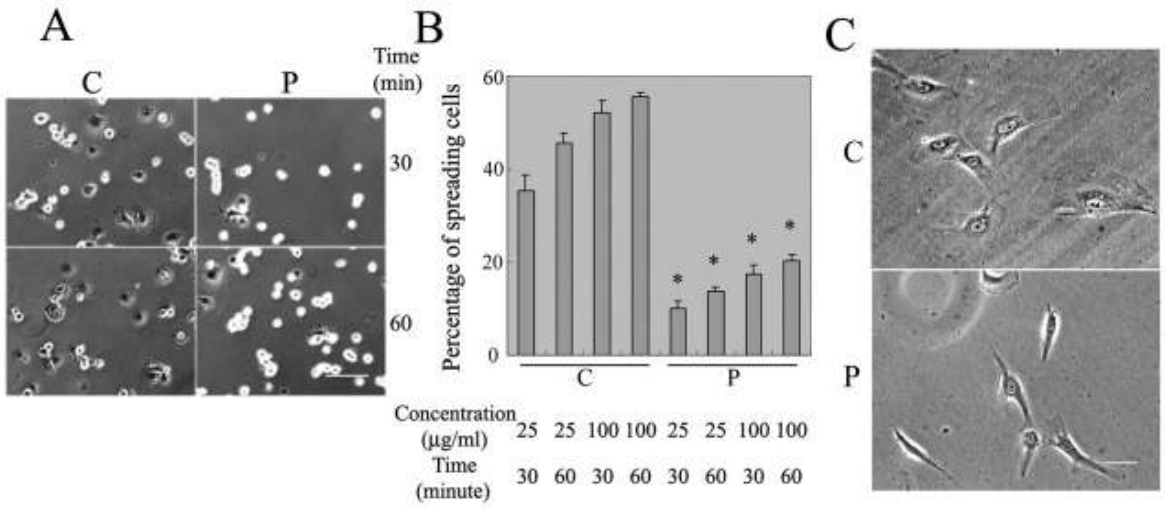

Figure 11. *Pfn1 Is Important for HUVEC Spreading*

(A) Phase contrast images of HUVECs seeded 1 hour after plating on a substrate that is pre-coated with 100 μg/ml matrigel show higher proportion of spreading cells (appear phase dense) in the control group (denoted by "C"). Impaired cell spreading was evident from round morphology of majority of Pfn1-deficient cells (denoted by "P"). Bar, 100 μm. **(B)** A bar graph plotting the percentage of spreading cells at two different time-points (30 minutes and 1 hour) and for two different coating concentrations of matrigel clearly shows increased spreading efficiency of the control cells (these data were pooled from the analyses of approximately 800-1000 cells for each experimental condition from two independent experiments). The asterisk indicates $P<0.001$. **(C)** Pfn1-deficient cells were still found to be much less flat and spread-out compared with the control cells at 22 hours after cell-seeding on matrigel-coated substrates. Bar, 50 μm.

3.2 DISCUSSION

3.2.1 Pfn1 and VEC Actin Cytoskeleton and Focal Adhesion Formation

Consistent with previous findings reported for smooth muscle (Tang and Tan, 2003) and alveolar epithelial cells (Bitko et al., 2003), we observed a significant reduction of F-actin level in HUVECs when Pfn1 expression was silenced thus implying that Pfn1 promotes actin polymerization in VECs. By contrast, a previous study had shown that gene deletion of Pfn1 and Pfn2 resulted in increased actin polymerization in *Dictyostelium* amoeba thus meaning Pfn's function as G-actin sequestering proteins in this organism (Haugwitz et al., 1994). Cell-specific difference in Pfn1's net action on actin cytoskeleton is not surprising since whether Pfn1 would function as a promoter of actin polymerization or a G-actin sequester depends on its concentration relative to that of available G-actin and free barbed ends of actin filaments. These parameters are controlled by other ABPs (sequestering, severing and capping), expression of which can vary between different cell types. Also, in mammalian cells, the intracellular concentration of Pfn1 does not appear to be sufficient for G-actin sequestration, which is primarily regulated by proteins belonging to the thymosin-ß family. From reduced actin stress-fibers in Pfn1-deficient cells, it is not immediately clear whether lack of Pfn1 inhibits only actin polymerization or affects the bundling of actin stress-fibers as well. It is known that Diaphanous-family proteins, such as mDia1, utilize Pfn1 to polymerize actin to form stress-fibers (Romero et al., 2004), which might partly explain reduced actin stress-fibers found in Pfn1-deficient cells. Bundling of actin stress-fibers, on the other hand, is facilitated by cell-contractility that requires actomyosin interactions. Pfn1-depletion may down-regulate VEC-contractility by partially suppressing actin polymerization [also shown previously for smooth muscle cells (Tang and Tan,

2003)] and therefore affect the bundling of actin stress-fibers. Consistent with the loss of actin stress-fibers, silencing Pfn1 expression also suppressed FA assembly in HUVECs. This data seems to be in qualitative agreement with previous findings by our laboratory and others where Pfn1-overexpression caused increased substrate-adhesion of breast cancer cells (Roy and Jacobson, 2004) and human aortic VECs (Moldovan et al., 1997). FA formation is initiated through integrin clustering, a process that is driven by both ECM-binding and contractility (Schoenwaelder and Burridge, 1999). Further assembly of FAs involves signaling through FAK (Focal adhesion kinase) and Src that recruit other molecular components to this adhesive structure. Integrin clustering can be diminished in Pfn1-deficient cells because of possible down-regulation of cell contractility. Surface recruitment of integrins can also be affected by Pfn1-dependent changes in cytoskeletal organization as postulated earlier (Moldovan et al., 1997). Furthermore, we earlier showed that Pfn1-overexpression up-regulates tyrosine phosphorylation of FAK and paxillin in breast cancer cells thus suggesting dependence of FAK-signaling on Pfn1's function (Roy and Jacobson, 2004). Thus, recruitment of molecular components to FA may also be influenced by altered FAK signaling in Pfn1-deficient VECs.

In previous studies, a common strategy is to use overexpression of Pfn1 mutants in a dominant-negative fashion. A general drawback of dominant negative approach is that one has to express a given mutant in large molar excess compared to the endogenous protein in order to assure dominant negative action and this could potentially result in experimental artifacts due to hyper-functionality of other ligand interactions which are not targeted by the mutation. In the present study, we for the first time have used a “knockdown and knock-in” strategy to evaluate the effect of expressing specific ligand-binding deficient mutants of Pfn1 in a near-null

endogenous background on lamellipodial dynamics, migration and capillary morphogenesis of VEC, and these are the novel aspects of this study.

3.2.2 Pfn1 and VEC Migration

We found significant inhibition of VEC migration as a result of loss of Pfn1 expression thus supporting our hypothesis that Pfn1 is an important player of VEC migration. The only previous study that directly evaluated how lack of Pfn1 affects the overall cell migration was performed in *Dictyostelium* and thus, the present work is the first demonstration of the effect of loss of Pfn1 function on the overall migration of any mammalian cell. In *Dictyostelium*, deletion of both Pfn1 and Pfn2 genes resulted in impaired cell motility, and knocking out Pfn1 gene alone failed to produce a phenotype because of functional compensation by Pfn2 gene product (Haugwitz et al., 1994). Since no Pfn2 expression was detected in HUVECs, we were able to see progressive inhibition of VEC migration with loss of Pfn1 expression.

The finding that both actin and polyproline interactions of Pfn1 are required for VEC migration is not surprising. Pfn1 was also found essential for migration in other organisms/cells, like *Dictyostelium* amoebae, *Drososphila*, and zebrafish (Haugwitz et al., 1994; Lai et al., 2008; Verheyen and Cooley, 1994). However, only few indirect studies have shown that the involvement of Pfn1's interaction with its ligands in cell migration. For example, the interaction between VASP and Pfn1 was suggested dispensable for cell migration (Loureiro et al., 2002). Loss of Pfn1 expression, but not Pfn2, synergistically inhibits gastrulation cell movement with loss of diaphanous-related formin 2 in zebrafish (Lai et al., 2008). The current study lays the foundation for future study of how ligand-interaction of Pfn1, especially how specific polyproline ligands coordinately regulate VEC migration with Pfn1.

3.2.3 Pfn1 and VEC Membrane Protrusion

We showed that loss of Pfn1 causes defects in membrane protrusion of VEC. This data is consistent with previous studies, where a model study of actin-based pathogen intracellular movement, which is a molecular mimicry of this actin-based protrusion of migrating cells, suggests Pfn1's involvement in this process (Grenklo et al., 2003; Mimuro et al., 2000; Theriot et al., 1994). Interestingly, a listeria-motility study showed that although Pfn1 increases the efficiency of listeria-induced actin polymerization and hence, the velocity of pathogen movement, it is not one of the essential cellular components needed for initiating motility of pathogens (Loisel et al., 1999). Similarly, our experiments showed that Pfn1-deficient cells are still able to protrude and spread, but clearly not to the same extent as displayed by the control cells. Conversely, a recent study showed overexpression of Pfn1 through protein transduction induces lamellipodia formation in bovine trabecular meshwork cells in a growth factor independent manner (Syriani et al., 2008).

We have also shown that abolishing either actin or polyproline interaction of Pfn1 leads to reduced velocity of lamellipodial protrusion in VEC. The effect of disrupting either actin or polyproline interaction of Pfn1 on lamellipodial protrusion is very similar to that of silencing the overall Pfn1 level in VEC, which suggests Pfn1 must interact simultaneously with actin and proline-rich ligands to generate efficient membrane protrusion. Our results with H119E mutant of Pfn1 is consistent with previous studies that showed cdc42/N-WASP-induced actin microspike formation, Rac-induced membrane ruffles, intracellular propulsion speed of bacterial pathogens (an indirect assessment of velocity of protrusion), and neurite outgrowth can be suppressed by disrupting Pfn1-actin interaction (Lambrechts et al., 2006; Mimuro et al., 2000; Suetsugu et al., 1998). Since the H119E substitution does not affect Pfn1's binding to any of the

proline-rich regulators of actin polymerization at the leading edge (example: VASP, WASP), the H119E mutant leads to slower protrusion of VEC most likely by trapping of these proline-rich proteins. Disruption of polyproline interactions of Pfn1, on the other hand, has been shown to have different responses on the generation of actin-based protrusion depending on the context. Overexpression of H133S mutant of Pfn1 dramatically inhibited intracellular movement of bacterial pathogens based on which it was postulated Pfn1's interaction with proline-rich ligands might be important for actin-based protrusion (Mimuro et al., 2000), and our present finding is consistent with this pathogen data. These results are further supported by existing biochemical data which show that the rate of F-actin elongation by some of the major proline-rich actin regulators is enhanced in the presence of Pfn1 (Barzik et al., 2005). Interestingly, neurite outgrowth, a process that is also driven by actin polymerization, was found to be actually facilitated when polyproline interaction of Pfn1 was abrogated by overexpression of W3A mutant (Lambrechts et al., 2006). Although the reason for this apparent discrepancy between the different studies is not clear, a few possibilities should be considered. First, a simple explanation could be that Pfn1's action on actin cytoskeleton can be cell-specific. This is not completely unlikely since *Dictyostelium* amoebae tend to have an increase in their F-actin content upon genetic deletion of Pfn1 while other mammalian cells including VEC show reduced F-actin after Pfn1 depletion. Second, the extent of actin-trapping by overexpression of polyproline mutant of Pfn1 and the resulting effect on actin cytoskeleton should be a function of the level of overexpression of the mutant, a factor which is likely to vary between different studies. Third, the two different polyproline-deficient mutants of Pfn1 may have subtle difference in their phospholipid binding, and particularly in an overexpression-based setting this can have a major influence on the functional status of Pfn1 if one considers phospholipid binding to be a critical

regulator of Pfn1 function. The last two points further exemplify the necessity of evaluating the effects of Pfn1-mutants at a close-to physiological level of expression which can be achieved in a "knockdown and knock-in" experimental system, as adopted in the present study.

Which of the proline-rich ABPs might cooperate with Pfn1 to regulate actin dynamics at the leading edge of VEC? It was previously shown that deleting polyproline domain of VASP, a region which binds to Pfn1, has no effect on actin cytoskeleton and membrane ruffling of HUVEC (Price and Brindle, 2000). Pfn1-VASP interaction was also found to be dispensable for fibroblast motility (Loureiro et al., 2002). WAVE, a proline-rich protein belonging to WASP family, utilizes Pfn1 to induce Rac-dependent actin clusters at the leading edge in fibroblasts (Miki et al., 1998). Whether Pfn1-WAVE interaction is one of the key interactions in driving lamellipodial protrusion and overall migration of VEC remains to be investigated in future studies.

3.3 CONCLUSION

Loss of Pfn1 expression causes a major change in actin cytoskeleton in VEC. Particularly, there is a significant depletion of actin filaments and focal adhesions in VEC when Pfn1 expression is silenced. Silencing Pfn1 expression also significantly impairs the migratory ability of VEC. Analyses of leading edge dynamics revealed that Pfn1 depletion results in decreased velocity and frequency of lamellipodial protrusion. Further experiments with point-mutants of Pfn1 showed that both actin and polyproline interactions of Pfn1 are required for lamellipodial protrusion and overall migration of VEC.

4.0 THE ROLE OF PROFILIN-1 IN CAPILLARY MORPHOGENESIS

This specific aim tests an overall working postulate that **loss of Pfn1 function impairs capillary morphogenesis of VEC (Working postulate #2).** Specifically, I will determine whether loss of Pfn1 function impairs:

a) Ability of VEC to form capillary-like structures *in vitro*.

b) ECM invasion of VEC.

c) VEC proliferation.

Some of the contents in this chapter have been or will be published in the following publications:

Ding, Z., Lambrechts, A., Parepally, M. and Roy, P. (2006). Silencing profilin-1 inhibits endothelial cell proliferation, migration and cord morphogenesis. *J Cell Sci* **119**, 4127-37.

Ding, Z., Gau, D., Deasy, B., Wells, A. and Roy, P. (2009) Both actin and polyproline interactions of profilin-1 are required for vascular endothelial cell migration and capillary morphogenesis. *(under review)*

4.1 RESULTS

4.1.1 Silencing Pfn1 Expression Inhibits Cord Formation by HUVEC

To determine whether Pfn1 plays any role in the early morphogenetic events of VEC, we examined the effect of silencing Pfn1 on matrigel-induced cord formation of VEC. HUVECs bearing the control siRNA started spreading as early as 1 hour after seeding on matrigel (data not shown) and formed prominent cord-like structures by 8 hours (Figure 12A). However, cord formation was significantly inhibited when Pfn1 expression was silenced as evident from the round morphology of majority of Pfn1-deficient cells at the indicated time-point. Quantitative analyses showed that early cord morphogenesis was inhibited by nearly 92% due to loss of Pfn1 expression (Figure 12B, data summarized from four independent experiments). Even at later time-points (18-22 hours after cell-seeding), we also found the number of cords formed by Pfn1-deficient cells to be still significantly less than that formed in the control culture (data not shown). These data suggest that Pfn1 is a key player in ECM-induced cord morphogenesis of VEC.

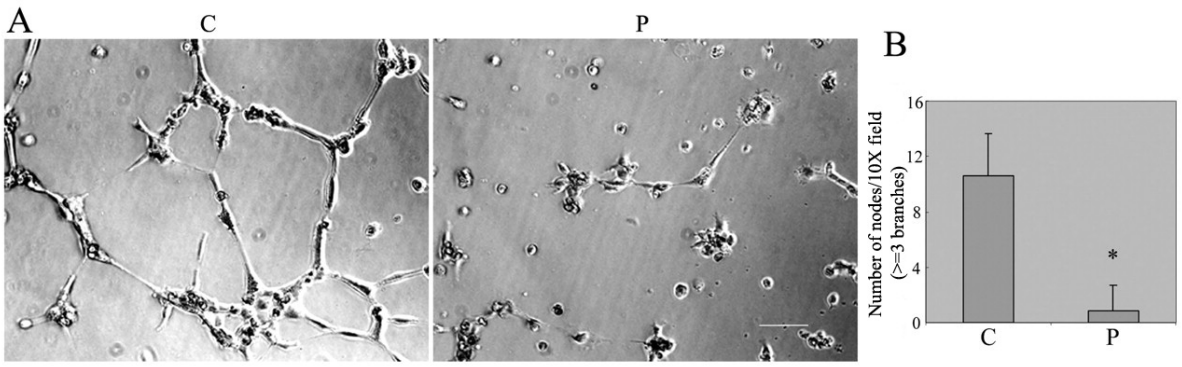

Figure 12. *Effect of Silencing Pfn1 on Early Cord Morphogenesis of HUVECs*

(A) Control HUVECs (denoted by "C") form prominent cord-like structures on polymerized matrigel by 8 hours after plating. Cord formation is significantly inhibited in the case of Pfn1-deficient cells (denoted by "P"). Bar, 100 µm. **(B)** A bar graph shows significantly higher number of nodes involving at least three branches in the control cells compared with the same in Pfn1-deficient cells per 10X field (the graph summarizes data from a total of four independent experiments; the asterisk indicates $P<0.001$).

4.1.2 Both Actin and Polyproline Interactions of Pfn1 Are Required For Cord Formation by HmVEC-1

To identify the roles of actin and polyproline interactions of Pfn1 in regulating VEC cord formation, we next evaluated the sensitivity of capillary morphogenesis of HmVEC-1 to disruption of ligand interactions of Pfn1 in a matrigel-induced planar cord-formation assay. Figure 13A depicts the representative cord formation by the different group of cells. To quantitatively represent the difference in cord forming ability between the various cell lines, we measured the total cord length/10X field of observation for each cell line, and these data are summarized in the form of a box and whisker plot in Figure 13B. Our data shows that the average cord length /field of GFP expressers bearing control siRNA (=5347±681 μm) is significantly higher than the value scored for the same cells in a Pfn1-depleted condition (=3136±972 μm), and this data is consistent with our previous observation with HUVEC (Ding et al., 2006). No statistically significant difference was found between the average cord length of GFP-Pfn1 re-expressers (=4941±1327 μm) and that of control GFP cells thus demonstrating that re-expression of GFP-Pfn1 can rescue cord morphogenesis defect of HmVEC-1 cells caused by Pfn1 depletion. However, the average cord lengths of both GFP-Pfn1-H119E (=3327±701 μm) and GFP-Pfn1-H133S (=2473±488 μm) were found significantly less compared to that of GFP-Pfn1 re-expressers. These data show that both actin and polyproline interactions of Pfn1 are indispensable for cord-morphogenesis of VEC.

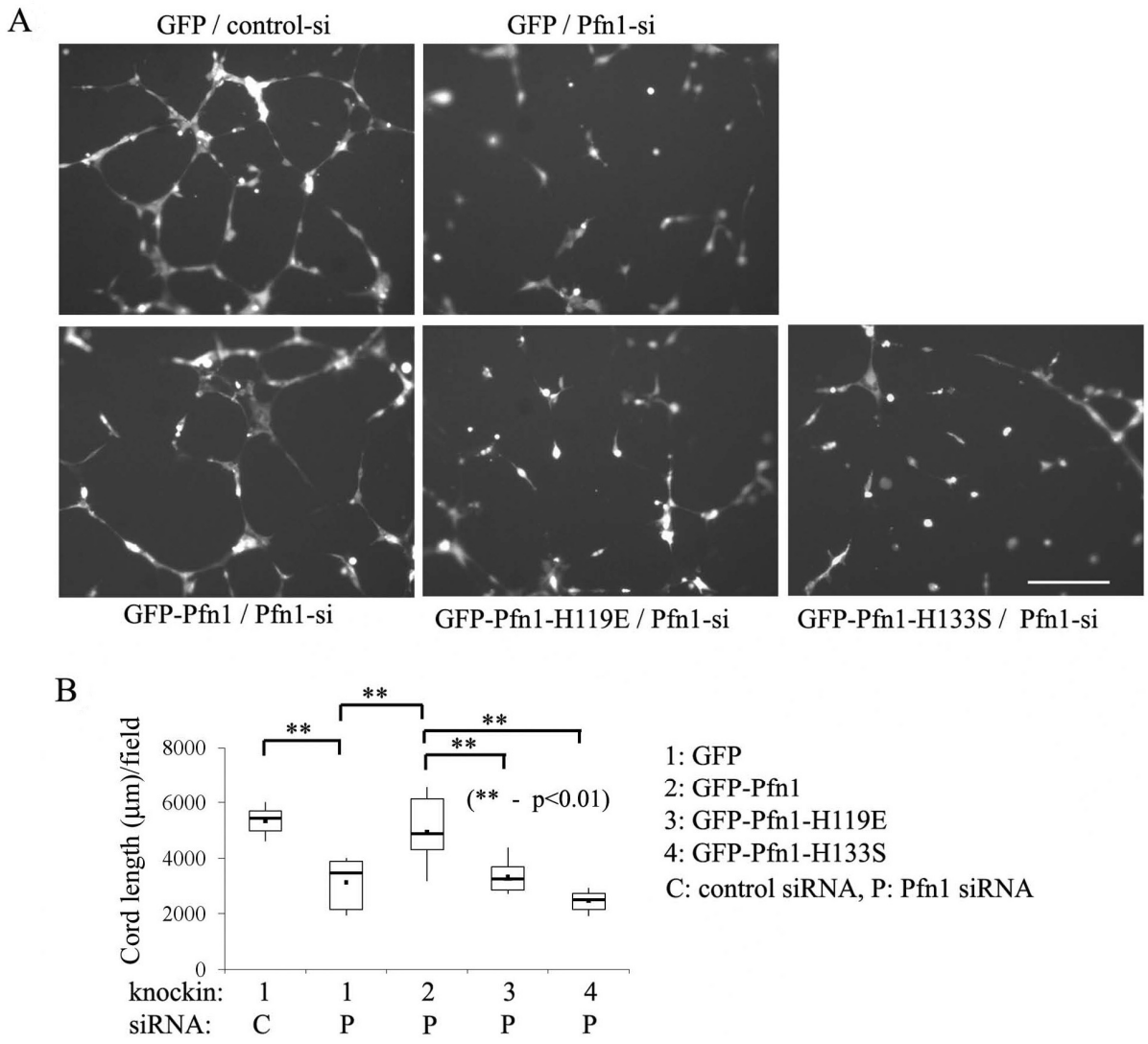

Figure 13. *Both Actin and Polyproline Interactions of Pfn1 Are Required For Cord Formation by HmVEC-1*

(A) Representative images of matrigel-induced cord formation by different groups of cells at 8 hrs after cell-seeding. **(B)** A box and whisker plot summarizing the cord morphogenesis data from a total of 2-3 independent experiments. (** indicates $P<0.01$).

4.1.3 Both Actin and Polyproline Interactions of Pfn1 Are Required For HmVEC-1 Spreading on Matrigel

VEC initially need to spread and then elongate to form cord-like structures on matrigel. It was apparent from Figure 13A that a significant fraction of HmVEC-1 displayed round morphology (suggesting spreading defect) when Pfn1 function was inhibited by either siRNA treatment or expression of the mutants. Since the images shown in Figure 13A represent end-point assessments, we also examined the spreading behavior of these different groups of cells in a time-course fashion within the first 4 hours after cell-seeding on matrigel, the results of which are shown in Figure 14. While for all experimental groups, there was a general trend of increase in % of spread cells (identified by elongated morphology) as a function of time, inhibiting Pfn1 function, either through silencing the endogenous expression or disrupting actin and polyproline interactions, clearly led to much reduced spreading efficiency when compared to control GFP or GFP-Pfn1 re-expressers at all time-points of evaluation. These data suggest that both actin and polyproline interactions of Pfn1 are required for VEC spreading.

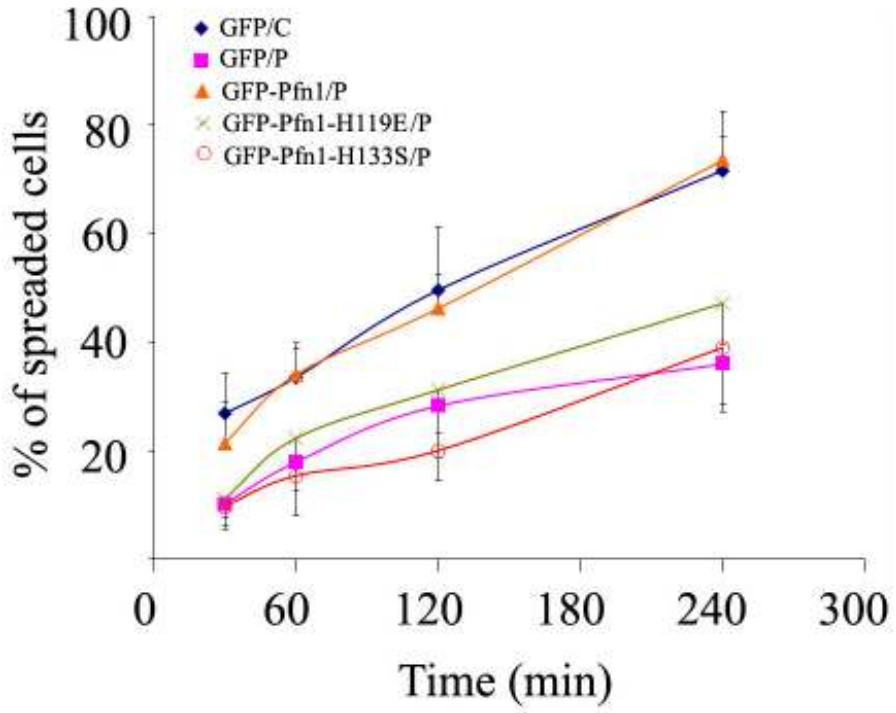

Figure 14. *Both Actin and Polyproline Interactions of Pfn1 Are Required For HmVEC-1 Spreading on Matrigel*

A line graph compares the relative spreading ability of different groups of cells at different time-points after seeding on matrigels. Data here are summarized from a total of two independent experiments with a duplicate set of samples for each experimental condition. (C: control siRNA; P: Pfn1-siRNA)

4.1.4 Loss of Pfn1 Expression Inhibits 3-D Morphogenesis of VEC

Capillary morphogenesis of VEC needs a 3-D environment for cells to execute a series of processes including spreading, migration, invasion, proliferation, and re-establishing cell-cell junctions. Pfn1 has been shown to be dramatically up-regulated in VECs during 3-D capillary morphogenesis (Salazar et al., 1999), however, whether Pfn1 is required for this process is unknown. To understand clearly whether Pfn1 plays a role in VEC morphogenesis in 3-D culture, an established 3-D collagen gel assay was performed (Salazar et al., 1999). After mixing either control or Pfn1-deficient HUVECs with 2.5 mg/ml collagen I gel, 72 to 96 hours were allowed for both cells to develop tube-like structures. After 96 hours, rhodamine-phalloidin staining of cells in 3-D culture, as shown in Figure 15A, demonstrates that HUVEC transfected with control siRNA form prominent network of capillary-like structures as expected. However, Pfn1 depletion severely inhibits capillary morphogenesis of HUVEC as judged by significant reduction in both number and length of capillary-like structures observed in this culture. This difference is quantitatively represented by a box and whisker plot in Figure 15B which shows that total length of capillary-like structures/ 10X field of observation formed by control siRNA treated cells (=3438 ± 546 μm) was nearly 2-fold greater than the same scored for Pfn1-depleted cells (=1820 ± 602 μm) thereby establishing that Pfn1 plays an indispensable role in 3-D capillary morphogenesis of VEC.

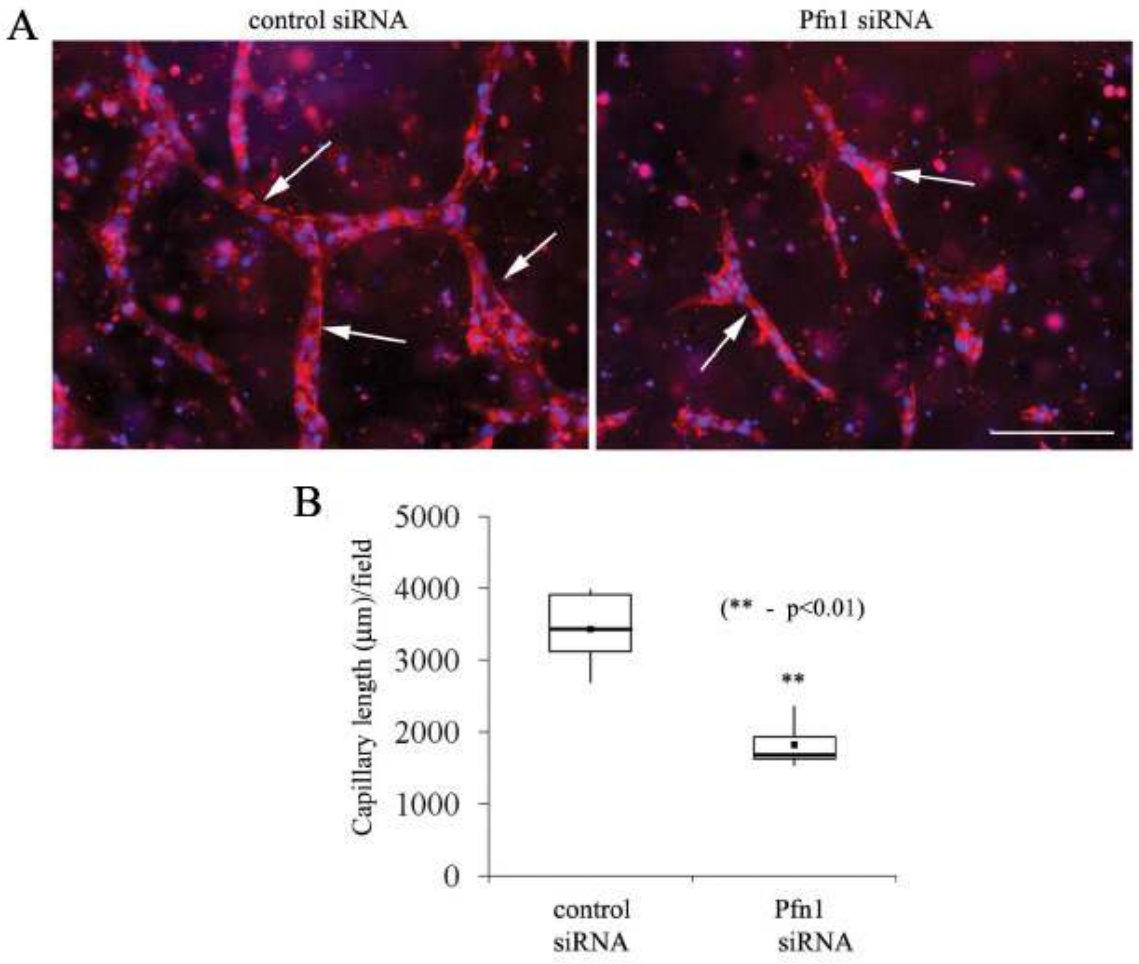

Figure 15. *Loss of Pfn1 Expression Inhibits 3-D Morphogenesis of VEC*

(A) Capillary formation (arrows) by control and Pfn1-siRNA treated HUVEC within collagen matrix (blue: DAPI, red: rhodamine-phalloidin) (scale bar – 200 μm) **(B)** A box and whisker plot comparing the mean values of total capillary length per 10X field of observation between the two transfection conditions (n=2 experiments). (** indicates $P<0.01$).

4.1.5 Pfn1 Plays an Important Role in VEC Invasion

Capillary morphogenesis requires activated VECs to invade both basement membrane and the underlying collagen matrix. VEC invasion involves cell motility and proteolytic degradation of ECM components. I utilized time-lapse imaging to track individual cell invasion in a 3-D collagen I gel. Both control and Pfn1-deficient HUVECs were allowed 72 to 96 hours to invade through 3-D matrix made of collagen I. A box and whisker plot in Figure 16 shows that the loss of Pfn1 expression is associated with a 37% decrease in the average speed of HUVEC invasion through collagen matrix, thus suggesting Pfn1 plays an important role in VEC invasion.

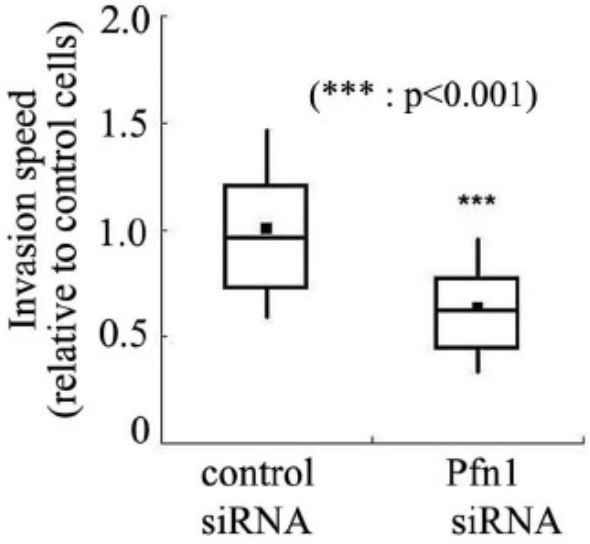

Figure 16. *Pfn1 Plays an Important Role in VEC Invasion*

A box and whisker plot showing the relative invasion speed of control and Pfn1-siRNA treated HUVEC through collagen I gel. These data are based on analyses of 86 control and 70 Pfn1-deficient cells pooled from 2 independent experiments. (*** indicates $P<0.001$).

4.1.6 Silencing Pfn1 Expression Inhibits VEC Proliferation

Since proliferation is an important part of VEC morphogenesis, Pfn1's role in regulating VEC proliferation was explored. First, growth curves of both control and Pfn1-deficient VECs were obtained. When I compared their growth at different time-points (24-72 hours) after replating both into sparse culture (to avoid contact inhibition), I found that Pfn1-deficient VECs exhibit a significant 40% inhibition in growth when compared to control cells (Figure 17A). Additionally, I performed DAPI staining of cells which revealed absolutely normal nuclear morphology (meaning lack of apoptosis) in either treatment groups (Figure 17B). To further the line of inquiry, I next performed flow cytometry-based analyses of cell cycle which showed that Pfn1-deficient VECs have a significant 10% higher fraction of cells in G1 phase when compared to control cells (Figure 17C). These data were further corroborated with immunoblot analyses that showed decreased cyclin D1 (a molecular marker of G1 progression) level in VECs associated with loss of Pfn1 expression (Figure 17D). These observations suggest that loss of Pfn1 expression does not affect VEC survival, at least, in the short-term, and further confirm that slower growth of Pfn1-deficient VECs is strictly due to their lower proliferative capacity.

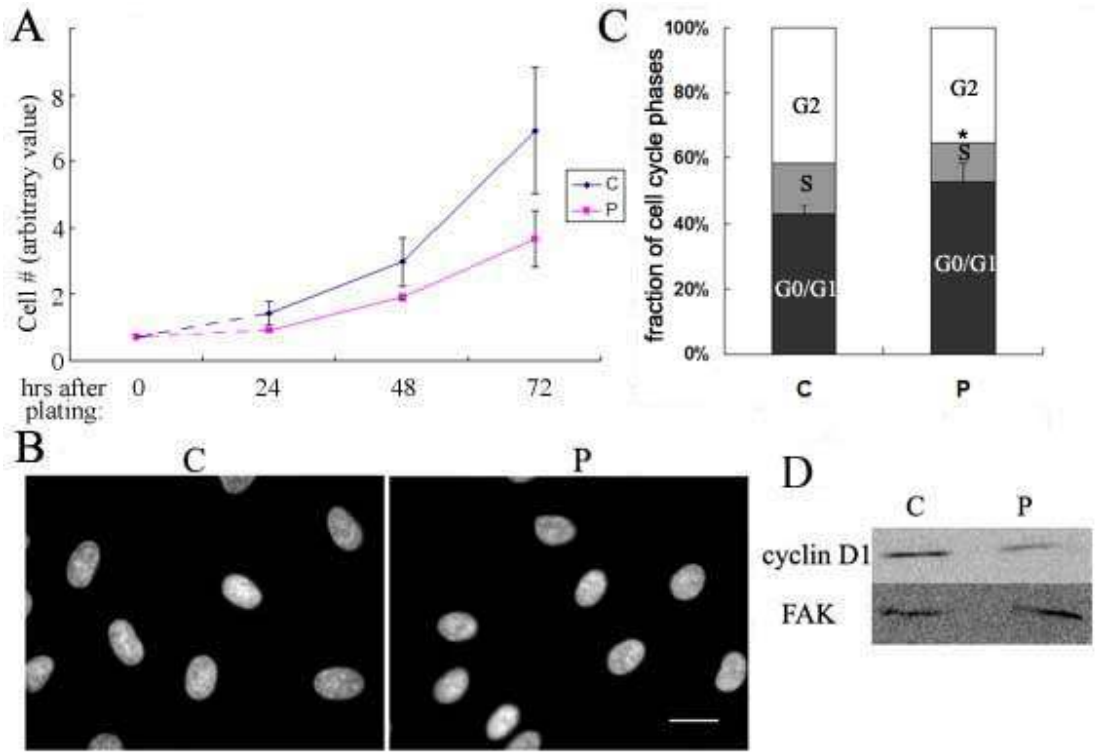

Figure 17. *Silencing Pfn1 Expression Inhibits VEC Proliferation*

(A) A line graph shows the growth curves of control (denoted by "C") and Pfn1-deficient cells (denoted by "P") in culture at 24, 48, and 72 after replating both cells for the assay. **(B)** DAPI staining of cells shows normal nuclear morphology under either experimental condition. Bar, 20 μm. **(C)** Flow cytometry shows significantly more cells in G0/G1 in Pfn1-deficient cells compared with control cells. (* indicate $P<0.05$) **(D)** Immunoblot of cyclin D1 shows a decreased expression in the Pfn1-deficient cells in subconfluent condition.

4.1.7 Loss of Pfn1 Expression Up-regulates p27 ^{Kip1} Expression

Based on my previous evidence of higher fraction of cells in G1 phase in Pfn1-deficient cells, I performed experiments to determine whether the expression level of p27 ^{Kip1}, a major cyclin-dependent kinase inhibitor (CDKI) at G1-to-S phase, is up-regulated as a result of silencing Pfn1 expression. The immunoblot data showed that p27 ^{Kip1} level is significantly higher in Pfn1-deficient VECs in both sparse and confluent culture conditions (Figure 18A). To further determine the sub-cellular distribution of p27 ^{Kip1}, I performed immunostaining which showed that Pfn1-deficient VECs not only display an overall increase, but also have higher nuclear staining of p27 ^{Kip1} compared to control cells (Figures 18B, C - note that nuclear localization of p27 ^{Kip1} inhibits CDK activity and progression to S-phase). These data suggest that Pfn1 up-regulates nuclear accumulation of p27 ^{Kip1}, which might inhibit G1-to-S phase progression of cell cycle in VEC.

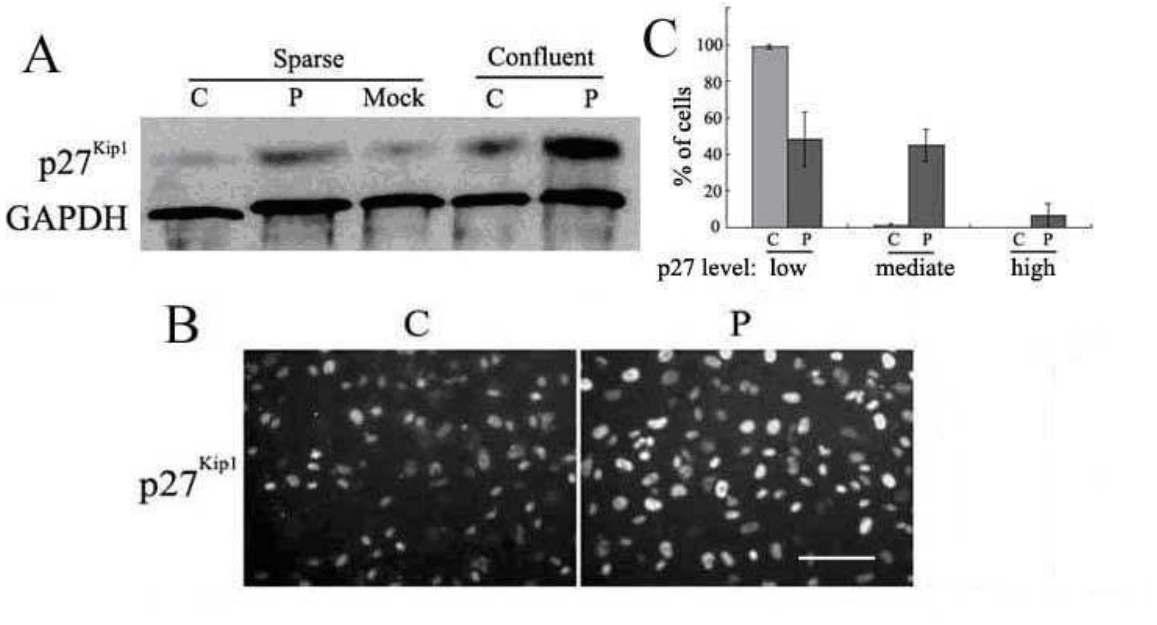

Figure 18. *Loss of Pfn1 Expression Up-regulates p27 Kip1 Expression*

(A) Western blot data shows increased expression of p27 Kip1 as a result of silencing Pfn1 in both sparse and confluent culture conditions. (C: control siRNA; P: Pfn1-siRNA) **(B)** Immunofluorescence staining of p27 Kip1 shows increased nuclear staining of p27 Kip1 in Pfn1-deficient VEC. Bar, 200 µm. **(C)** A histogram shows 97% of control VECs have low p27 Kip1 level in nuclei, while 42%, 49%, and 9% of Pfn1-deficient VECs have low, moderate, and high nuclear p27 Kip1 level, respectively.

4.1.8 Silencing Pfn1 Expression Inhibits HUVEC Scattering

Previous study showed that at least in epithelial cells, the up-regulation of $p27^{Kip1}$ level is the result of increased cell-cell adhesion mediated by E-cadherin (St Croix et al., 1998). Interestingly, we found our Pfn1-deficient VECs are significantly less scattered compared with control cells when plated sparsely in culture (Figure 19).

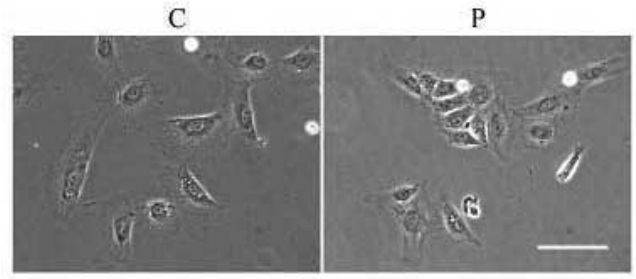

Figure 19. *Silencing Pfn1 Expression Inhibits HUVEC Scattering*

Representative phase contrast images shows morphology of sparsely-cultured control (denoted by "C") and Pfn1-deficient (denoted by "P") HUVECs. Bar, 100μm.

4.1.9 Silencing Pfn1 Expression Stabilizes VEC Adherens Junction

Cadherin-based adherens junction plays a negative role in cell scattering. Since my previous data showed significant less scattering of Pfn1-deficient VECs, I asked whether silencing Pfn1 expression suppresses the dynamics of adherens junction in VECs. To address this question, I challenged monolayer of HUVECs, bearing either control or Pfn1 siRNA, with vascular endothelial growth factor (VEGF, a potent disruptor of intercellular junctions). Immunostaining data showed that VEGF treatment induces a significant loss of junctional distribution of VE-cadherin (arrow) with concomitant formation of paracellular holes (arrowhead) in the control VEC monolayer as expected; however, VEGF-induced junctional disruption is completely inhibited in Pfn1-deficient VECs (Figure 20A). Further immunoblot data showed similar expression level for VE-cadherin between the two treatment groups (Figure 20B), which indicates Pfn1 stabilizes adherens junction not through regulating its expression level. In another experiment that a monolayer of control or Pfn1-deficient HUVECs was challenged with a specific VE-cadherin neutralizing antibody (BV9) targeting its extracellular region overnight. In control experiments a monolayer of control or Pfn1-deficient cells were incubated with same concentration of a species-matched control IgG. Immunostaining of VE-cadherin shows control IgG did not affect the distribution of VE-cadherin in both cells (Figure 20C). However, BV9 disrupted cell-cell junctions and induced significant loss of VE-cadherin at adherens junction in control cells, while BV9 only affects a very small part of the monolayer (Figure 20C). Overall, these data suggest Pfn1 plays a critical role stabilizing VEC adherens junction.

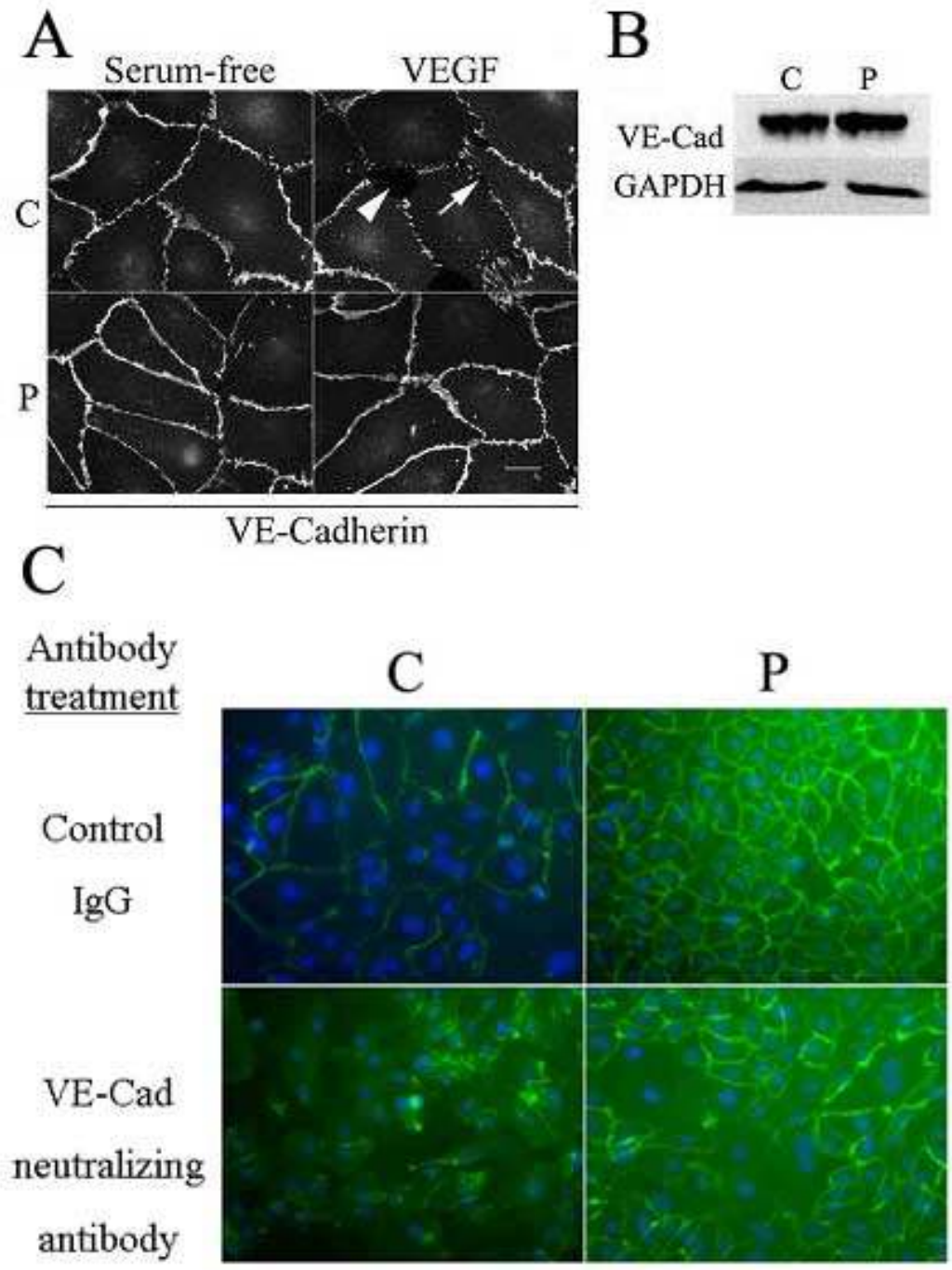

Figure 20. *Silencing Pfn1 Expression Stabilizes VEC Adherens Junction*

(A) VEGF-induced loss of intercellular junctional staining of VE-cadherin (arrow) and formation of paracellular holes (arrowhead) is inhibited in Pfn1-deficient VECs. Bar, 20 μm. **(B)** Immunoblot data show no appreciable difference of VE-cadherin expression between both

treatments. **(C)** Immunostaining shows VE-cadherin (green) and DAPI (blue) after overnight treatment of a VE-cadherin neutralizing antibody (BV9) and species-matched control IgG in both control (denoted by “C”) and Pfn1-deficient VECs (denoted by “P”).

4.1.10 Pfn1 Regulates Nuclear p27 ^{Kip1} Accumulation Secondary to Changes in Cell-cell Adhesion

To determine whether there is an association between increased stability of VE-cadherin and higher nuclear staining of p27 ^{Kip1} in Pfn1-deficient VECs, I carried out experiments where I treated VECs with either a VE-cadherin neutralizing antibody (BV9) or a species-matched, control IgG, and followed by immunostaining of sub-cellular distribution of p27 ^{Kip1}. As shown in Figure 21, in control VECs, the nuclear distribution of p27 ^{Kip1} completely disappears with concomitant loss of adherens junction specifically in response to BV9 treatment. Pfn1-deficient cells are still highly resistant to junctional disruption in response to BV9 treatment; however in those regions of the culture, where junctional disruption was observed there was a concomitant loss of nuclear staining of p27 ^{Kip1} (Figure 21, arrows). While control and Pfn1-deficient cells both maintained good VE-cadherin staining at cell-cell junctions with intact nuclear p27 ^{Kip1} staining in the treatment of control IgG. Overall, these data suggest Pfn1 regulates nuclear p27 ^{Kip1} accumulation secondary to changes in cell-cell adhesion.

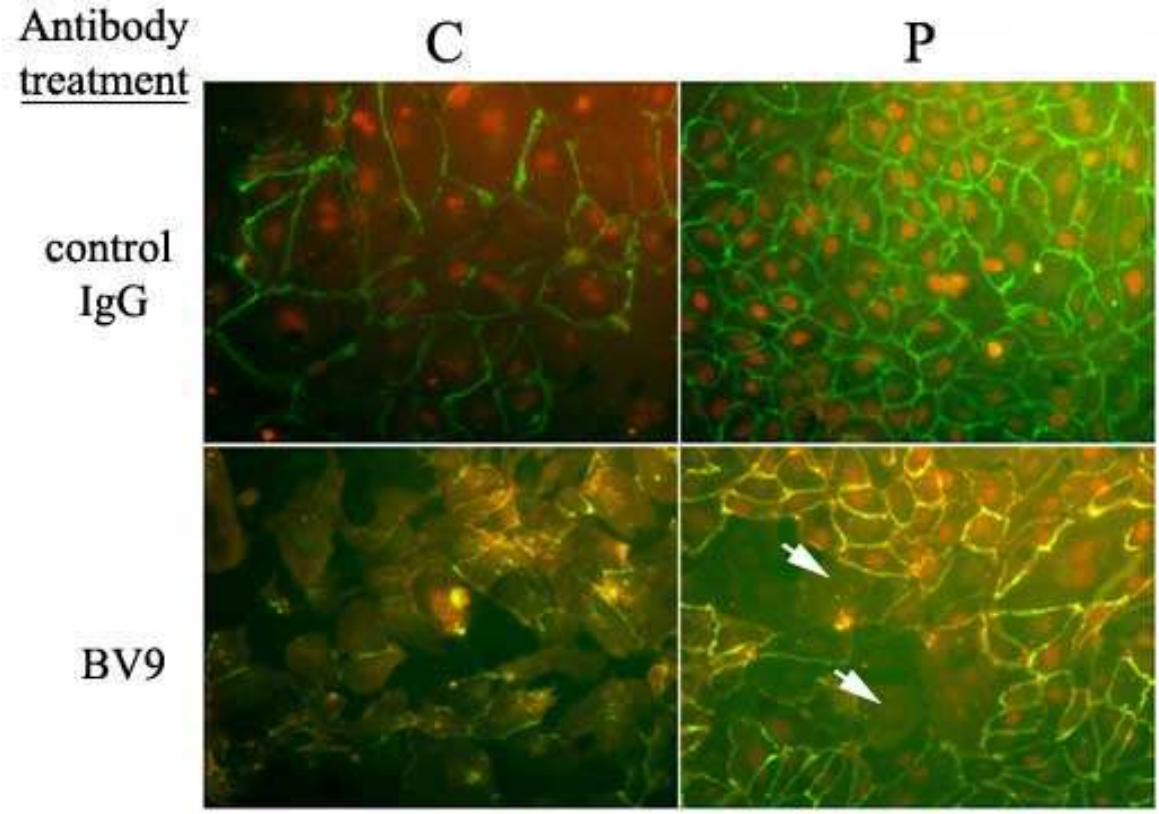

Figure 21. *Pfn1 Regulates Nuclear p27 ^{Kip1} Accumulation Secondary to Changes in Cell-cell Adhesion*

Immunostaining shows p27 ^{Kip1} (red) and VE-cadherin (green) after overnight treatment of BV9 and species-matched control IgG in both control (denoted by "C") and Pfn1-deficient VECs (denoted by "P"). Arrows indicate loss of nuclear p27 ^{Kip1} staining in Pfn1-deficient VECs with concomitant loss of VE-cadherin at cell-cell junctions in the treatment of BV9.

4.2 DISCUSSION

4.2.1 Pfn1 and Capillary Mophogenesis of VEC

We demonstrated that perturbing Pfn1 function significantly inhibits ECM-induced capillary morphogenesis of VECs. Network formation by VECs on ECM proceeds through several stages including (1) cell adhesion on ECM, (2) cell migration, (3) cell-induced mechanical remodeling of ECM that further defines matrix guidance tracks to allow directed VEC migration to the neighboring cells, and (4) cell proliferation (Cascone et al., 2003; Davis et al., 2002; Davis and Camarillo, 1995; Liu and Senger, 2004; Whelan and Senger, 2003). Early impairment in cord-forming ability of either Pfn1-deficient VECs or VECs with ligand-binding deficient Pfn1 is most likely a result of defect in cell spreading and migration, both of which we showed impaired as a result of perturbing Pfn1 function.

We observed less tube formation by Pfn1-deficient cells in 3-D collagen matrix 72~96 hours after cell seeding, in which case, however, additional contribution of inhibited cell proliferation cannot be ruled out. This data suggests that Pfn1's up-regulation in 3-D capillary morphogenesis published earlier is critical in the process (Salazar et al., 1999). VEC spreading and directed migration on ECM is not only influenced by the intrinsic protrusive and invasive ability of cells, but also governed by the ECM-remodeling capacity of cells. Reduced formation of actin stress-fibers and FAs might render Pfn1-deficient cells less efficient in ECM remodeling because of possible down-regulation of contractility. In conclusion, we found that Pfn1 plays an important role in capillary morphogenesis probably through both actin and polyproline interactions.

4.2.2 Pfn1 and VEC Proliferation

Loss of Pfn1 expression inhibits VEC growth by 42% without compromising cell survival, at least in the short-term, thus suggesting that Pfn1 plays an important role in VEC proliferation. Previous studies showed that Pfn1 localizes at the cleavage furrow during cytokinesis and its function is important for cleavage furrow regression (Dean et al., 2005; Suetsugu et al., 1999). Since Pfn1-deficient cells did not display multinuclei (>2) phenotype, our data suggests that Pfn1's function is not essential for cytokinesis of mammalian VECs as it is for some other species including yeast and amoeba (Haugwitz et al., 1994; Lu and Pollard, 2001). A recent study showed that in mouse chondrocytes disruption of Pfn1 gene causes defects in abscission during late cytokinesis (Bottcher et al., 2009). Thus, Pfn1's role in mammalian cell proliferation may be cell type dependent. The fact that we observed only a partial inhibition of VEC proliferation after near complete silencing of Pfn1 gene is intriguing since it has been shown that gene deletion of Pfn1 arrests developing mouse embryo at the two-cell stage and produces embryonic lethality (Witke et al., 2001). Although it is tempting to speculate that embryonic stem cells might be more sensitive to loss of Pfn1 expression, a more detailed work is necessary to address whether persistent suppression of Pfn1 expression completely arrests cell-growth and/or affects the long-term survival of VECs.

An interesting finding of the present study is that in addition to affecting cell-matrix adhesion, loss of Pfn1 expression also influences cell-cell adhesion where, in particular, VEGF-induced dynamics of intercellular junctions is suppressed. Previous studies showed that agonist-induced disruption of intercellular junctions requires Rho-based VEC contraction (Alexander and Elrod, 2002; Bates et al., 2002). If depletion of Pfn1 reduces VEC contractility as observed previously for smooth muscle cells (Tang and Tan, 2003), VECs will become resistant to VEGF-

induced disruption of cell-cell adhesion. It has been shown that activation of receptor tyrosine kinases, such as VEGF receptor, is stimulated by integrin clustering and cell-contraction (Gingras et al., 2000; Sundberg and Rubin, 1996). Thus, reduced contractility of Pfn1-deficient cells may also modulate the spatial distribution of VEGF-receptors and hence, suppress VEGF signaling by decreasing the receptor activation. In addition, our morphological analyses show that HUVECs lacking Pfn1 have a higher tendency to form clusters in a sub-confluent culture condition, which is probably also due to the stablized adherens junction as a result of loss of Pfn1 expression.

It is known that for at least epithelial cells, increased cadherin engagement leads to contact inhibition (a process by which proliferation of cells is arrested upon forming stable contacts with neighboring cells) (Kandikonda et al., 1996; Takahashi and Suzuki, 1996). It has been shown that p27 ^{Kip1} is abundant in quiescent cells (Coats et al., 1996), and its degradation is required for G1-to-S progression in mammalian cells (Sutterluty et al., 1999). E-cadherin-dependent contact inhibition is mediated by up-regulation of p27 ^{Kip1} that ultimately leads to cell-cycle arrest at G1 phase (St Croix et al., 1998). The present study also showed that p27^{Kip1} is significantly up-regulated, especially in VEC nuclei, probably through VE-cadherin engagement as a result of silencing Pfn1 expression, thus raising an intriguing possibility that a similar cadherin-dependent p27 ^{Kip1} up-regulatory mechanism might also be operative in Pfn1-depleted VECs.

In addition to its involvement in cell cycle, p27 ^{Kip1} has also been implicated in regulating Rho activity that is important for stress fiber formation and cell migration. It was reported in cytosol p27 ^{Kip1} can bind to RhoA and block its activation by its guanine-nucleotide exchange factors (GEFs) (Besson et al., 2004). So in our Pfn1-deficient VEC, up-regulated p27 ^{Kip1} might

also bind to RhoA and inhibit its activity. In fact, in Pfn1-deficient VEC fewer actin stress fibers were observed, which is consistent with the literature. Further experiments are needed to determine whether Pfn1's effect on actin stress fibers is $p27^{Kip1}$ dependent.

4.3 CONCLUSION

Loss of Pfn1 expression is associated with reduced dynamics of VE-cadherin dependent cell-cell adhesion, which was also found to be correlated with increased nuclear accumulation of $p27^{Kip1}$ (a major cell-cycle inhibitor) and reduced VEC proliferation. We found that loss of overall expression of Pfn1 significantly impairs collagen gel invasion and three-dimensional (3-D) capillary morphogenesis of VEC. Abolishing either actin or polyproline interaction of Pfn1 also leads a dramatic inhibition of capillary mophogenesis of VEC. Taken together, these results demonstrate that Pfn1 plays a critical role in capillary morphogenesis of VEC through its interactions with both actin and polyproline ligands.

5.0 CONCLUSIONS

In the present study, we showed that loss of Pfn1 expression causes a major change in actin cytoskeleton in VEC. Particularly, there is a significant depletion of actin filaments and focal adhesions in VEC when Pfn1 expression is silenced. Silencing Pfn1 expression also significantly impairs the migratory ability of VEC. Analyses of leading edge dynamics revealed that Pfn1 depletion results in decreased velocity and frequency of lamellipodial protrusion. Further experiments with point-mutants of Pfn1 showed that both actin and polyproline interactions of Pfn1 are required for overall migration of VEC. Loss of Pfn1 expression is associated with reduced dynamics of VE-cadherin dependent cell-cell adhesion, which was also found to be correlated with increased nuclear accumulation of $p27^{Kip1}$ (a major cell-cycle inhibitor) and reduced VEC proliferation. Finally, we found that loss of overall expression of Pfn1 significantly impairs collagen I gel invasion and 3-D capillary morphogenesis of VEC. Abolishing either of actin or polyproline interactions of Pfn1 also leads a dramatic inhibition of cord formation of VEC. Taken together, these results demonstrate that Pfn1 plays a critical role in capillary morphogenesis of VEC through its interactions with both actin and polyproline ligands (Figure 22).

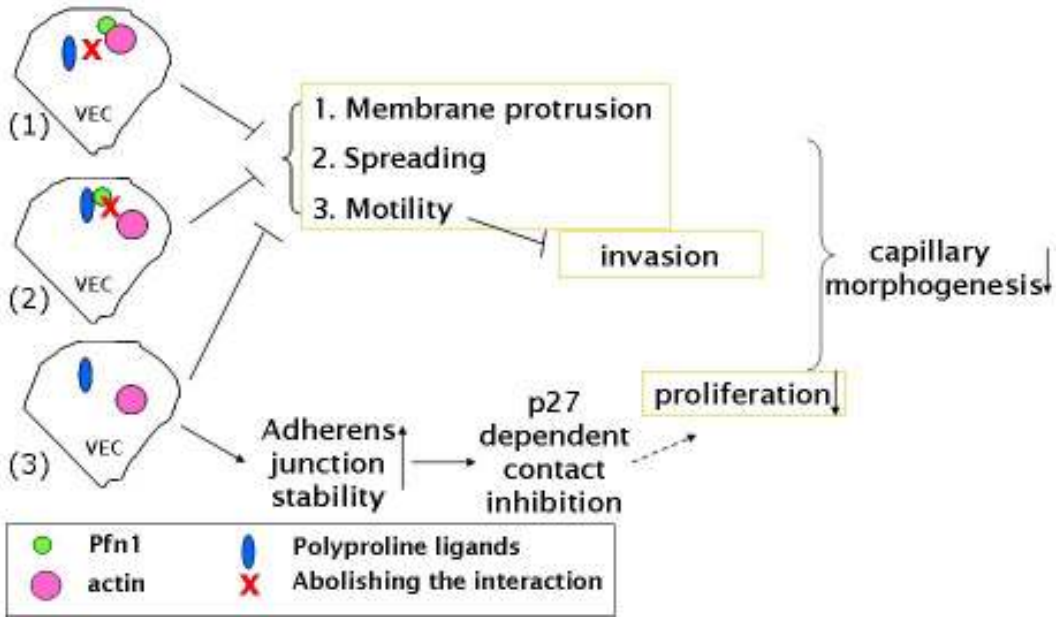

Figure 22. *Proposed Role of Pfn1 in Capillary Morphogenesis*

In VEC, abolishing Pfn1's interaction with either polyproline ligands (1) or actin (2) leads to impaired membrane protrusion, spreading, motility, and cord formation of VEC. Silencing Pfn1 expression in VEC (3) leads to impaired membrane protrusion, spreading, motility, and collagen I invasion. Silencing Pfn1 also results in VE-cadherin dependent $p27^{Kip1}$ up-regulation in VEC, which is associated with reduced cell proliferation. These effects of silencing Pfn1 in VEC might eventually contribute to compromised capillary morphogenesis. Taken together, these results demonstrate that Pfn1 plays a critical role in capillary morphogenesis of VEC through its interactions with both actin and polyproline ligands.

5.1 FUTURE DIRECTIONS

5.1.1 To Study Phospholipid-binding of Pfn1 in VEC Migration and Morphogenesis

In addition to interacting with actin and polyproline ligands, Pfn1 also has an ability to interact with phosphoinositides, such as phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$], and phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] (Lu et al., 1996). It is known that Pfn1 is able to inhibit $PI(4,5)P_2$ hydrolysis *in vitro* (Goldschmidt-Clermont et al., 1990), however, very little at best is known about in vivo significance of Pfn1's interactions with membrane phosphoinositides. A recent study from our laboratory showed that overexpression of Pfn1 in breast cancer cells dramatically inhibits growth factor induced generation of $PI(3,4,5)P_3$ (Das et al., 2009), which suggests Pfn1 might play a similar role regulating phosphoinositide metabolism in VEC. Since membrane phosphoinositides play important roles in signal transduction and regulating actin cytoskeleton, it will be very interesting to determine whether and how abolishing Pfn1's interaction with membrane phosphoinositides affects cell migration and VEC morphogenesis.

5.1.2 To Study Pfn1- Dependent Modulation of Angiogenesis *in Vivo*

Although the present study clearly demonstrates that Pfn1 plays a critical role in capillary morphogenesis of VEC in vitro, angiogenesis in vivo is a much more complex process involving multi-cellular interactions that cannot be faithfully represented in *in vitro* model systems. Therefore, it remains to be seen whether Pfn1 has a similar indispensable role in angiogenesis *in vivo* and this will be our immediate future direction of research. Several different strategies can

be adopted to evaluate Pfn1's role in angiogenesis *in vivo*. First, studying the effect of endothelial cell specific-silencing of Pfn1 expression on angiogenic outgrowth during vascular development in a zebrafish model system will be an ideal approach. If results from these studies are promising, one could translate those findings into a mammalian system by evaluating subcutaneously-implanted matrigel-induced angiogenic response in transgenic mice expressing dominant-negative Pfn1 constructs. If expressing dominant negative Pfn1 constructs appear to be embryonic lethal, an alternative approach could be subcutaneous injection of adenovirus carrying various dominant-negative Pfn1 constructs with matrigel in mice and evaluating the subsequent changes in host angiogenic response. If findings from these *in vivo* studies indicate indispensable role of Pfn1 in angiogenesis *in vivo*, one can conceive targeting Pfn1 as a potential anti-angiogenic strategy. It will further justify development and use of small molecule inhibitors of Pfn1 as possible therapeutic agents against diseases involving aberrant angiogenesis. It will be also interesting to know whether increasing Pfn1 function in endothelial cells has an augmenting effect on angiogenesis. This could pave the way for future interventional strategies in certain cardiovascular diseases.

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