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Abstract

Positive and Negative Modulatory Roles of Platelet Endothelial Cell Adhesion Molecule-1 signaling in endothelial cell migration: coordination of Rho signaling and targeting of SHP-2 tyrosine phosphatase activity

Dita Gratzinger

2003

Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), an immunoglobulin family adhesion molecule, is involved in endothelial migration and angiogenesis. I found that PECAM-1 deficient endothelial cells exhibit enhanced nondirected single cell motility and extension formation at the expense of wound-healing migration. These *in vitro* endothelial behaviors model aspects of highly developmentally and pathophysiologically relevant processes. Wound healing migration may model the reendothelialization of denuded vasculature following balloon angioplasty or the remodeling of vascular endothelium under variant flow conditions; single cell motility is prominently involved in developmental processes such as endocardial-mesenchymal transition within the cardiac cushion. A specific deficiency of RhoGTP in PECAM-1 deficient endothelial cells accounted for their migratory phenotype. The serum sphingolipid sphingosine-1-phosphate (S1P) drives Rho-dependent migration and angiogenesis. Indeed PECAM-1 proved necessary for an appropriate wound healing

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response to S1P. PECAM-1 localizes to rafts, and in its absence heterotrimeric G protein components are differentially recruited to rafts, providing a potential mechanism for PECAM-1-mediated coordination of S1P signaling.

PECAM-1 activates the tyrosine phosphatase SHP-2 via its phosphorylated immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIM signaling is not required for the positive Rho-activating role of PECAM-1. Instead I found that ITIM signaling retards wound healing migration, and that during migration the second ITIM tyrosine becomes selectively dephosphorylated. The second ITIM tyrosine proved important for recruitment but not activation of SHP-2. Any SHP-2 that remains transiently associated with partially dephosphorylated PECAM-1 could in theory amplify the migration signal by dephosphorylating adjacent PECAM-1 ITIMs. PECAM-1 also helps localize SHP-2 to the actin cytoskeleton, and to a subset of phosphoproteins. In fact, interfering with PECAM-1 ITIM signaling not only increased wound healing migration but decreased dephosphorylation of beta catenin and focal adhesion kinase. SHP-2 targeting is instrumental in controlling endothelial wound healing migration, potentially via effects on adherens junction stability and focal contact dynamics.

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Positive and negative modulatory roles of Platelet Endothelial Cell Adhesion Molecule-1 signaling in endothelial cell migration: coordination of Rho signaling and targeting of SHP-2 tyrosine phosphatase activity

A Dissertation

Presented to the Faculty of the Graduate School

Of

Yale University

In Candidacy for the Degree of

Doctor of Philosophy

by

Dita Gratzinger

Dissertation Director: Joseph A. Madri

Chairman of Dissertation Committee: David L. Rimm

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·» *My gratitude is always with my family and with my love JBF* «

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Abbreviations

ADP: adenosine diphosphate

ANOVA: analysis of variance

BAEC: bovine aortic endothelial cells

BD: binding domain

CS-FBS: charcoal-stripped fetal calf serum

CXCR: cysteine-x-cysteine motif-containing chemokine receptor

EDG: endothelial differentiation gene

EMT: epithelial mesenchymal transition

Eoma: murine endothelioma

ERK: extracellular signal regulated kinase

exoC3: *C. botulinum* exoenzyme C3

FACS: fluorescence activated cell sorting

F-actin: filamentous actin

FBS: fetal bovine serum

FGF-2: fibroblast growth factor-2

GAP: guanosine triphosphate phosphatase activating protein

GFP: green fluorescent protein

GST: glutathione *S*-transferase

GDP: guanosine diphosphate

GTP: guanosine triphosphate

HGF: hepatocyte growth factor, or scatter factor

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HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

hpf: high power field

HUVEC: human umbilical vein endothelial cells

IP: immunoprecipitation

ITIM: immunoreceptor tyrosine-based inhibitory motif

kD: kilodalton

LDL: low density lipoprotein

LMW-PTP: low molecular weight protein tyrosine phosphatase

LPA: lipophosphatidic acid

mßcd: methyl ß cyclodextrin

PECAM-1: platelet endothelial cell adhesion molecule-1

PecamKO: immortalized PECAM-1 knockout mouse lung endothelial cells

PecamRC: PecamKO cells reconstituted with full-length PECAM-1

S1P: sphingosine 1 phosphate

SDF-1: stromal derived factor 1

PAGE: polyacrylamide gel electropheresis

PAK: p21 activated kinase

PAR: protease activated receptor

PBS: phosphate-buffered saline

pNPP: *p* nitrophenyl phosphate

PTP-LAR: protein tyrosine phosphatase – leukocyte common antigen related

PTX: pertussis toxin

PY: phosphotyrosine

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r:	radius
ROCK:	Rho coiled coil kinase
ROS:	reactive oxygen species
RPTP:	receptor-type protein tyrosine phosphatase
SDF-1:	stromal derived factor-1
SDS:	sodium dodecyl sulfate
STAT:	signal transducer and activator of transcription
TBS:	Tris-buffered saline
VCAM:	vascular cell adhesion molecule
VEGF:	vascular endothelial growth factor
Y27632:	synthetic inhibitor of p160ROCK

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Chapter 1: Background

PECAM-1 signaling in vascular biology

Platelet Endothelial Cell Adhesion Molecule-1, or PECAM-1, is a 130 kD type I transmembrane adhesion molecule expressed on endothelial cells, platelets, neutrophils, monocytes, and subsets of T cells (Albelda et al., 1991; Albelda et al., 1990; Newman et al., 1990). Although PECAM-1 knockout (KO) mice are viable, fertile, and overtly developmentally normal (Duncan et al., 1999), abnormalities of vascular functioning become apparent in response to certain insults. Aberrant endothelial cell-platelet thrombus interactions result in prolonged bleeding times attributable to endothelial rather than platelet dysfunction (Mahooti et al., 2000). In a murine model of multiple sclerosis (experimental autoimmune encephalitis) PECAM-1 KO mice exhibit neurologic deficits early, and infiltration of their central nervous system by mononuclear cells is similarly accelerated (Graesser et al., 2002). This abnormality too localizes to the vasculature rather than the bone marrow; indeed these mice have a defect in restoration of junctional integrity in response to a permeability-promoting stimulus.

In fact even angiogenesis is impaired in the PECAM-1 knockout mouse under certain conditions. PECAM-1 deficient endothelial cells display poor tube formation in three dimensional collagen culture. Moreover, a markedly diminished vascular infiltration into a sterile polyvynil acetate sponge is seen in the PECAM-1 knockout mouse during the chronic phase of a model of foreign body inflammation (Solowiej et al.,

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2002). PECAM-1 in fact plays multiple roles in vascular endothelial biology. Anti-PECAM-1 antibodies block endothelial wound healing migration, whereas expression of PECAM-1 mutated in its immunoreceptor tyrosine based inhibitory motif enhances endothelial sheet migration (Cao et al., 2002; Kim et al., 1998). Treatment with antibodies against PECAM-1 inhibits both endothelial tube formation and angiogenesis (Cao et al., 2002; DeLisser et al., 1997).

PECAM-1 has roles to play in the physiology of established vasculature as well. Both anti-PECAM-1 treated wildtype endothelium and PECAM-1 deficient endothelium has a defect in permeability to macromolecules (Ferrero et al., 1995; Graesser et al., 2002). PECAM-1 may even play a role in regulation of vascular tone, with endothelial PECAM-1 engagement mediating vasoactive mediator release, which may then act on subjacent vascular smooth muscle cells (Gurubhagavatula et al., 1998). In the area of leukocyte-endothelial interaction as well PECAM-1 has a role to play in leukocyte adherence to (Chiba et al., 1999; Piali et al., 1995; Tanaka et al., 1992) and transmigration across (Graesser et al., 2002; Gumina et al., 1996; Vaporciyan et al., 1993) vascular endothelium.

The PECAM-1 extracellular domain consists of six immunoglobulin-like repeats (**Figure 1**) and can mediate homophilic, and perhaps heterophilic, binding interactions (Baldwin et al., 1994). The heterophilic binding partner for PECAM-1 remains unclear; after initial reports of interaction with glycosaminoglycans (Vaporciyan et al., 1993), the model of PECAM-1 as a heparin-binding protein has been effectively ruled out (Sun et al., 1998). Interaction with CD38, an adenosine diphosphate ribosyl cyclase, (Deaglio et al., 1998) has also been suggested, although no further work has been published to

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confirm this. PECAM-1 integrin heterophilic interactions, on the other hand, appear to be real, if indirect; they generally involve an initial outside-in signaling step experimentally induced by PECAM-1 engagement accompanied by tyrosine phosphorylation of the cytoplasmic domain, followed by inside-out signaling in the form of integrin activation (increased adhesive activity). This model holds for several families of integrins including T cell ß1 integrins (Tanaka et al., 1992), natural killer cell β2 integrins (Berman et al., 1996), and endothelial αvβ3 (Chiba et al., 1999).

The cytoplasmic domain of PECAM-1 has no intrinsic enzymatic activity but instead associates with several signaling molecules including the tyrosine phosphatase SHP-2, beta catenin, gamma catenin or plakoglobin, and STAT family proteins (**Figure 1**). The 106 residues of the PECAM-1 cytoplasmic domain are encoded by seven short exons which are alternatively spliced during development (Baldwin et al., 1994; Sheibani et al., 1999). Tyrosines 663 and 686 (Lu et al., 1997) in exons 13 and 14 constitute an ITIM, or immunoreceptor tyrosine–based inhibitory motif (Newman, 1999), which binds and modulates the activity of SH2-domain-containing proteins when phosphorylated (Pumphrey et al., 1999).

Homophilic engagement of PECAM-1 using recombinant PECAM-1 extracellular domain to mimic the cell-cell interactions found in confluent endothelium results in tyrosine phosphorylation of the PECAM-1 cytoplasmic domain (Bird et al., 1999). Antibody-mediated or homophilic PECAM-1 engagement independent of PECAM-1 clustering likewise causes src-dependent PECAM-1 tyrosine phosphorylation, resulting in calcium influx and prostacyclin release (Gurubhagavatula et al., 1998; O'Brien et al., 2001). PECAM-1 engagement-mediated integrin activation, on the other hand, requires

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concomitant PECAM-1 *cis*-dimerization or clustering (Leavesley et al., 1994; Tanaka *et al.*, 1992; Varon et al., 1998). PECAM-1 extracellular domains have been shown to *cis*-dimerize with a dissociation constant of ~12 μM (Newton et al., 1999). Elegant studies manipulating PECAM-1 cytoplasmic domain oligomerization state have validated the model of PECAM-1-mediated inside-out signaling. Forced dimerization and oligomerization of the PECAM-1 cytoplasmic domain caused changes in integrin activation state and homophilic cell-cell adhesion (Zhao and Newman, 2001).

In addition to homophilic engagement, endothelial PECAM-1 is tyrosine phosphorylated in response to a number of physiologic stimuli. Increased flow and osmotic shock (Masuda et al., 1997); the atherosclerotic plaque component lysophosphatidylcholine (Ochi et al., 1998); and exposure to vascular endothelial growth factor (VEGF), a major angiogenic and permeability factor (Esser et al., 1998), all induce PECAM-1 tyrosine phosphorylation. Phosphorylation is likely mediated by Src and/or Csk family protein tyrosine kinases (Cao et al., 1998); in endothelial cells the protein tyrosine phosphatase SHP-2 is a major binding partner at the phosphorylated PECAM-1 ITIM (Jackson et al., 1997; Masuda et al., 1997).

PECAM-1 also interacts with signaling proteins via other, as yet undefined portions of its cytoplasmic domain. Endothelial PECAM-1 binds two *armadillo* family proteins, beta catenin and plakoglobin, in a phosphorylation-regulated manner (Ilan et al., 2000; Ilan et al., 1999). These intriguing signaling proteins shuttle between the plasma membrane, where they link adherens junctions with the actin and the intermediate filament cytoskeleton, respectively; the cytoplasm, where they are subject to degradation via the proteasome pathway; and the nucleus, where they have transcriptional regulatory

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effects through interactions with LEF/TCF family transcription factors (Ben-Ze'ev and Geiger, 1998).

PECAM-1 also binds and modulates the phosphorylation of STAT5a (Ilan et al., 2001), a member of the family of signal transducers and activators of transcription. Like the catenins, these multifunctional proteins shuttle between the plasma membrane and the nucleus in a phosphorylation dependent manner to propagate transcriptional signals in response to growth factor and cytokine signaling (Pellegrini and Dusanter-Fourt, 1997). In short, PECAM-1, while certainly involved in cell-cell interactions, is much more than a simple adhesion molecule: it is a signaling scaffold sensitive to multiple physiologic inputs and capable of coordinating complex endothelial behaviors.

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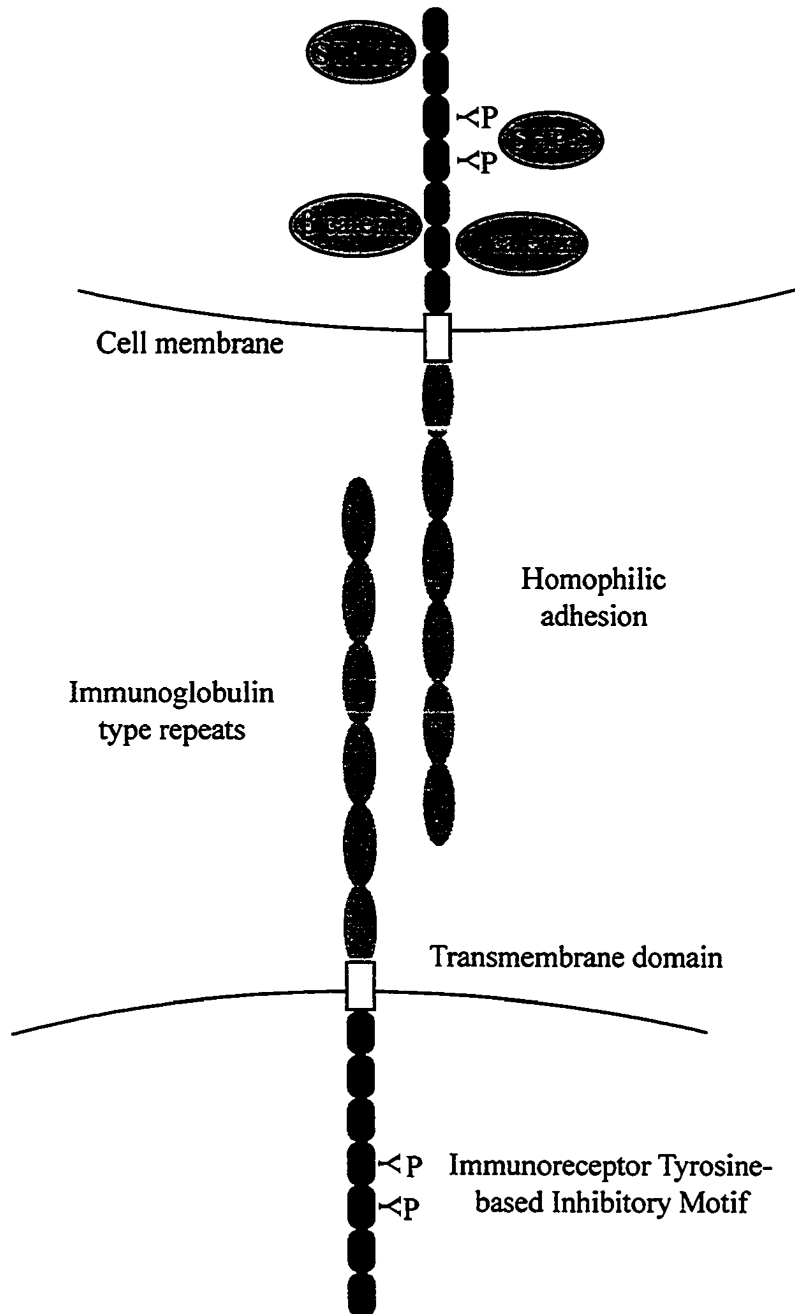

Figure 1: Schematic of Platelet-Endothelial Cell Adhesion Molecule-1.

Two adjacent endothelial cells with homophilically adhesive PECAM-1 molecules and interacting signaling proteins are depicted. (Y = tyrosine, P = phospho)

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Research Aims

- To characterize the positive role of Platelet Endothelial Cell Adhesion Molecule-1 in the coordinated migration of endothelial cells

- To elucidate the signals and signaling pathways that mediate this positive role

- To clarify the negative modulatory role of the PECAM-1 immunoreceptor tyrosine-based inhibitory motif and its interaction with the protein tyrosine phosphatase SHP-2 in endothelial migration

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Chapter 2: PECAM-1 dependent migratory characteristics of endothelial cells

Summary

Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), an immunoglobulin family vascular adhesion molecule, is involved in endothelial cell migration and angiogenesis (DeLisser et al., 1997; Lu et al., 1996). Endothelial cells lacking PECAM-1 expression exhibit increased single cell motility and extension formation. Their coordinated wound healing migration is however retarded in comparison to PECAM-1 expressing endothelium. This migratory phenotype is reminiscent of cells in which Rho activity has been suppressed by overexpressing a GTPase Activating Protein (Arthur et al., 2000). The ability of PECAM-1 to restore wound healing migration to PECAM-1-deficient cells was independent of both its extracellular domain and of signaling via its immunoreceptor tyrosine-based inhibitory motif. PECAM-1-deficient endothelial cells had a selective defect in RhoGTP loading, and inhibition of Rho activity mimicked the PECAM-1-deficient phenotype of increased nondirected motility at the expense of coordinated migration.

The wound healing advantage of PECAM-1-positive endothelial cells was not only Rho-mediated but pertussis-toxin inhibitable. Both pertussis treatment (Lee et al., 2000a) and inhibition of Rho-mediated signaling (Paik et al., 2001) likewise inhibits wound healing migration mediated by the serum sphingolipid sphingosine-1-phosphate (S1P), raising the question of whether PECAM-1 deficient endothelial cells would be impaired in their coordinated wound healing migration response to S1P. Indeed the

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difference in wound-healing migration between PECAM-1 null and PECAM-1-positive endothelial cells is minimized in sphingolipid-depleted media; moreover PECAM-1 null endothelial cells fail to increase their migration in response to S1P.

Effective S1P-mediated endothelial migration requires coordinated signaling via Galphai2 and Galpha13; these pathways are dysregulated in the absence of PECAM-1. Signaling pathways downstream of Galphai2 such as ERK and FAK are inappropriately activated even in non-migrating PECAM-1 deficient endothelial cells. Rho signaling, which is downstream of Galpha13, is in contrast depressed in PECAM-1 null endothelium. PECAM-1 localizes to caveolin-positive lipid raft domains and relative recruitment of Galphai2 versus Galpha13 to rafts is decreased in PECAM-1-deficient endothelial cells. PECAM-1 may thus support the effective S1P/RhoGTP signaling required for wound healing endothelial migration by allowing for the spatially directed, coordinated activation of Galpha signaling pathways.

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Introduction

PECAM-1 modulates migration *in vitro* and angiogenesis both *in vitro* and *in vivo*. Transfection of non-endothelial cells has revealed a role for PECAM-1 in modulating both coordinated, or wound healing, migration and single cell motility, although results have been cell type-dependent. PECAM-1 expression for example inhibits coordinated migration in fibroblast (NIH/3T3) or epithelial (ECV304) cells (Kim et al., 1998; Schimmenti et al., 1992). In mesothelial (REN) cells PECAM-1 instead promotes coordinated migration and single cell motility (Cao et al., 2002). Anti-PECAM-1 antibodies on the other hand block in vitro endothelial tube formation, wound healing and single cell motility. PECAM-1 antibody treatment also retards angiogenesis in multiple in vivo models, including FGF-2 (fibroblast growth factor-2) induced angiogenesis, corneal neovascularization and tumor angiogenesis (Cao et al., 2002; DeLisser et al., 1997).

The PECAM-1 knockout mouse is viable and fertile, with no evident angiogenic or vasculogenic abnormalities at baseline (Duncan et al., 1999). However, defects in angiogenesis into a polyvinyl acetate sponge are seen in a model of the chronic phase of foreign body inflammation in the PECAM-1 knockout mouse, and endothelial cells derived from the knockout mouse perform poorly in an *in vitro* assay of tube formation (Solowiej et al., 2002). At the same time some aspects of endothelial cell motility are enhanced in the absence of PECAM-1. In an *in vitro* model of cardiac cushion development (Bernanke and Markwald, 1982), migration of individual endocardial cells onto a type I collagen gel represents one step of the developmental process of epithelial-

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mesenchymal transition (EMT). Exposure to high glucose interferes with endocardial cell motility in this cardiac cushion explant model, secondary to a decrease in VEGF-mediated signaling in hyperglycemic cultures. Single endocardial cell motility however is preserved in the presence of high glucose in explants derived from the PECAM-1 knockout mouse (Enciso et al., 2003).

Rho family small G proteins act in a coordinated manner to regulate cell extension formation, motility and directionality by modulating actin cytoskeletal and focal contact dynamics. Rac promotes formation of immature focal complexes, membrane ruffling, and lamellipodial extension formation. Rho then contributes to adhesion during migration, with maturation of focal complexes into focal contacts and suppression of further ruffling or focal complex formation (Nobes and Hall, 1999; Rottner et al., 1999; Sastry and Burridge, 2000). The sequential activation of Rac and suppression of Rho at the leading edge of a cell to promote lamellipodial protrusion is followed by renewed Rho activation and firm focal contact adhesion to allow directed forward cell movement.

Rho family small G proteins play prominent roles in vascular physiology, which have recently been reviewed (van Nieuw Amerongen and van Hinsbergh, 2001). Rearrangement and migration of confluent bovine aortic endothelial cells (BAECs) in response to pulsatile flow is Rho-dependent (Yano et al., 1996), as is coordinated, wound healing migration of human umbilical vein endothelial cells or HUVEC (Aepfelbacher et al., 1997). Rho signaling is likewise required for HUVEC tube formation *in vitro* and VEGF-induced chorioallantoic membrane angiogenesis and tumor angiogenesis *in vivo* (Uchida et al., 2000). In fact therapeutic levels of HMG-CoA (3-hydroxy-3-

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methylglutaryl coenzyme A) reductase inhibitors, statin compounds which interfere with cholesterol synthesis, block tube formation and angiogenesis by blocking the geranylgeranylation of Rho (Park et al., 2002a).

Sphingosine-1-phosphate (S1P) is a platelet-released lipid mediator of endothelial chemotaxis, wound-healing endothelial migration, angiogenesis, and vascular maturation (English et al., 2001). Platelets phosphorylate sphingosine and store the resulting S1P (Fukuhara et al., 1999). Platelet-released S1P accounts for most of the chemotactic activity of serum; S1P isolated from serum supports chemotaxis and FGF-2-induced angiogenesis (English et al., 2000) S1P signals through Endothelial Differentiation Gene (EDG) seven-transmembrane receptors to mediate endothelial cell migration and angiogenesis through heterotrimeric G-protein signaling (Lee et al., 2000a; Lee et al., 1998; Lee et al., 1999b). S1P signaling via EDG-1 activates Galphai2, localizing and activating focal contact components Focal Adhesion Kinase (FAK) and src at the leading edge to promote lamellipodial extension formation (Rosenfeldt et al., 2001). EDG-3 meanwhile activates Galpha13 to appropriately remodel the actin cytoskeleton and strengthen focal contacts via Rho signaling, allowing coordinated migration to occur (Buhl et al., 1995; Offermanns et al., 1997; Paik et al., 2001).

Endothelial caveolin-rich membrane subdomains, or caveolae, represent a subset of low density, cholesterol-enriched lipid rafts (Sowa et al., 2001). These caveolin-positive rafts are enriched for certain signaling proteins, including heterotrimeric G proteins as well as Src family kinases and many others (Lisanti et al., 1994). Caveolin family proteins act as scaffolds which associate with signaling proteins and modulate their activity (Galbiati et al., 2001). In particular, caveolin-1 negatively regulates

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heterotrimeric G protein signaling by preferentially binding inactive, GDP (guanosine diphosphate)-bound Galpha proteins and inhibiting the GDP/GTP (guanosine triphosphate) exchange that would reactivate them (Li et al., 1995). EDG-1 too is recruited to lipid rafts in response to S1P treatment, and according to one report, EDG-receptor-mediated signaling may be inhibited by interaction with caveolin-1 (Igarashi and Michel, 2000). I have therefore examined the role of PECAM-1 in promoting S1P-EDG-driven coordinated wound-healing migration over nondirected motility of individual endothelial cells via modulation of Rho signaling.

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Results

Immortalized PECAM-1 deficient and reconstituted endothelial cells

In order to efficiently explore the role of PECAM-1 in endothelial migration I needed a model system of matched PECAM-1 null and PECAM-1 expressing endothelial cells that would survive through multiple passages and tolerate multiple manipulations. It is possible to isolate primary mouse endothelial cells from PECAM-1 knockout and wildtype animals, but the procedure is time-consuming and difficult and the resulting endothelial cells last for very few passages in culture. An immortalized lung endothelial cell line has been derived from the PECAM-1 knockout mouse (PecamKO). This line has also been reconstituted with full-length PECAM-1 (PecamRC) to provide a control immortalized line resembling wildtype, PECAM-1 expressing endothelium (Graesser et al., 2002; Wong et al., 2000).

I confirmed PECAM-1 surface expression levels and maintenance of endothelial cell markers by fluorescence activated cell sorting (FACS) analysis (**Figure 2**). A mouse hemangioendothelioma-derived cell line (Eoma) which maintains characteristics of microvascular endothelial cells (Obeso et al., 1990) was used as a positive control. FACS analysis revealed no PECAM-1 expression in PecamKO endothelial cells, as expected. PecamRC endothelial cells have surface levels of PECAM-1 similar to those in the Eoma positive control. Both PecamRC and PecamKO endothelial cell lines maintain expression of the endothelial marker VE-cadherin.

PecamRC endothelial cells form the cobblestone-like monolayers typical of endothelial cells in culture. PecamKO endothelial cells form similar tightly compact

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monolayers, but the cells are more spindle-shaped. **Figure 3a** demonstrates this morphology at the migrating front of a wound-healing endothelial monolayer. These morphologic differences are seen more clearly in individually plated cells that have been allowed to spread on fibronectin. Individually plated PecamKO cells form multiple extensions whereas PecamRC cells spread more evenly (**Figure 3b**). As a surrogate measure of extension formation or deviation from circularity, I derived theoretical radii (r) from the measured perimeter and the measured area, respectively, as if they had been perfect circles. For a perfect circle the ratio of $r_{perimeter}$ to r_{area} will be 1.0; any deviation from circularity will produce higher ratios. This number is dimensionless and therefore independent of any variation in size between cell populations. The ratio of $r_{perimeter}$ to r_{area} is in fact significantly increased in PecamKO cells, confirming the apparent increase in extension formation seen when cells are individually plated (**Figure 4**).

Given the morphological differences between PecamKO and PecamRC endothelial cells, I next assessed them for gross differences in actin cytoskeletal architecture and focal contact or adhesion formation (**Figure 5**). Cells were cultured on fibronectin-coated slides, then fixed and stained with rhodamine-conjugated phalloidin to visualize filamentous or F-actin and with anti-phosphopaxillin to highlight focal contacts and adhesions (Nakamura et al., 2000). Phalloidin staining revealed stress fibers throughout the cytoplasm of PecamRC cells, as compared to sparser, cortical actin bands in PecamKO cells. Immunostaining for phosphopaxillin revealed typical elongated focal adhesions in PecamRC cells; in PecamKO cells, phosphopaxillin staining prominently highlighted the leading edges of lamellipodial extensions, sites of newly forming focal complexes and contacts (Parsons et al., 2000).

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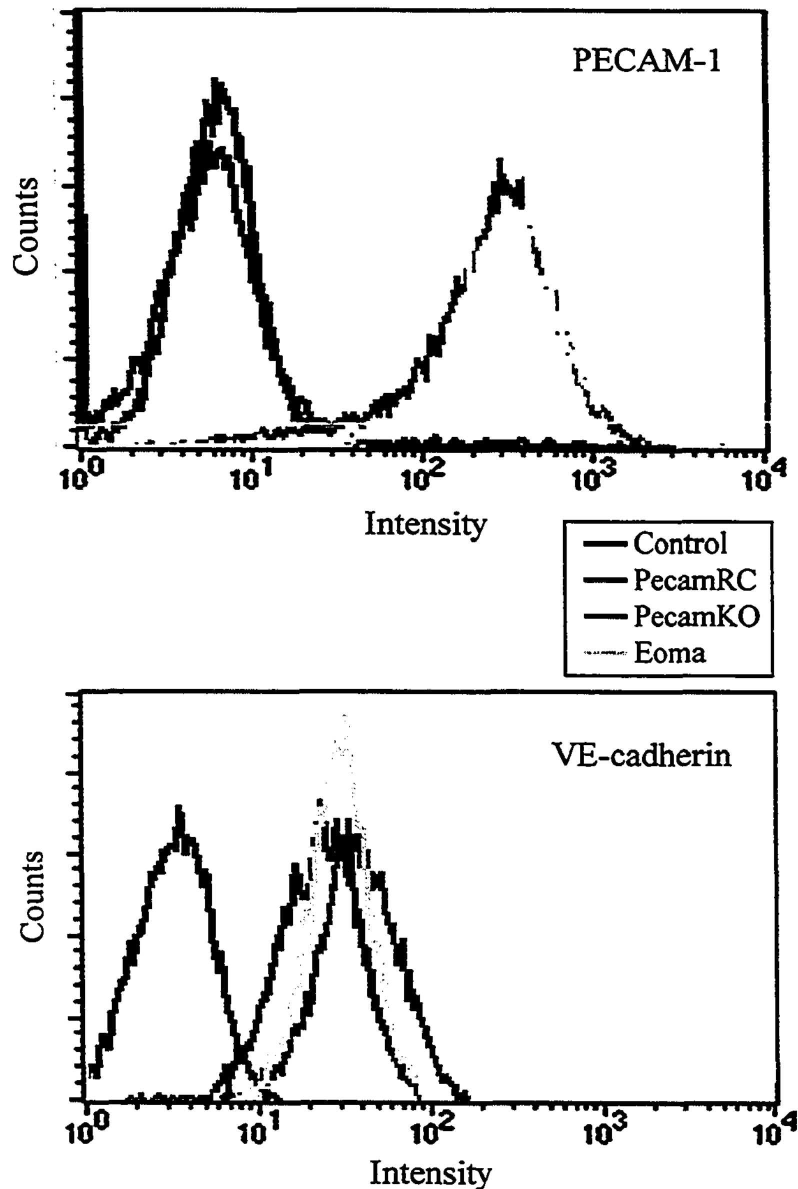

Figure 2: FACS analysis of PecamRC and PecamKO cells.

Surface expression of PECAM-1 is absent in PecamKO cells; the level of expression on PecamRC cells is similar to that in Eoma cells. Surface expression of endothelial marker VE-cadherin on both PecamRC and PecamKO cells is similar to that in Eoma cells. (control = secondary antibody)

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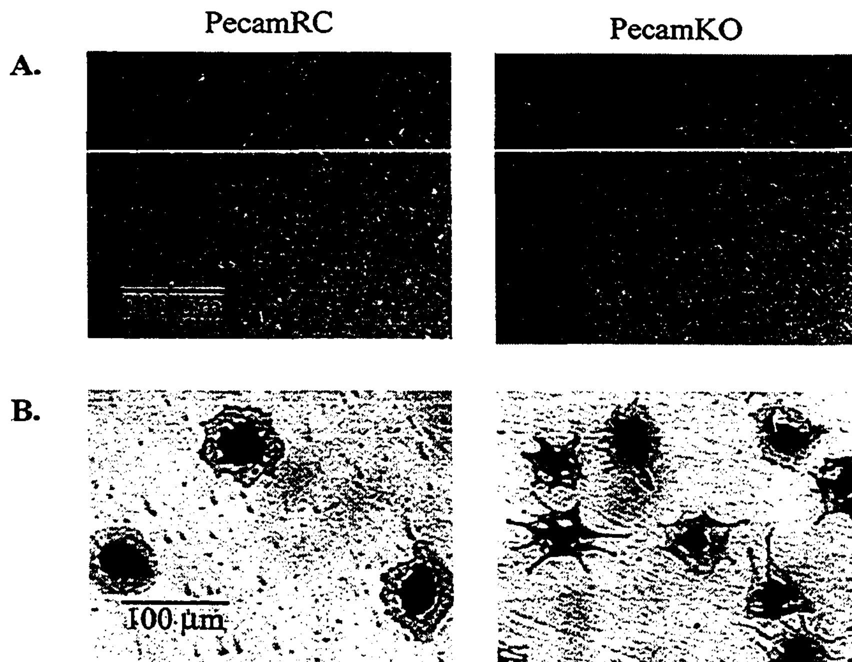

Figure 3: Morphology of PecamRC and PecamKO endothelial cells.

A. Confluent endothelial cells were scraped, then allowed to migrate for 24 hours.

B. Cells were allowed to spread for four hours, fixed and stained with crystal violet.

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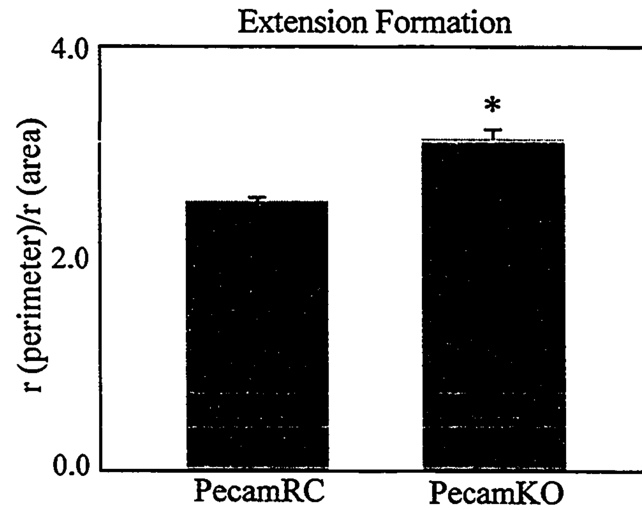

Figure 4: Extension formation is increased in the absence of PECAM-1. Quantitation of ratios of r derived from perimeter versus r derived from area as a measure of the deviation from circularity confirms significantly higher extension formation by PecamKO cells. (* $p < 0.000000005$ vs RC, $n = 89$)

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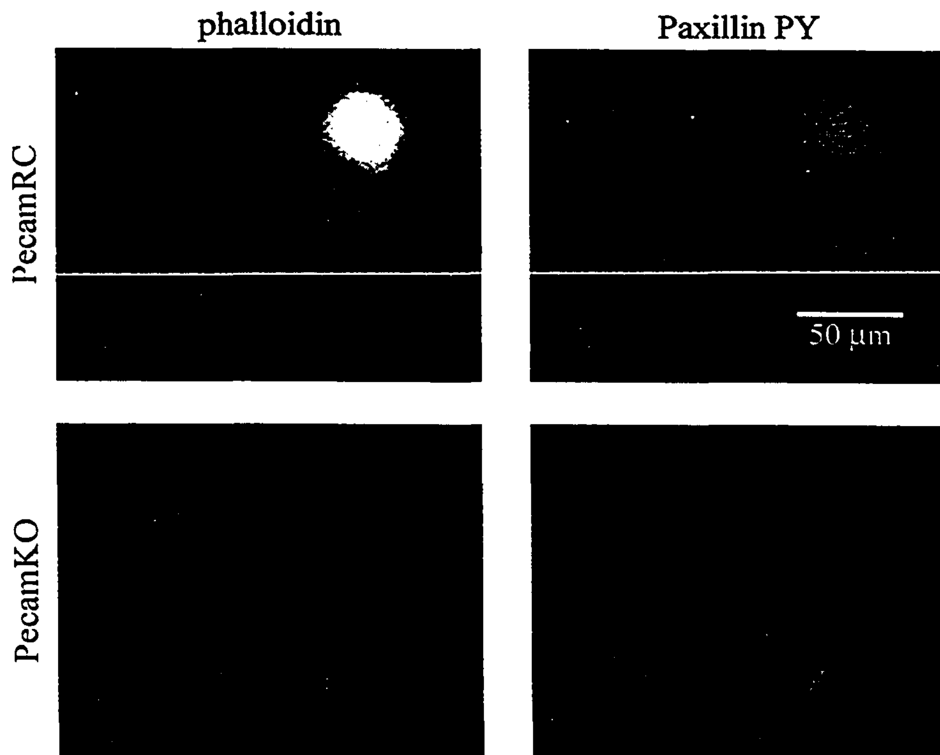

Figure 5: Cytoskeletal and focal contact immunostaining.

Immunofluorescence demonstrates rounded cell shape and increased stress fibers in PecamRC cells (upper left panel, phalloidin stain) and distinct dash-like focal adhesion contacts with the underlying fibronectin matrix (upper right panel, anti-phosphopaxillin stain). PecamKO cells exhibit sparse cortical actin staining (lower left panel) and extensions whose leading edges are highlighted with the focal contact component phosphopaxillin (lower right panel).

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PECAM-1 promotes wound-healing migration over nondirected motility

I set out to address the role of PECAM-1 in two aspects of endothelial cell motility: the intrinsic nondirected movement of individual cells, and the coordinated movement of endothelial monolayers. Nondirected cell motility, or chemokinesis, was assessed by quantitating the cells that had reached the underside of a fibronectin-coated 8 μm pore Transwell® membrane 2.5 hours after plating on top of the membrane. There was no chemotactic or haptotactic stimulus involved in this assay: the membranes were fibronectin coated on both sides, and no additional growth factors were added to the serum-containing media on either side of the membrane. Coordinated migration of endothelial cells was assessed using a wound-healing assay: endothelial cells were allowed to reach confluence on a fibronectin-coated culture dish, then scraped to leave alternating cell-free areas in a bull's eye pattern. The distance migrated by the leading edge of monolayer cells into the scraped wound was measured after 24 hours.

Previous studies have come to conflicting conclusions about the effect of PECAM-1 expression on migration. Ectopic expression of PECAM-1 has been reported to decrease wound-healing migration in epithelial cells (Schimmenti et al., 1992) on the one hand, and to increase both wound-healing and nondirected motility in mesothelial cells (Cao et al., 2002) on the other. In endothelial cells, where PECAM-1 expression is physiologic, the role of PECAM-1 in the two types of migration turns out to be discordant. I found that PecamKO endothelial cells have significantly enhanced nondirected motility (**Figure 6a**) but decreased wound-healing migration (**Figure 6b**) as compared to PecamRC endothelial cells.

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To rule out the possibility that decreased wound healing in PecamKO cells is secondary to a proliferation rather than a migration defect, the wound healing assay was also carried out in the presence of mitomycin C to block proliferation (**Figure 6b**). A concentration of mitomycin C that blocked the appearance of all mitotic figures as visualized by DAPI staining but that was minimally toxic was determined empirically (data not shown). Proliferation does contribute to wound healing migration, and overall distance migrated during mitomycin C treatment dropped by ~50% for both PecamRC and PecamKO endothelial cells. However the differential in migration between the two cell types remained, implying that a proliferation defect does not account for the retardation of wound healing migration in PECAM-1-deficient endothelial cells.

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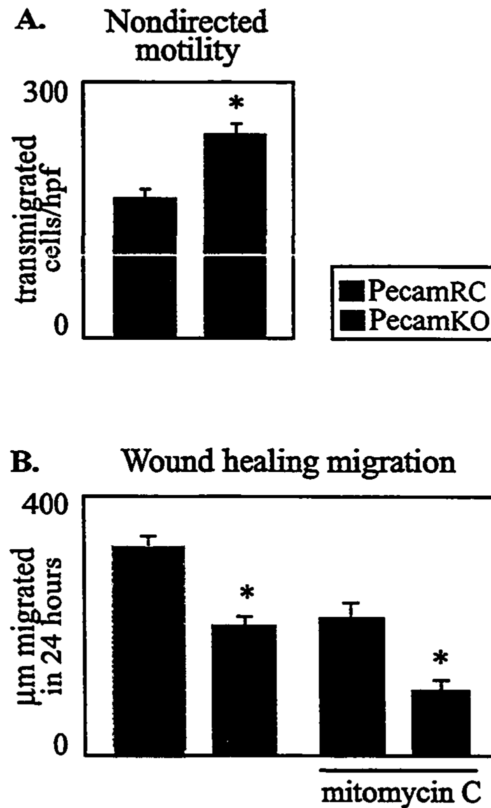

Figure 6: Migration characteristics of PecamKO and PecamRC cells.

A. PecamKO cells display enhanced nondirected motility through 8 μm fibronectin coated membranes but **B.** poor wound-healing migration on fibronectin in the 24 hour scrape assay as compared to PecamRC cells. The wound migration defect was not eliminated by blocking proliferation with mitomycin C (mito C). (* $p < 0.0005$ vs PecamRC; $n = 8$ for wound healing; $n = 9$ for transmigration)

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Parsing the contribution of PECAM-1 to wound healing

The PecamKO and PecamRC endothelial cells I have used to characterize the role of PECAM-1 in endothelial migration have been easy to work with largely because they are immortalized. By the same token, however, immortalization with polyoma middle T antigen is known to affect signaling through multiple pathways (Wagner and Risau, 1994), many of which could affect migration as well as survival and proliferation. To rule out the possibility that the migration differences between PecamKO and PecamRC were due to the interaction of PECAM-1 with ectopically expressed polyoma middle T antigen rather than to the expression of PECAM-1 itself, I chose to confirm our findings in a primary endothelial cell line.

I used antisense oligonucleotides (Baker et al., 2001) to decrease PECAM-1 expression in human umbilical vein endothelial cells (HUVEC). Western blotting analysis confirmed stable depletion of PECAM-1 protein at both 24 and 48 hours in HUVEC treated with antisense oligonucleotide, as compared to stable PECAM-1 expression in HUVEC treated with the scrambled oligonucleotide control **(Figure 7a)**. Nondirected motility was significantly increased and wound healing migration decreased in PECAM-1 depleted HUVEC as opposed to control oligonucleotide treated HUVEC **(Figure 7b)**, supporting the role of PECAM-1 itself in modulating endothelial migratory behavior. The fact that a similar phenotype was seen in primary endothelial cells derived from human as opposed to murine tissue, and from a macrovascular umbilical vein bed as opposed to a microvascular lung vascular bed, confirmed the wider applicability of the findings in PecamKO and PecamRC endothelial cells.

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I next set out to ascertain which domains of PECAM-1 were needed to mediate its positive role in wound healing migration. The effect of PECAM-1 on wound-healing could simply involve the ectodomain, which mediates homophilic cell-cell interactions. It might involve outside in and inside out signaling via both the ectodomain and the cytoplasmic domain by modulating integrin activation, with engagement and clustering of the ectodomain leading to signaling via tyrosine phosphorylation of the PECAM-1 ITIM (Chiba et al., 1999; Sun et al., 1996). The ITIM domain becomes dephosphorylated during wound healing migration (Lu et al., 1996) but phosphorylated during flow-induced rearrangement (Osawa et al., 1997), modulating binding and activation of SH2-domain containing proteins, and has been the principal target of studies of PECAM-1 mediated signaling to date. This signaling modality can be inactivated through site-specific mutagenesis of the tyrosines within the PECAM-1 ITIM. Alternately, the role of PECAM-1 in wound healing may call on neither the ITIM nor the ectodomain, but instead may involve signaling via other portions of the cytoplasmic domain and through interacting signaling proteins, known or unknown.

To determine the relative contributions of each domain of PECAM-1 to endothelial wound-healing migration, I expressed three different PECAM-1 constructs in originally PECAM-1 deficient endothelial cells. PecamKO cells were transiently transfected with constructs encoding either full-length, wildtype PECAM-1; wildtype PECAM-1 lacking its ectodomain; or PECAM-1 mutated in its ITIM domain to eliminate phosphorylation (**Figure 8a**). At 24 hours post transfection the endothelial cells were replated to fibronectin-coated dishes, allowed to become confluent overnight, and then assessed for their wound healing ability.

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Unlike control transfection with green fluorescent protein (GFP) or vascular cell adhesion molecule (VCAM), transfection of full-length PECAM-1 restored wound healing migration to PecamKO cells (**Figure 8b**). In fact all three PECAM-1 constructs were equally capable of supporting wound healing migration. Absolute rates of migration between transfection and nontransfection experiments are not comparable, since the toxicity of the transfection itself decreases wound healing migration. Nevertheless the degree of rescue of wound healing with any of the PECAM-1 constructs of ~60% roughly approximates the ~50% wound healing advantage of PecamRC versus PecamKO endothelial cells. Thus neither the ectodomain nor the intact ITIM domain is required to confer the PECAM-mediated wound-healing advantage. The PECAM-1 cytoplasmic domain itself appears to signal to modulate wound healing migration independent of tyrosine phosphorylation-mediated recruitment of SH2-domain containing proteins. Further truncation mutant transfection experiments will be necessary to localize the responsible portion of the PECAM-1 cytoplasmic domain.

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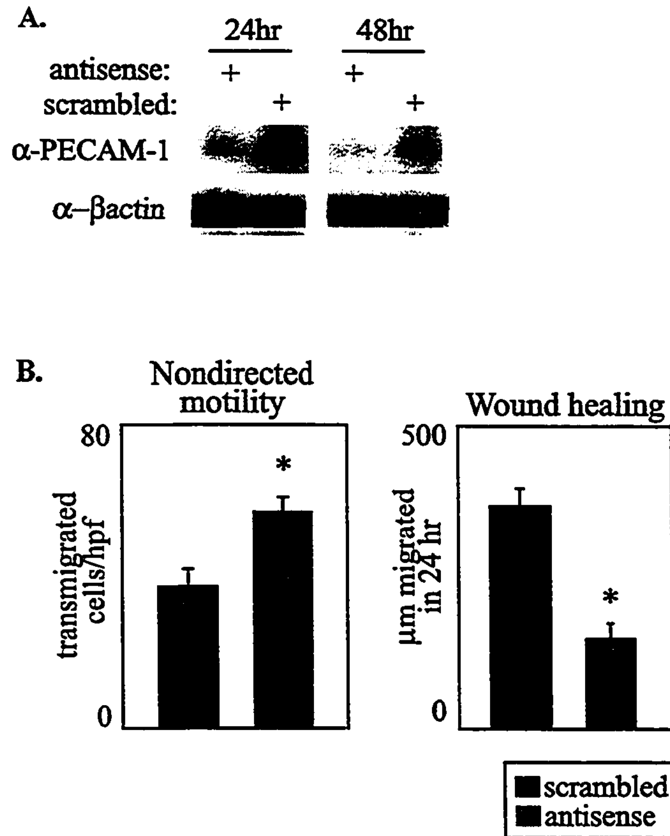

Figure 7: PECAM-1 expression modulates endothelial migration.

A. Antisense oligonucleotides decrease PECAM-1 expression in HUVEC as compared to the scrambled oligonucleotide control.

B. Antisense-treated HUVEC have significantly increased motility but decreased wound healing migration (* $p < 0.005$ vs scrambled; $n = 6$ for wound healing; $n = 9$ for motility)

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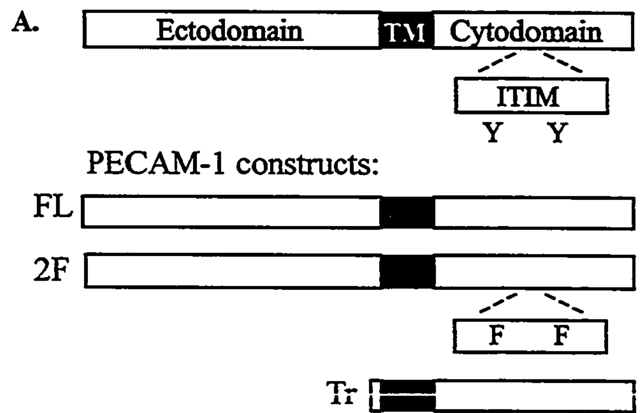

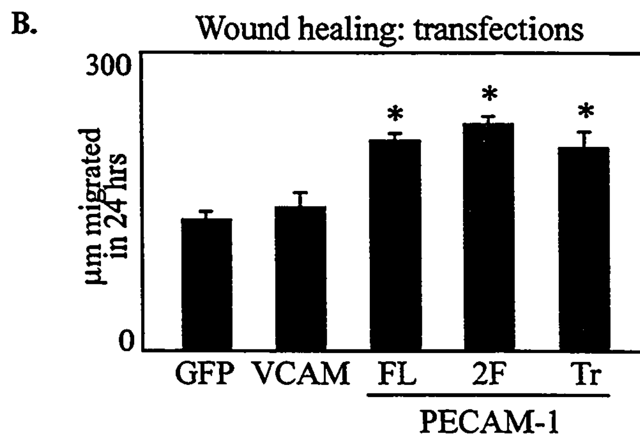

Figure 8: Contribution of PECAM-1 domains to wound healing.

A. The constructs used in transient transfections of PecamKO endothelial cells include full-length PECAM-1 (FL), PECAM-1 mutated in its ITIM domain (2F), and PECAM-1 lacking the ectodomain (Tr).

B. Transient transfection of PecamKO with FL, 2F, and Tr PECAM-1 contstructs but not with GFP or VCAM restores wound healing migration to PecamKO cells.

(* $p < 0.0005$ vs GFP; $n = 8$)

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PECAM-1-null endothelial cells are selectively deficient in RhoGTP

PecamKO endothelial cells show pronounced extension-formation and enhanced nondirected motility, but blunted wound-healing migration. Lamellipodia are RacGTP-driven membrane extensions (Nobes and Hall, 1999) involved in initiation of cell motility. Endothelial wound healing in contrast is RhoGTP-mediated and is sensitive to inhibition of Rho signaling (Aepfelbacher et al., 1997). The two-sided migratory phenotype of PECAM-1 null endothelial cells may thus reflect an imbalance of Rho and Rac signaling. I used selective pulldown assays to assess levels of activated or GTP-bound Rho and Rac. RhoGTP was pulled down with a recombinant GST (glutathione-S-transferase) - Rhotekin Rho binding domain fusion protein (Ren and Schwartz, 2000). GST-p21 Activated Kinase (PAK) Rac binding domain fusion protein was used for RacGTP pulldowns (Royal et al., 2000). Glutathione agarose-bound fusion protein was used to selectively pull down GTP-bound Rho or Rac from whole cell lysates; the pulled down protein was quantitated and normalized to total G protein expression.

PecamKO endothelial cells proved to have a > 50% deficiency in RhoGTP levels as compared to Pecam RC endothelial cells (**Figure 9a**). There was by comparison no significant difference between the two cell types in RacGTP loading (**Figure 9b**). Lack of PECAM-1 expression in endothelial cells thus upsets the balance of small G protein signaling, with normal levels of Rac activation and concomitant decreased Rho signaling. The balance appears to tilt in favor of Rac-mediated control of actin cytoskeletal and focal contact dynamics, lowering the threshold for Rac-mediated extension formation and motility while interfering with Rho-dependent wound healing migration.

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The trio of well-studied small G proteins involved in modulation of actin cytoskeletal and focal contact dynamics in cell motility include not only Rho and Rac but also Cdc42. Cdc42 appears to cooperate with or act upstream of Rac in the initiation of extension formation in the form of membrane ruffling and lamellipodial protrusions (Clark et al., 1998; Nobes and Hall, 1999). The PAK binding domain fusion protein should pull down the GTP-bound form of the related small G protein Cdc42 in addition to GTP-bound Rac; however I was never able to detect Cdc42 in the pulldown fraction, whether due to the small fraction of activated Cdc42 present in these cell lysates or due to technical difficulties. Thus any potential additional role for Cdc42 in preferentially promoting extension formation in PecamKO cells in concert with Rac remains to be evaluated.

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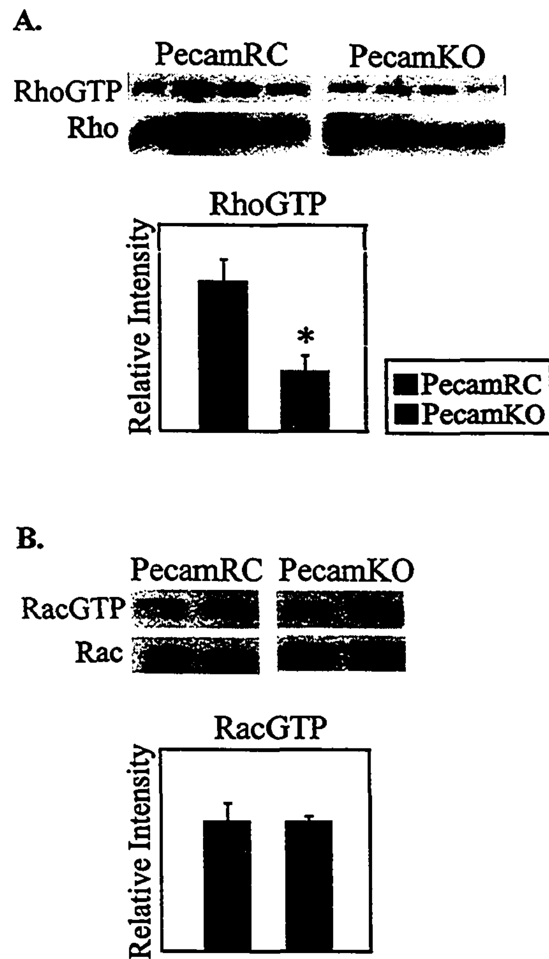

Figure 9: PecamKO cells have a selective defect in RhoGTP loading.

A. Pulldowns with the recombinant RhoGTP-binding domain of Rhotekin reveal significantly decreased levels of RhoGTP in PecamKO cells (* $p < 0.05$ vs PecamRC)

B. Pulldowns with the recombinant RacGTP binding domain of PAK reveal no difference in RacGTP loading in PecamRC vs PecamKO cells (shown in duplicate).

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Activation of p190RhoGAP contributes to RhoGTP deficiency

RhoGTP levels during migration are regulated by a GTPase activating protein, or GAP, which promotes the dephosphorylation of Rho-bound GTP to GDP and consequently inhibits Rho-mediated signaling. p190RhoGAP was cloned and characterized as a p120RasGAP-associated protein which colocalizes with alpha5beta1 integrin and Rho upon integrin clustering (Burbelo et al., 1995). The importance of p190RhoGAP in directed migration became evident with the generation of the p190RhoGAP knockout mouse, which has a severe neural morphogenetic defect and excessive filamentous actin (F-actin) accumulation indicative of hyperactive RhoGTP signaling (Brouns et al., 2000).

p190RhoGAP inhibits Rho signaling upon integrin engagement in a src-dependent manner (Arthur et al., 2000), disinhibiting extension formation and cell motility (Arthur and Burridge, 2001). **Figure 10a** provides a schematic of src-mediated p190RhoGAP activation. Src-mediated tyrosine phosphorylation of p190RhoGAP mediates association with p120RasGAP via its SH2 domains. This association in turn promotes the GTPase activity of p190RhoGAP toward RhoGTP (Haskell et al., 2001). The consequent depression of local RhoGTP-mediated signaling then promotes extension formation and initiation of directed wound healing migration (Kulkarni et al., 2000). Low molecular weight protein tyrosine phosphatase (LMW-PTP) dephosphorylates p190RhoGAP, blocking its inhibition of Rho signaling (Raugei et al., 2002).

To determine whether the low baseline levels of RhoGTP in PECAM-1 deficient endothelial cells are at least partly attributable to increased activation of p190RhoGAP,

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p120RasGAP was immunoprecipitated to determine levels and tyrosine phosphorylation of associated p190RhoGAP. Quantitation revealed significantly increased association of p120RasGAP with p190RhoGAP in PecamKO as compared to PecamRC endothelial cells (**Figure 10b**). The tyrosine phosphorylation of that associated p190RhoGAP was also significantly increased. In the converse experiment immunoprecipitation of p190RhoGAP also revealed increased p190 tyrosine phosphorylation and p120RasGAP association (data not shown). These findings are consistent with increased p190RhoGAP activity and may in part account for the observed decrease in RhoGTP levels in PecamKO endothelial cells.

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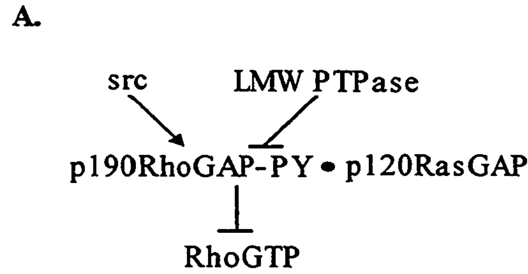

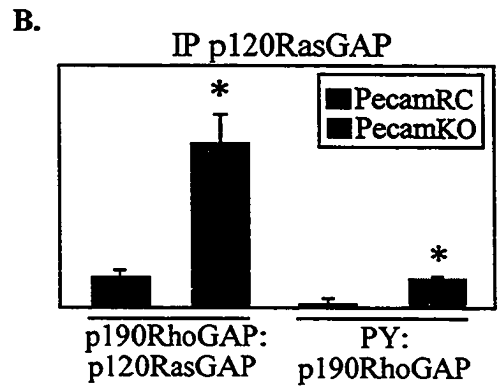

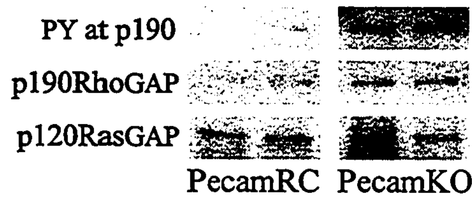

Figure 10: RhoGTP regulation by p190RhoGAP.

A. The ability of p190RhoGAP to inhibit Rho signaling is regulated by its tyrosine phosphorylation (PY) and association with p120RasGAP.

B. Immunoprecipitation (IP) of p120RasGAP reveals higher association with p190 RhoGAP (first two columns) and increased PY of that p190RhoGAP (second two columns) in PecamKO cells (* $p < 0.05$ vs PecamRC).

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RhoGTP deficiency mimics the PECAM-1 null motility phenotype

PecamKO endothelial cells, then, are selectively deficient in RhoGTP, and excess tyrosine phosphorylation of p190RhoGAP and association with p120RasGAP may at least in part account for this deficiency. Furthermore, the divergent migratory characteristics of PECAM-1 deficient endothelial cells, including increased extension formation and nondirected single cell motility as opposed to decreased coordinated wound healing migration, are consistent with depressed Rho signaling, as when p190RhoGAP is overexpressed (Arthur et al., 2000). To determine whether in fact decreased Rho activity accounts for the migration characteristics of PecamKO endothelial cells, the Rho signaling pathway was blocked at the level of Rho itself and of its effector p160 Rho coiled-coil kinase, or p160ROCK, which mediates the RhoGTP-induced increase in stress fibers and focal adhesions (Ishizaki et al., 1997).

The C3 exoenzyme of *C. botulinum* (exoC3) ADP-ribosylates Rho and directly inhibits its activity, interfering with endothelial migration (Aepfelbacher et al., 1997). ExoC3 abrogated the wound-healing advantage of PecamRC as compared to PecamKO endothelial cells (**Figure 11a**). Moreover, it actually significantly increased nondirected endothelial cell motility (**Figure 11b**). Y27632 is a synthetic inhibitor of the Rho effector p160ROCK, which inhibits endothelial tube formation *in vitro* and angiogenesis *in vivo* (Uchida et al., 2000). Y27632 had effects similar to those of ExoC3 in PecamKO and PecamRC cells, with increased nondirected cell motility at the expense of diminished coordinated wound healing migration (**Figure 12**).

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PECAM-1 deficient endothelial cells exhibit increased extension formation to parallel their increased nondirected single cell motility. Quantitation of $r_{perimeter}$ to r_{area} ratios confirmed that extension formation was also significantly increased upon inhibition of Rho signaling (**Figure 13a**). Hence decreased basal RhoGTP levels can account for both the migratory and the morphologic characteristics of the PecamKO endothelial cell phenotype. To reaffirm that Rho signaling levels are important determinants of endothelial motility in general, I assessed the effect of Y27632 on primary bovine aortic endothelial cells (BAECs). Inhibition of p160ROCK significantly increased both nondirected motility and extension formation in BAECs, confirming the general applicability of these findings to endothelial cell behavior (**Figure 13b**).

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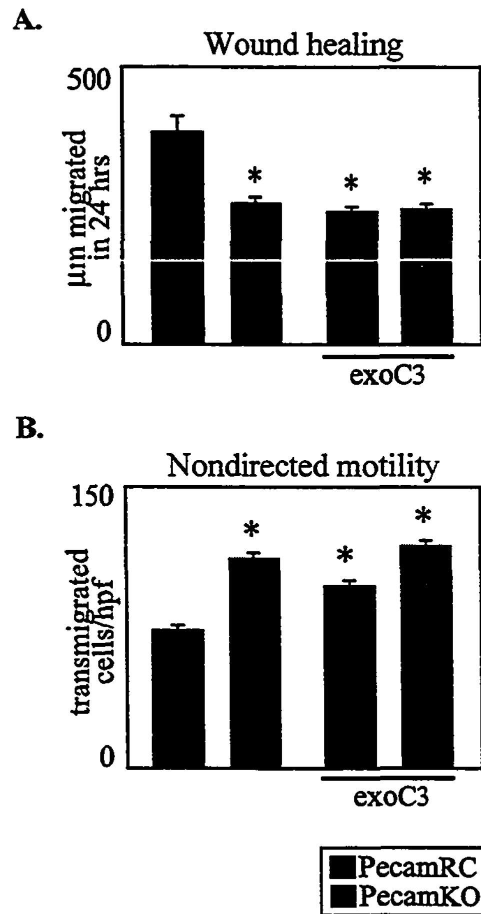

Figure 11: Rho inhibition mimics the PecamKO migratory phenotype.

Cells were treated with exoC3 to directly inactivate Rho.

A. Wound migration was significantly inhibited and **B.** nondirected motility was significantly promoted by exoC3 treatment. (* $p < 0.005$ vs PecamRC control; $n = 8$ for wound healing; $n = 9$ for motility)

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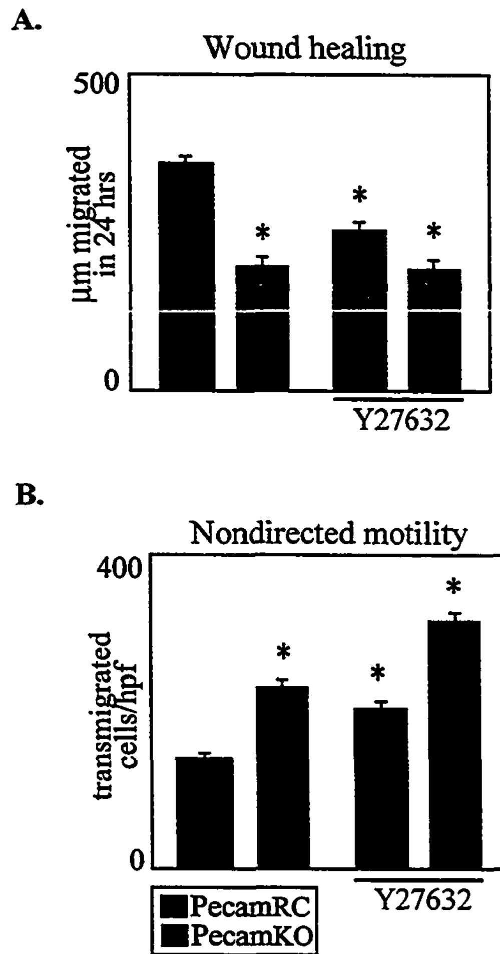

Figure 12: p160ROCK inhibition resembles Rho inhibition.

Cells were treated with Y27632 to inactivate p160ROCK.

A. Wound migration was significantly inhibited and **B.** nondirected motility was significantly promoted by Y27632 treatment. (* $p < 0.005$ vs PecamRC control; $n = 8$ for wound healing; $n = 9$ for motility)

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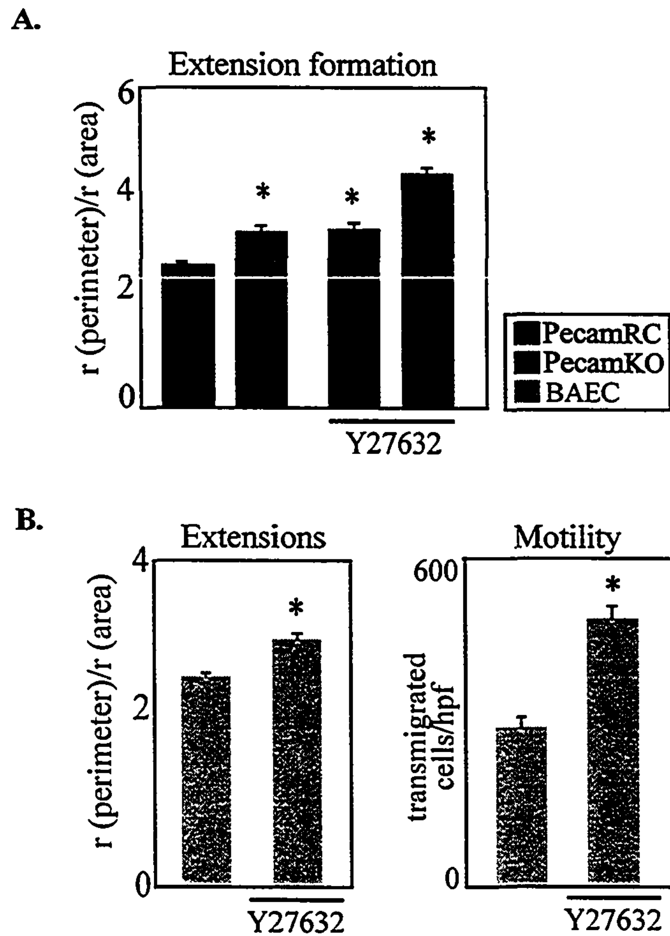

Figure 13: Rho signaling inhibits endothelial extension formation.

A. Quantitation of ratios of r derived from perimeter versus r derived from area as a measure of the deviation from circularity confirms that extension formation was significantly increased in Y27632-treated cells.

B. BAECs respond to inhibition of Rho signaling with increased nondirected motility and extensions. (* $p < 0.005$ vs untreated control; $n = 9$ for motility; $n = 98$ for extensions)

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PECAM-1 confers sensitivity of single cell motility to high glucose

Children of diabetic mothers are at increased risk for heart defects, particularly defects of the outflow tract and atrioventricular septum (Loffredo et al., 2001). Many of these can be traced to incomplete epithelial-mesenchymal transformation (EMT) within the endocardial cushion. Individual endocardial cells in this thickened area of the cardiac tube migrate down into the underlying cardiac jelly. They assume a mesenchymal phenotype and contribute to the incipient atrial and ventricular membranous septae and atrioventricular valves. In a model of EMT using the explanted murine endocardial cushion (Bernanke and Markwald, 1982), endocardial cells initially spread out from the explant as an endothelioid sheet. They then disperse out over the type I collagen gel substrate as they take on an elongated, fibroblast-like morphology, gain expression of mesenchymal markers such as alpha smooth muscle actin, and lose expression of endothelial markers including PECAM-1.

Previous work from our lab has demonstrated that culturing endocardial cushion explants under conditions of increased glucose concentration, at levels similar to those found in poorly controlled maternal diabetes, interferes with EMT. Single cell motility is blocked; endocardial cells instead continue to grow out as a PECAM-1 positive, alpha smooth muscle actin negative endothelioid sheet. Endocardial cells originating from the PECAM-1 knockout mouse however are resistant to this effect: they continue to disperse out as individual cells even in the presence of high glucose (Enciso et al., 2003).

PECAM-1-deficient endocardial cells, then, are resistant to inhibition of single cell motility by high glucose. Given our findings of increased nondirected motility in

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PECAM-1-deficient endothelial cells, I asked whether cultured endothelial cells could model this physiologically relevant behavior. Endothelial culture media contains 5 mM glucose, which approximates euglycemia. The nondirected motility of endothelial cells was determined in the presence of 30 mM glucose, a concentration consistent with poorly-controlled diabetes mellitus. I found that PecamRC endothelial cells were indeed retarded in their motility in the presence of high glucose as compared to a mannose osmotic control (**Figure 14**). However, as had been the case in the cardiac cushion culture system, the motility of PecamKO cells was unaffected by high glucose. Thus the behavior of endothelial cells in culture faithfully mirrors the behavior of explanted endocardial cushion tissue, and may provide useful starting points for modeling complex vascular behaviors.

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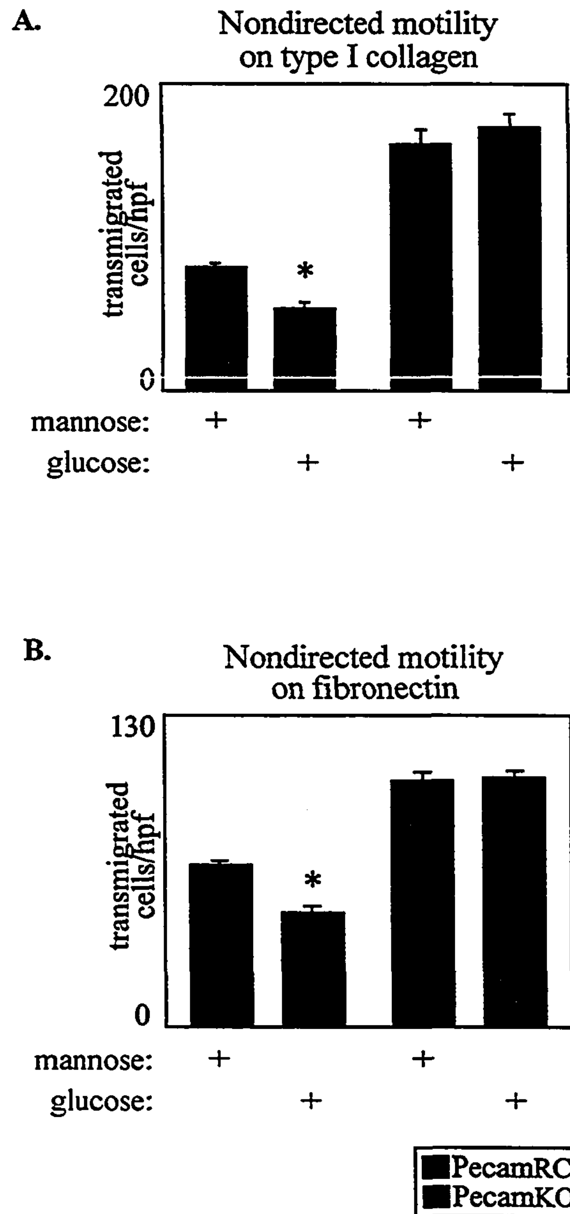

Figure 14: PecamKO cells resist glucose-mediated retardation of motility. On both collagen **A.** and on fibronectin **B.** endothelial cell motility is significantly decreased in the presence of high glucose as compared to mannose osmotic control (* $p < 0.005$ vs mannose, $n = 9$). PecamKO cells are resistant to the glucose effect.

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Decreased Rho signaling provides resistance to high glucose

Having determined that endothelial cell motility models the behavior of endocardial cells in the cardiac cushion explant assay, I determined to explore a potential mechanism for the resistance of PECAM-1 null endocardial cells to inhibition of their motility. The glucose-mediated inhibition of endocardial cell motility is attributable to decreased expression of Vascular Endothelial Growth Factor (VEGF). Normoglycemic cultures depleted of VEGF exhibit a migratory phenotype very similar to that of endocardial cushions cultured in the presence of high glucose. Conversely, VEGF supplementation is sufficient to rescue single cell motility in high glucose cardiac cushion cultures (Enciso et al., 2003). The correct balance and timing of VEGF signaling appears to be crucial for successful endocardial cushion development: too much VEGF too early has likewise been shown to inhibit EMT (Dor et al., 2001).

Interestingly, VEGF-mediated endothelial motility is Rac-mediated and is inhibited by a constitutively-active mutant of Rho (Soga et al., 2001). I therefore hypothesized that the resistance of PECAM-1-deficient endocardial cells to glucose-mediated retardation of single cell motility is secondary to low Rho activation levels at baseline, with a consequent disinhibition of Rac-mediated lamellipodial extension and nondirected motility. To determine whether the decreased sensitivity of PECAM-1-deficient cells to glucose-mediated retardation could in fact be attributed to their decreased Rho activation at baseline, I quantitated endothelial cell motility in the presence of glucose plus or minus the p160ROCK inhibitor, Y27632, to block signaling downstream of Rho. Indeed, concomitant treatment with Y27632 blocked the effect of

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high glucose on motility in PecamRC cells (**Figure 15a**). Two way analysis of variance (ANOVA) revealed a significant cell type by treatment effect for high glucose which was abrogated by inhibition of the Rho effector pathway with Y27632. The ANOCA results in effect confirm that the migratory response of PecamRC cells and PecamKO cells to high glucose is significantly different, and that this differential response requires intact signaling via the Rho effector pathway.

VEGF signaling in endothelial cells is mediated by Rac signaling and production of reactive oxygen species (ROS) (Colavitti et al., 2002). **Figure 15b** presents a model of VEGF-mediated induction of Rac-mediated motility, and inhibition of that motility by way of the PECAM-1-dependent stimulation of basal Rho activity. The small GTPase Rac contains an insert region which contributes to the production of ROS in the form of hydrogen peroxide (H_2O_2) (Deshpande et al., 2000); this region is required for lamellipodial induction (Karnoub et al., 2001). LMW-PTP, the low molecular weight protein tyrosine phosphatase which dephosphorylates p190RhoGAP (**Figure 10a**), promotes Rho signaling by blocking the dephosphorylation of Rho-associated GTP (Raugei et al., 2002). In fact overexpression of a human isozyme of LMW-PTP has been shown to block VEGF-mediated endothelial migration and angiogenesis (Huang et al., 1999). LMW-PTP is physiologically inhibited by H_2O_2, which oxidizes two active site cysteine residues to form a disulfide bond (Caselli et al., 1998), providing a likely mechanism for local Rac-mediated inhibition of Rho signaling.

Thus VEGF-mediated signaling acts to promote extension formation and initiate motility by locally inhibiting RhoGTP. The major source of VEGF during endocardial cushion morphogenesis appears to be the layer of myocardium underlying the cardiac

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jelly (Enciso et al., 2003). This localized RhoGTP inhibition likely serves to direct endocardial cells down into the cardiac jelly, where they will go on to contribute to the incipient atrioventricular septum and valves. The low baseline levels of RhoGTP in PECAM-1 deficient endothelial or endocardial cells should therefore lower the barrier for Rac-mediated initiation of single cell motility. Lowering the threshold for motility, however, must by definition come at the cost of losing sensitivity to directional information, due to a lowered signal to noise ratio for initiation of extension formation. This lowering of the barrier for Rac-mediated extension formation in turn potentially explains the robust nondirectional single cell motility of PECAM-1 deficient cells in the face of decreased VEGF-Rac signaling secondary to hyperglycemia.

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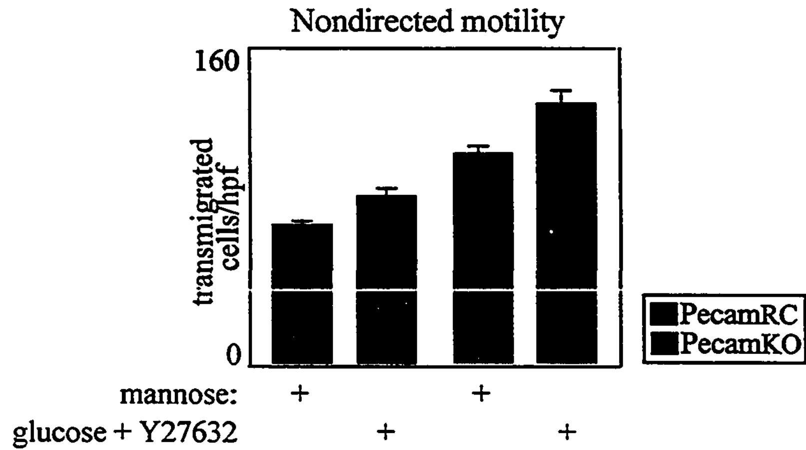
A.



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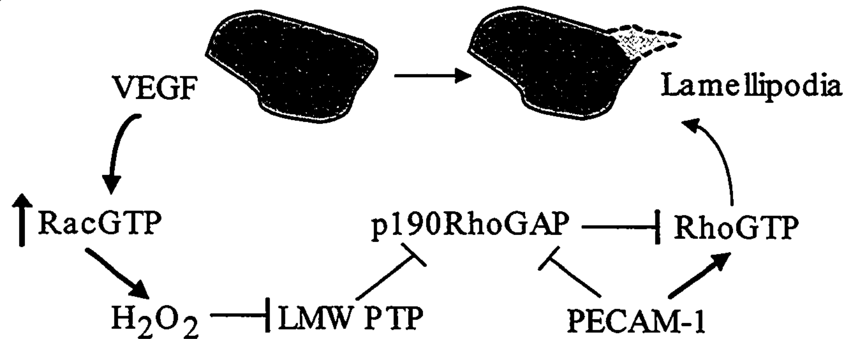

Figure 15: Inhibition of Rho signaling abrogates the effect of glucose on motility.

A. Treatment with the p160ROCK inhibitor Y27632 blocks the inhibitory effect of glucose on motility. Two way ANOVA reveals a significant cell type by treatment effect ($p < 0.0005$) for glucose, which is abrogated in the presence of Y27632.

B. Induction of lamellipodial extension through repression of Rho signaling.

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PECAM-1 is required for effective Galphai2-mediated signaling

S1P, a serum sphingolipid, signals through heterotrimeric G proteins associated with its seven-transmembrane EDG-family receptors. S1P-mediated endothelial wound-healing and directed migration is both Rho-dependent (**Figure 11**) and pertussis toxin (PTX) inhibitable. Pertussis toxin subunit A inhibits signaling via heterotrimeric G proteins by catalyzing ADP ribosylation and inactivation of Galphai family subunits, including Galphai2, downstream of EDG-1 (Lee et al., 1996). S1P signaling via EDG-1 and EDG-3 potentiates capillary morphogenesis, adherens junction formation, and angiogenesis in a Rho-dependent and pertussis toxin-inhibitable manner (Lee et al., 1999a). S1P-induced endothelial tube formation and matrigel plug angiogenesis (Lee et al., 1999b) as well as wound healing migration (Lee et al., 2000a) and directed, chemotactic motility (Lee et al., 2000b; Liu et al., 2001) have all been shown to be PTX-inhibitable as well.

S1P is one of an array of signaling molecules, including chemokines such as stromal derived factor-1 (SDF-1) (Salvucci et al., 2002) as well as thrombin (Maragoudakis et al., 2002), that promote endothelial migration and angiogenesis through heterotrimeric G protein signaling. Defects in heterotrimeric G protein signaling downstream of S1P may therefore be representative of dysfunctional responses to a broad array of seven-transmembrane receptor mediated signals in PECAM-1 deficient endothelial cells. Given the Rho-dependence of PECAM-1-mediated endothelial wound healing migration, I set out to see whether this effect might in part reflect modulation heterotrimeric G protein signaling by PECAM-1. Indeed, I found that PTX treatment

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abrogated the wound-healing advantage of PecamRC cells; no significant difference in distance migrated remained between PTX-treated PecamRC and PecamKO endothelial cells (**Figure 16a**). This, together with the key role of Rho signaling in the wound healing advantage conferred by PECAM-1, strongly implicates a dysfunction in heterotrimeric G protein-mediated signaling in the migratory phenotype of PECAM-1 null endothelial cells.

PTX inhibits Galphai2-mediated activation of Extracellular signal Regulated Kinase (ERK) (Lee et al., 1996) and FAK as well as src (Liu et al., 2000; Rosenfeldt et al., 2001) downstream of the EDG-1 receptor (**Figure 16b**). To uncover potential disturbances in Galphai2-mediated signaling in PecamKO endothelial cells, I performed Western blots with phosphospecific antibodies to threonine/tyrosine phosphorylated ERK and to tyrosine 397-phosphorylated FAK (FAK PY397). These revealed significantly increased activation of both ERK signaling and of FAK PY397 in confluent PecamKO endothelial cells as compared to PecamRC cells (**Figure 17**). ERK phosphorylation is normally increased only at the free edge of an endothelial monolayer as a part of the normal migratory response to mechanical wounding (Pintucci et al., 2002). Indeed, ERK phosphorylation increased as expected in wounded, migrating PecamRC monolayers, while no further increase was observed in the already high levels of ERK activation in migrating PecamKO monolayers (data not shown). Inappropriate activation of FAK and ERK in confluent PecamKO cells indicates dysfunctional signaling in the absence of an appropriate migratory stimulus, and this signaling proves ineffective in coordinating migration of PecamKO monolayers when that wounding stimulus is actually provided.

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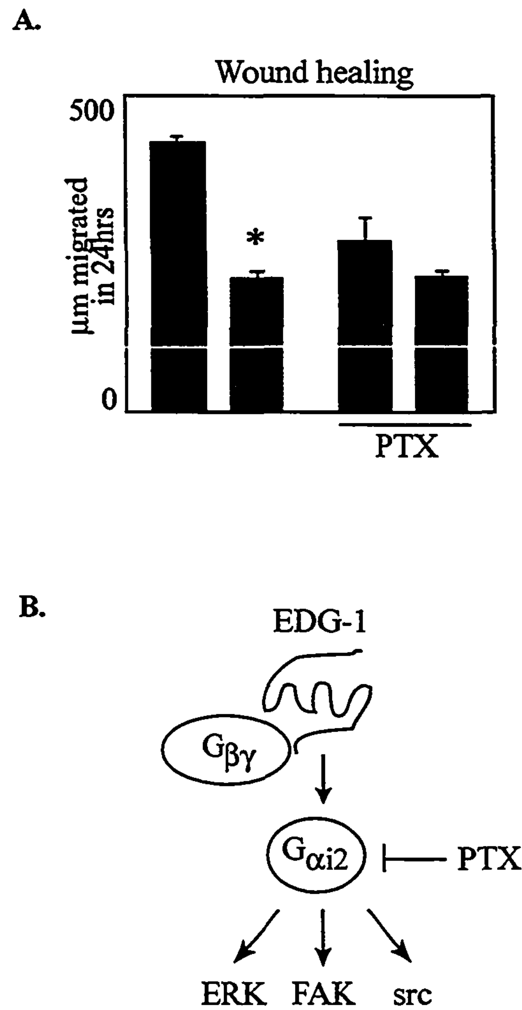

Figure 16: Galphai2 signaling in PECAM-1 mediated migration.

A. PTX abrogates the wound-healing advantage of PecamRC endothelial cells (* $p < 0.005$ vs control, $n = 8$).

B. EDG-1 receptor mediated signaling releases active GTP bound Galphai2, which activates ERK, FAK and src. Pertussis toxin (PTX) directly inhibits Galphai2.

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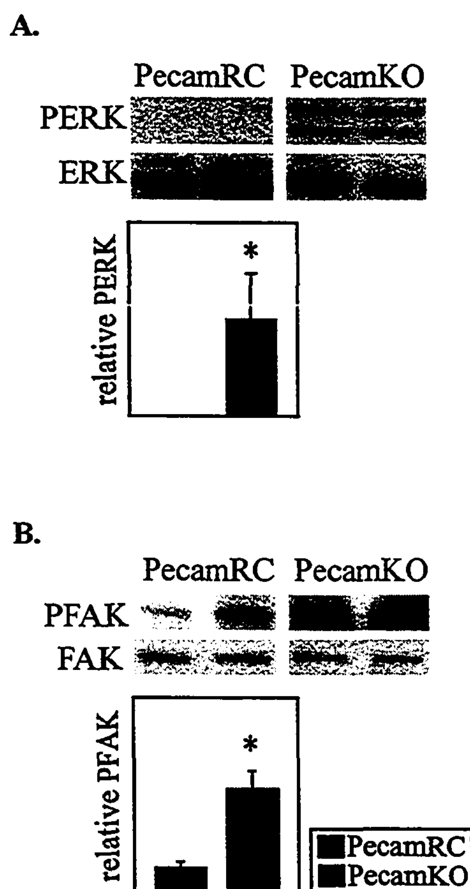

Figure 17: Increased ERK and FAK signaling in PecamKO cells.

Confluent endothelial cells were evaluated for:

A. Activated ERK (PERK) and **B.** FAK phosphorylated at Y397 (PFAK). Quantitation of PERK normalized to total ERK and PFAK normalized to total FAK revealed significantly increased activation of both FAK and ERK in PecamKO (* $p < 0.05$ vs PecamRC; $n = 4$).

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PECAM-1 promotes coordinated migration to sphingosine-1-phosphate

PECAM-1-mediated wound healing is Rho-dependent and sensitive to PTX, consistent with heterotrimeric G protein-mediated signaling such as that which occurs in response to sphingosine-1-phosphate (S1P). Sphingosine-1-phosphate (S1P) is a major serum-derived mediator of endothelial chemotaxis, wound-healing endothelial migration, angiogenesis, and vascular maturation (English et al., 2001). To assess S1P-mediated migration, I measured wound healing in the absence of serum-derived sphingolipids, with physiologic levels of S1P added back, and in baseline sphingolipid-replete endothelial culture medium containing 10% FBS. Sphingolipid-free medium was prepared using FBS that had been charcoal-stripped to deplete it of sphingolipids (CS-FBS). Such treatment depletes S1P without significantly affecting levels of non-lipid growth factors such as VEGF and FGF-2 (English et al., 2000).

Wound healing migration in sphingolipid-depleted CS-FBS medium was compared to migration in medium supplemented with 100 nM S1P [about half the concentration of S1P in normal human plasma (Yatomi et al., 1997)] as well as to migration in baseline sphingolipid-replete medium (**Figure 18**). PecamRC cells responded to S1P supplementation and to sphingolipid-replete medium with significantly increased migration, whereas PecamKO cells experienced no such increase in wound healing migration, whether in the presence of sphingolipids in general or S1P specifically. Two-way ANOVA revealed a statistically significant cell type by treatment effect, the difference in the response of PecamKO versus PecamRC cells to S1P. In fact

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there was even a nonsignificant decrease in migration of PecamKO cells with S1P supplementation and a significant decrease in wound healing in sphingolipid-replete media. It is tempting to speculate that this decrease may be due to the high nondirected motility seen in PecamKO endothelial cells; a further increase in random initiation of motility in response to S1P may further interfere with the coordinated, directional nature of wound healing migration. One would predict that while the single cell Transwell™ motility of PecamKO cells may increase somewhat over an already high baseline in the presence of S1P, their directed motility in response to an S1P gradient would be severely compromised.

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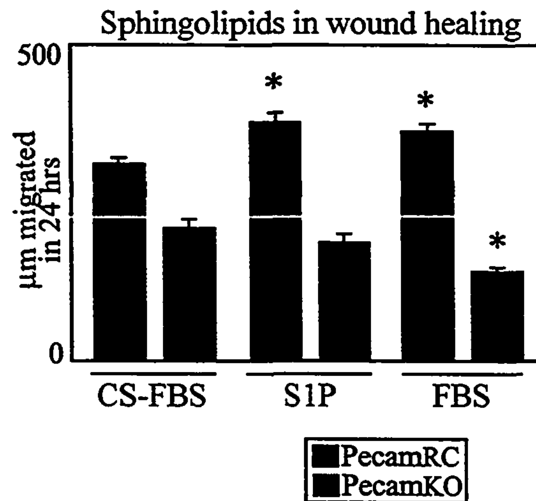

Figure 18: Lack of appropriate S1P response in PecamKO cells.

Wound healing migration was assessed in sphingolipid-depleted media (CS-FBS), in CS-FBS supplemented with 100 nM S1P, and in non-depleted media (FBS). PecamRC cells had increased migration from in response to S1P or sphingolipid-replete media, whereas PecamKO cells had no such increase. (* $p < 0.005$ vs CS-FBS, $n = 8$). Two-way ANOVA reveals a significant cell type by treatment effect ($F = 16.86$, $p < 0.000005$)

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PECAM-1 localizes to caveolin-positive lipid rafts

Lipid rafts are cholesterol-rich liquid-ordered subdomains within the liquid-disordered plasma membrane (Galbiati et al., 2001). Such lipid raft domains have been found to help segregate associated proteins to the leading edge versus the rear of certain polarized migrating cells (Gomez-Mouton et al., 2001). A subset of lipid rafts which contain oligomerized caveolin-1 form small invaginations or caveolae which are particularly enriched in vascular endothelium. A number of signaling proteins including heterotrimeric G proteins, src and H-ras localize to these endothelial subdomains (Bar-Sagi, 2001; Lisanti et al., 1994), providing spatially localized signaling information that could potentially contribute to polarized cellular behaviors including migration. Although caveolae as such are not concentrated at cell-cell junctions, caveolin-positive low density lipid raft domains have also been found to interact with components of adherens junctions (Galbiati et al., 2000), tight junctions (Nusrat et al., 2000), glycophosphoinositol-linked adhesion molecules (Harris and Siu, 2002), and even gap junctions (Schubert et al., 2002), and as such could play a role in the loosening of cell-cell adhesions that permits coordinated migration of cells into a wound to occur.

I used sucrose density gradient fractionation to isolate the low density caveolin-positive lipid raft fraction of PecamRC and PecamKO endothelial cell monolayers **(Figure 19a)**. Methyl ß cyclodextrin (mßcd) disperses low density lipid rafts by depleting them of cholesterol. Treatment with mßcd eliminated the low density caveolin positive fraction, confirming that it consists of lipid rafts including caveolae.

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Interestingly, PECAM-1 was found to colocalize with caveolin-1 to the lipid raft fraction (**Figure 19b**); mßcd treatment also disrupted the low density fractionation of PECAM-1.

To determine whether the raft localization of PECAM-1 might relate to its role in promoting coordinated endothelial migration, I assessed the effect of raft disruption on wound healing and single cell motility. It must be stressed that the cellular effects of mßcd treatment are not limited to raft disruption, but that membrane cholesterol depletion additionally has a wide range of nonspecific effects including plasma membrane depolarization and a nonspecific depletion of Ca^{2+} stores (Pizzo et al., 2002). Thus these experiments are best interpreted as a way to rule out (or not) rather than a way to rule in (or not) the possibility of a role of raft localization of PECAM-1 in migration.

Additional complications arise from the fact that raft disruption interferes with multiple signaling pathways, not just those potentially associated with PECAM-1. In particular, mßcd disrupts the raft localization of H-ras (Bar-Sagi, 2001) and hence its ability to activate MEK and subsequently ERK. Treatment with 2 mM mßcd inhibits ERK activation in wound healing PecamRC and PecamKO endothelial cells (**Figure 20a**). Treatment with 10 μm U0126, a MEK inhibitor, to inhibit ERK signaling confirmed its necessary role in both wound healing and single cell motility (**Figure 20b**), meaning that mßcd-induced inhibition of ERK signaling must be accounted for in the interpretation of raft disruption experiments.

In order to differentiate the effect of raft disruption from that of the concomitant inhibition of ERK signaling, endothelial cells were treated with 10 μm U0126 plus or minus 2 mM mßcd to eliminate ERK signaling even in the absence of raft disruption. Interestingly, mßcd treatment in the absence of ERK signaling decreased endothelial

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wound healing (**Figure 21a**) but increased single cell motility (**Figure 21b**). Thus the effect of raft disruption on endothelial migration is analogous to the inhibition of Rho signaling or the depletion of PECAM-1 expression of endothelial cells. (Similar results were also obtained in the absence of U0126 to inhibit MEK). While any experiments utilizing mßcd to disrupt rafts are necessarily nonspecific and may be attributable to non-raft-mediated effects of mßcd, these experiments at least fail to rule out the possibility that rafts may play a role in promoting coordinated wound healing migration, and that the raft localization of PECAM-1 may be pertinent to its role in mediating these effects.

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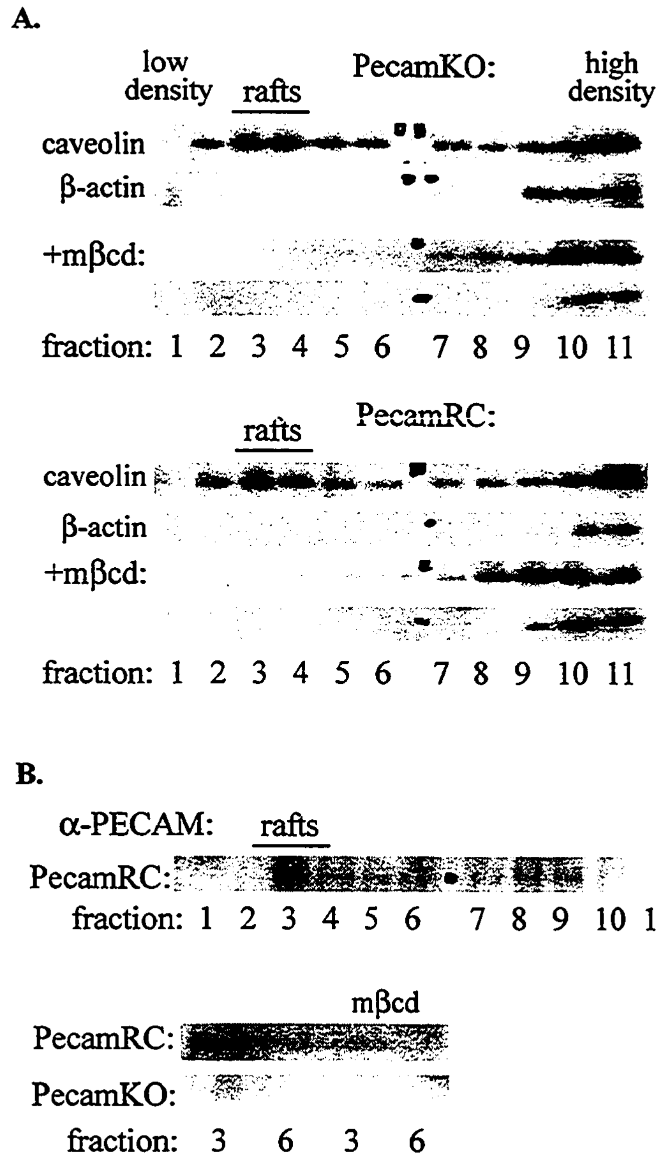

Figure 19: PECAM-1 localizes to a low density raft fraction.

A. Low density, caveolin positive lipid rafts separate from most other proteins by sucrose gradient fractionation. Methyl β cyclodextrin (mβcd) disrupts lipid rafts and disperses the low density caveolin positive fraction.

B. PECAM-1 cofractionates with lipid rafts in PecamRCcells, and disappears from the low density fraction upon raft disruption.

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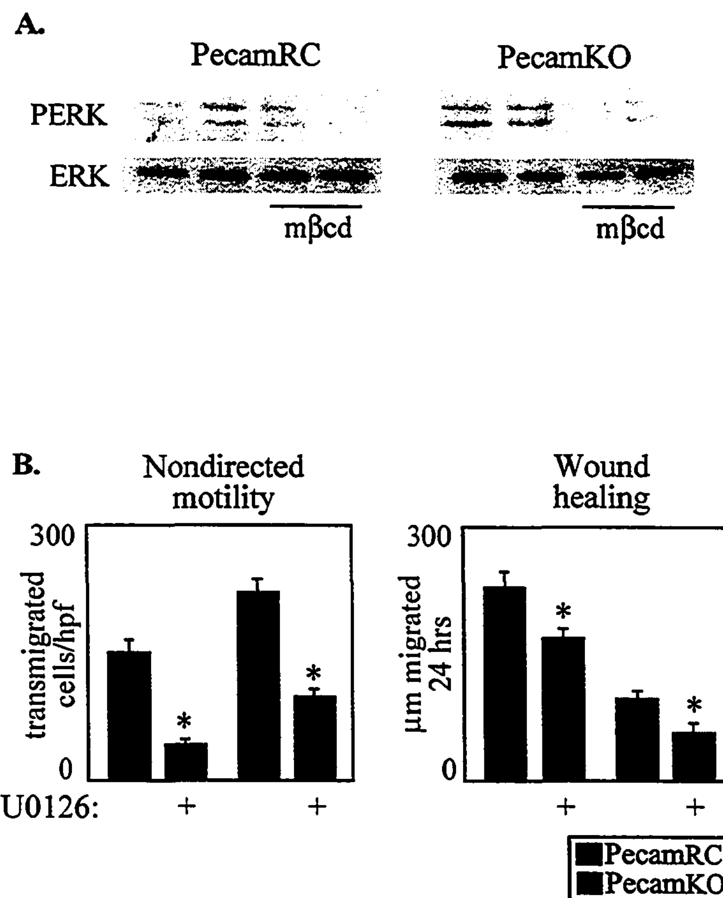

Figure 20: ERK signaling supports both motility and wound healing:

A. Raft disruption interrupts ERK signaling in wound healing endothelial cells.

B. Treatment with the MEK inhibitor U0126 at 10 µm to suppress ERK activation interferes with both single cell and wound healing motility in PecamRC and PecamKO cells. (n = 16 for wound healing, n = 9 for motility; *p < 0.005)

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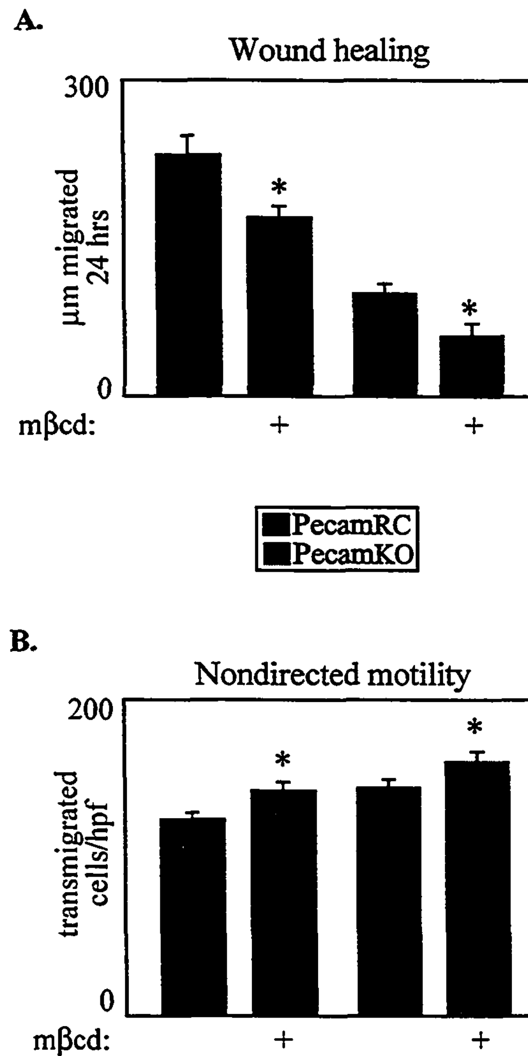

Figure 21: Methyl ß cyclodextrin inhibits wound healing and increases motility.

Cells were pretreated with U0126 to block ERK activation independent of raft disruption; the effect of mßcd treatment and raft disruption on wound healing migration was evaluated.

A. Wound healing migration decreases significantly upon mßcd treatment. (n = 8, *$p < 0.05$ vs U0126 alone)

B. Single cell motility increases upon mβcd treatment (n = 9, *$p < 0.05$ vs U0126 alone).

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PECAM-1 modulates the relative recruitment of Galphai2 and Galpha13 to rafts

PECAM-1 cofractionates with low density caveolin-positive lipid rafts, and raft integrity promotes coordinated wound healing migration over nondirected single cell motility. PECAM-1 expression is needed for an effective coordinated migration response to S1P. Galpha subunits also localize to low density lipid rafts and move in and out of caveolae (Oh and Schnitzer, 2001). Caveolin-1 interactions may modulate heterotrimeric G protein signaling: either directly by binding the inactive, GDP-bound version of Galpha heterotrimeric protein subunits and inhibiting GDP/GTP exchange (Li et al., 1995), or indirectly by transiently sequestering dissociated Galpha subunits, desensitizing signaling via heterologous seven transmembrane receptors that share the same G protein (Murthy and Makhlouf, 2000). Finally, overexpression experiments in several non-endothelial cell lines have demonstrated that EDG-1 localizes to caveolin-positive low density lipid rafts (Igarashi and Michel, 2000), a process that is required for ligand-induced receptor internalization and resensitization (Kohno et al., 2002). Therefore the relative recruitment of Galpha subunits to caveolin positive rafts, and their accessibility to either EDG family receptors or to inhibitory associations with caveolin-1, may have a direct bearing on Galpha subunit localization and degree of activation.

S1P-mediated migration has a pertussis-inhibitable, EDG-1/Galphai2 mediated component which results in activation of ERK signaling and FAK phosphorylation, and an EDG-3 mediated component that couples to Galpha13, contributing to Rho activation. The ERK, FAK, and Rho signaling characteristics of PecamKO as compared to those of PecamRC endothelial cells suggest a model in which the Galphai2-mediated signal is

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promiscuously activated, and the Galpha13 component is defective, in the absence of PECAM-1. Given the potential role of raft localization in modulating heterotrimeric G protein mediated signaling, I assessed the relative targeting of Galphai2 versus Galpha13 to rafts in PecamRC versus PecamKO endothelial cells (**Figure 22a**). The results of these preliminary experiments were consistent with a role for PECAM-1 in supporting the targeting of Galpha subunits to lipid rafts: the ratio of Galphai2 to Galpha13 in the low density caveolin positive fraction was decreased by more than half in PecamKO as compared to PecamRC endothelial cells (**Figure 22b**).

To begin to explore how the raft localization of PECAM-1 may contribute to Galpha mediated Rho signaling, I assessed the tyrosine phosphorylation of p120RasGAP -associated p190RhoGAP in PecamRC and PecamKO endothelial cells that had been treated with mßcd to disperse rafts (**Figure 23a**). PecamKO endothelial cells have increased levels of p190RhoGAP tyrosine phosphorylation and p120RasGAP association at baseline, which should in turn promote p190RhoGAP activation, and therefore likely contributes to the low baseline RhoGTP levels in the absence of PECAM-1 (**Figure 10**). Raft dispersion (mßcd treatment) increased p120RasGAP-associated p190RhoGAP tyrosine phosphorylation in PecamRC endothelial cells to levels similar to those seen in PecamKO cells (**Figure 23b**). Cholesterol depletion via mßcd treatment is a fairly nonspecific treatment, as discussed in the preceding section. Nevertheless, it should prove fruitful to explore the potential role of PECAM-1 in promoting Galphai2-mediated src activation and p190RhoGAP tyrosine phosphorylation.

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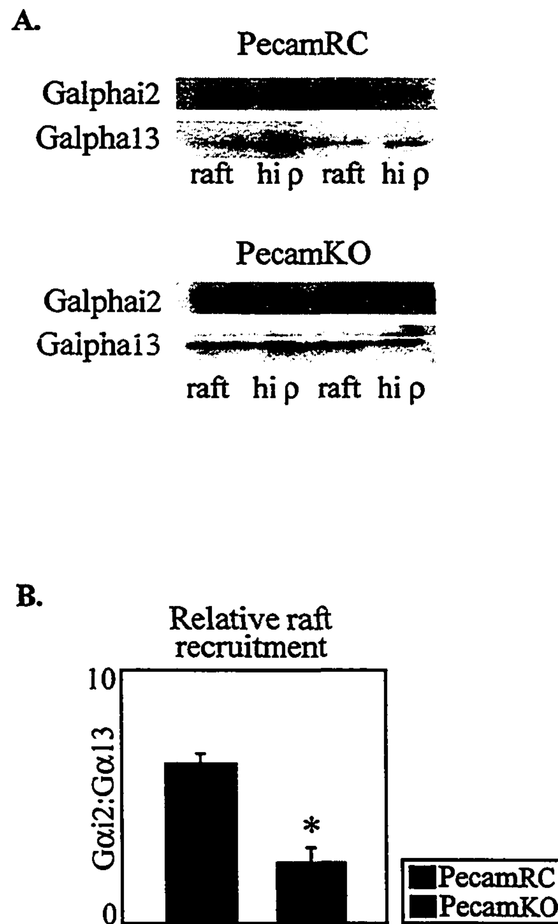

Figure 22: PECAM-1 modulates raft recruitment of Galpha subunits.

A. Western blots demonstrate raft localization of Galphai2 and Galpha13 subunits.

B. Quantitation reveals higher ratio of Galphai2:Galpha13 in the presence of PECAM-1 ($n = 4$, $*p < 0.05$).

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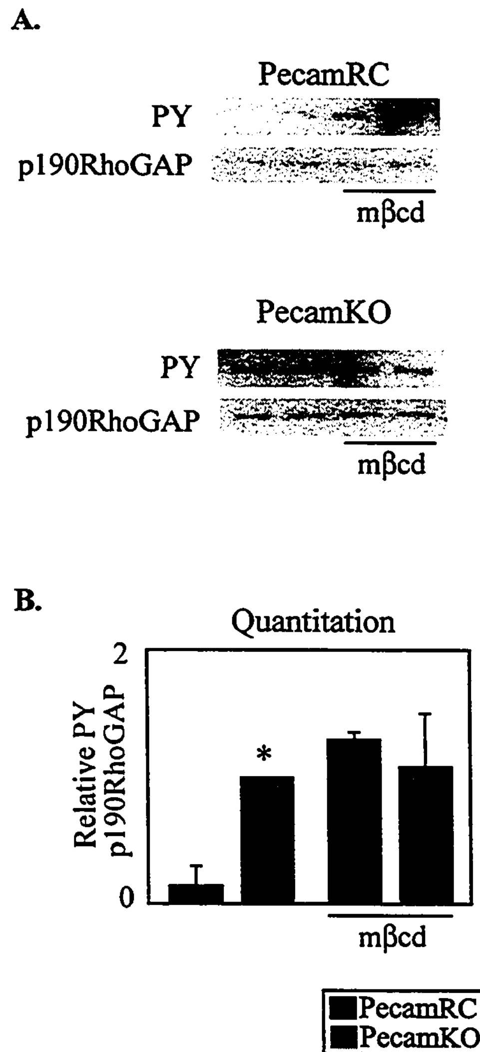

Figure 23: Methyl ß cyclodextrin treatment increases p190RhoGAP PY.

A. Western blot showing tyrosine phosphorylation of p190RhoGAP coprecipitating with p120RasGAP.

B. Quantitation reveals significant increase in p190RhoGAP PY in response to mβcd treatment in PecamRC cells, to levels comparable to PecamKO cells.

(n = 2, *p < 0.05 vs PecamRC)

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Discussion

The data presented above suggest a significant new role for PECAM-1 in balancing coordinated wound healing migration and nondirected motility of endothelial cells. The PECAM-1 knockout mouse is overtly developmentally normal, but abnormalities of endothelial function become apparent in response to appropriate insult. During the chronic phase of foreign body inflammation, for example, angiogenesis is severely compromised in the PECAM-1 knockout mouse (Solowiej et al., 2002). An *in vitro* model of angiogenesis likewise reveals a prominent defect in tube formation in immortalized lung endothelial cells derived from the PECAM-1 knockout mouse (Solowiej et al., 2002). Embryonic endocardial cells derived from the cardiac cushion of the PECAM-1 knockout mouse, on the other hand, are aberrantly resistant to glucose-mediated inhibition of VEGF-induced scattering motility (Enciso et al., 2003). PECAM-1 deficient endothelial cells, then, are on the one hand defective in forming a stable new vasculature, and conversely are resistant to inhibition of endocardial cell motility.

Consistent with their dichotomous migratory behavior *in vivo*, PecamKO endothelial cells exhibit enhanced nondirected single cell motility and lamellipodial extension formation at the expense of wound-healing migration. The contribution of PECAM-1 to this migratory phenotype was confirmed by depleting PECAM-1 in primary endothelial cells using antisense technology. The migratory differences are accounted for by a selective deficiency of RhoGTP as opposed to RacGTP in PecamKO cells, since inhibition of either Rho itself or the Rho effector p160ROCK reproduced the PECAM-1-deficient migratory phenotype.

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The decrease in RhoGTP loading could be at least partially attributed to increased activation of p190RhoGAP, a GTPase activating protein, as marked by tyrosine phosphorylation and association of p190RhoGAP with p120RasGAP. **Figure 24a** delineates growth factor and integrin engagement-mediated lamellipodial extension and initiation of motility: some growth factors such as VEGF (Soga et al., 2001) activate Rac, which itself inhibits Rho (Sander et al., 1999); other growth factors (Haskell et al., 2001) and integrins (Arthur et al., 2000) activate src family kinases which phosphorylate and activate p190RhoGAP, inhibiting Rho indirectly. In fact, overexpression of p190RhoGAP alone derepresses Rac-mediated lamellipodial extension formation and enhances motility (Arthur et al., 2000). The insensitivity of PECAM-1-deficient endocardial cells to glucose-mediated suppression of VEGF-induced motility may thus be attributable to baseline low RhoGTP. In fact, like primary PECAM-1-deficient endocardial cells, PecamKO endothelial cells are resistant to glucose-mediated inhibition of single cell motility; pretreatment with an inhibitor of the Rho effector p160ROCK abrogates the inhibitory effect of glucose on PecamRC endothelial cell motility.

S1P is a major endothelial chemotactic component of serum which engages EDG-1 and EDG-3 seven-transmembrane receptors to mediate endothelial migration and angiogenesis (Lee et al., 1999a; Lee et al., 1998) via downstream signaling through Galphai2 to FAK, ERK, and src (Lee et al., 1999b; Rosenfeldt et al., 2001) and through Galpha13 to Rho (**Figure 24b**) (Buhl et al., 1995; Offermanns et al., 1997). The wound-healing advantage of PecamRC cells is not only Rho-dependent but PTX-inhibitable, a pattern characteristic of heterotrimeric G protein-mediated endothelial migration such as that mediated by S1P. Physiologic levels of S1P increase wound healing migration of

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PecamRC endothelial cells, as expected; PecamKO endothelial cells in contrast are not responsive to S1P. Indeed the relative wound-healing advantage of PecamRC cells is much decreased in sphingolipid-depleted serum, implicating the heterotrimeric G protein-mediated sphingolipid signaling pathway in the promotion of wound healing migration by PECAM-1. Elevated threonine/tyrosine phosphorylation of ERK and tyrosine phosphorylation of FAK in confluent PecamKO cells is consistent with dysregulated and ineffective Galphai2 signaling downstream of EDG-1 receptor. Src activity is also likely increased in PecamKO cells: increased FAK at PY397 indicates that more src is likely bound and activated at focal contacts (Eide et al., 1995); and tyrosine phosphorylation of p190RhoGAP, which is also increased, is likewise mediated by src (Arthur et al., 2000).

Global activation of these pathways in and of itself is insufficient to permit directed migration and coordinated wound-healing. EDG-1 null fibroblasts, like PecamKO endothelial cells, display aberrant FAK and src activation but a decreased wound healing response to S1P; local FAK and src activation at the leading edge promotes the directed extension formation and migration of wildtype fibroblasts in response to S1P (Rosenfeldt et al., 2001). Interestingly, another immunoglobulin family molecule containing ITIM sequences in its cytoplasmic domains, SHPS-1, may play a similar role to that of PECAM-1 in fibroblasts: expression of SHPS-1 lacking its cytoplasmic domain in fibroblasts blocks RhoGTP activation and wound healing migration, promotes extension formation, and increases ERK and FAK phosphorylation (Inagaki et al., 2000).

A model of defective sphingosine-1-phosphate-mediated signaling and Rho activation in PECAM-1-deficient endothelial cells incorporates these observations

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(Figure 24b,c). In PECAM-1-positive endothelial cells S1P signals through Galphai2 and Galpha13 in a coordinated manner to produce directed extension formation and directed wound healing migration rather than nondirected motility **(Figure 24b)**. In PECAM-1-deficient endothelial cells EDG-1 and Galphai2 are activated in a non-coordinated manner, leading to baseline activation of ERK, FAK, and src **(Figure 24c)**. Phosphorylation activates p190RhoGAP and suppresses RhoGTP; EDG-3-mediated signaling to Galpha13 is insufficient to activate Rho, favoring extension formation and nondirected motility over directed wound healing migration. This lack of coordinated responsiveness to serum sphingolipid chemotactic signals may account for the poor angiogenesis into polyvinyl acetate sponge implants in the PECAM-1 knockout mouse in a model of the chronic phase of foreign body inflammation (Solowiej et al., 2002). PECAM-1 modulates the selective recruitment of Galphai2 versus Galpha13 to caveolin positive low density lipid raft fractions, where their activity might be modulated via association with EDG receptors and with caveolin-1. It will be rewarding to investigate the circumstances under which PECAM-1-mediated modulation of heterotrimeric G-protein signaling to Rho family small G proteins becomes pathophysiologically relevant.

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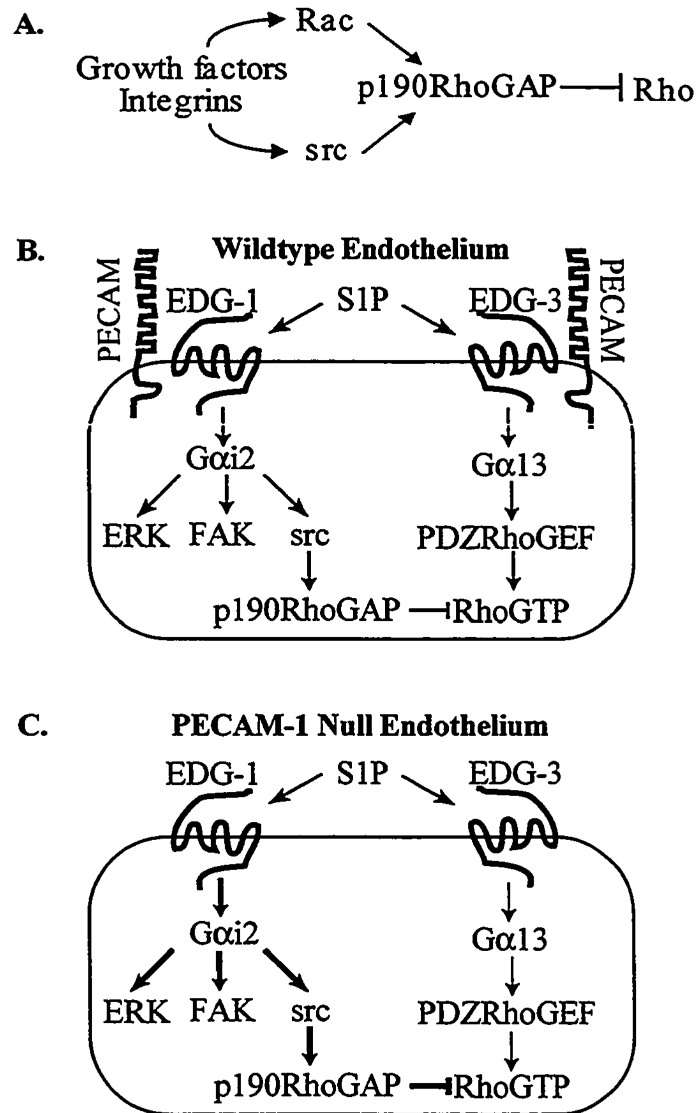

Figure 24: Model for role of PECAM-1 in endothelial motility.

A. Growth factor or integrin signaling coordinately activates Rac and inhibits Rho via src mediated phosphorylation of p190RhoGAP, allowing lamellipodial extension and motility.

B. In PECAM-positive cells S1P signals in a coordinated manner to produce directed extension formation and wound healing rather than nondirected motility.

C. In PECAM-null endothelium noncoordinated EDG receptor signaling favors extension formation and nondirected motility over directed wound healing.

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Chapter 3: The PECAM-1 ITIM in endothelial wound healing migration

Summary

Platelet-Endothelial Cell Adhesion Molecule-1, PECAM-1, a transmembrane endothelial adhesion protein, binds and activates the tyrosine phosphatase SHP-2 via phosphotyrosines 663 and 686. PECAM-1 phosphorylation and recruitment of SHP-2 is regulated by cell-cell and cell-substrate adhesion. PECAM-1 is dephosphorylated on tyrosine 686 during endothelial migration, resulting in diffuse dispersal of PECAM-1 and SHP-2. Overexpression of native PECAM-1 slowed, and nonphosphorylatable PECAM-1 increased, endothelial migration, implying that the SHP-2-regulatory phosphotyrosines negatively regulate migration. Using differentially phosphorylated recombinant proteins I found that phosphotyrosine 686 preferentially mediates binding and 663 mediates activation of SHP-2 by PECAM-1. In PECAM-1-null endothelial cells, SHP-2 bound and dephosphorylated an alternative set of phosphoproteins and its distribution to a Triton-insoluble, likely cytoskeletal fraction was significantly decreased. Tyrosine phosphorylation of beta catenin and focal adhesion kinase was increased in endothelial cells overexpressing nonphosphorylateable PECAM-1. Thus homophilically engaged, tyrosine-phosphorylated PECAM-1 locally activates SHP-2 at cell-cell junctions; with disruption of the endothelial monolayer, selective dephosphorylation of PECAM-1 leads to redistribution of SHP-2 and pro-migratory changes in phosphorylation of cytoskeletal and focal contact components.

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Introduction

Platelet-Endothelial Cell Adhesion Molecule, or PECAM-1, is a homophilically adhesive transmembrane glycoprotein (Albelda et al., 1990; Newman et al., 1990) which modulates endothelial migration, tube formation, and angiogenesis *in vivo* and *in vitro* (Cao et al., 2002; DeLisser et al., 1997; Kim et al., 1998). Many of these functions localize to the Immunoreceptor Tyrosine-based Inhibitory Motif or ITIM (Newman, 1999), which binds and activates SH2-domain-containing proteins (Pumphrey et al., 1999). A model of the PECAM-1 ITIM seen in **Figure 25a** shows the orientation of ITIM tyrosines 663 and 686 within a bend in the cytoplasmic domain (Kim et al., 1998). ITIM tyrosine 686 is dephosphorylated in endothelial cells upon engagement of 1 integrins (Lu et al., 1996) and during normal yolk sac vasculogenesis (Pinter et al., 1997); dephosphorylation is abrogated during abnormal vasculogenesis in glucose-induced embryopathy (Pinter et al., 1999). Conversely, PECAM-1 mutated at tyrosine 686 increases endothelial wound healing migration (Kim et al., 1998). PECAM-1 phosphorylation at tyrosine 686 also increases homophilic cell-cell adhesion, supporting a role for the ITIM in inside-out signaling (Famiglietti et al., 1997; Yan et al., 1995)

In endothelial cells the tyrosine phosphatase SHP-2 is a major PECAM-1 ITIM partner (Jackson et al., 1997; Masuda et al., 1997). SHP-2 was identified as a homologue of a *Drosophila* gene, *csw*, involved in early development downstream of a receptor tyrosine kinase (Freeman et al., 1992). It is a ubiquitously-expressed protein tyrosine phosphatase which is activated by occupancy of its two tandem SH2 domains (Dechert et al., 1994; Sugimoto et al., 1993). The crystal structure of SHP-2 (**Figure 26a**) has

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revealed that a portion of the N-terminal SH2 domain (N-SH2) sterically blocks access to the active site (Barford and Neel, 1998). Phosphotyrosine engagement of the N-SH2 domain shifts it out of the active site, sterically activating the enzyme (Hof et al., 1998). Engagement of the C-terminal SH2 domain contributes to binding specificity of appropriately spaced phosphotyrosines, but does not contribute to enzyme activation. The spacing of the SH2 domains of SHP-2 (Eck et al., 1996) confers on it high specificity for engagement by the tandem phosphotyrosines located in ITIMs (Ottinger et al., 1998), including that of PECAM-1 (Jackson et al., 1997), as opposed to the more closely spaced bisphosphorylated ITAMs (immunoreceptor tyrosine-based activation motifs) (Ottinger *et al.*, 1998). Thus the phosphorylation of one or both PECAM-1 ITIM tyrosines may be expected to have differential effects both on SHP-2 recruitment and on modulation of its tyrosine phosphatase activity.

Coordinated migration of endothelial cell monolayers involves loosening of adherens junctions and focal contact turnover. Vascular endothelial cadherin (VE-cadherin) plays a large role in maintaining tight endothelial cell-cell interactions at adherens junctions (Corada et al., 1999). These junctions undergo major reorganization at the migrating front of a wounded endothelial monolayer (Lampugnani et al., 1995). Beta catenin becomes tyrosine phosphorylated in response to stimuli that induce an increase in vascular permeability and angiogenesis, such as vascular endothelial growth factor or VEGF (Cohen et al., 1999). Linkage of VE-cadherin to the actin cytoskeleton via beta catenin association with alpha catenin (Provost and Rimm, 1999), and hence the stability of cadherin-mediated cell-cell adhesion, is regulated by beta catenin tyrosine phosphorylation (Ukropec et al., 2002).

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A number of protein tyrosine phosphatases, including LMW-PTPase (Taddei et al., 2002), RPTPase (receptor-type protein tyrosine phosphatase) beta/zeta (Meng et al., 2000), PTP1B (Balsamo et al., 1998), and PTP-LAR (leukocyte common antigen related) have been implicated in the maintenance of cadherin-mediated cell-cell adhesion through dephosphorylation of beta catenin by overexpression in non-endothelial cells. In endothelial cells, and in the absence of overexpression, SHP-2 is the only protein tyrosine phosphatase that has been implicated in beta catenin dephosphorylation. Endothelial PECAM-1 binds tyrosyl-phosphorylated beta catenin (Ilan et al., 1999); beta catenin is therefore in a position to be targeted for dephosphorylation by PECAM-1 ITIM activated SHP-2. Although no direct evidence of SHP-2-mediated dephosphorylation of beta catenin is available, the loss of SHP-2 from adherens junction complexes in response to thrombin (Ukropec et al., 2000) or shear stress (Ukropec et al., 2002) correlates with increased beta catenin phosphorylation and loss of cadherin-cytoskeletal linkage.

Focal adhesion kinase (FAK) activity, mediated by autophosphorylation (Owen et al., 1999), is also required for migration (Gilmore and Romer, 1996). SHP-2 modulates tyrosine phosphorylation of FAK and thus focal contact turnover and cell motility (Gilmore and Romer, 1996; Yu et al., 1998), and FAK tyrsoine phosphorylation is increased in PECAM-1 deficient endothelial cells (**Figure 17b**), providing another potential target for PECAM-1 ITIM-associated SHP-2 dephosphorylation. The functional role of PECAM-1-mediated modulation of SHP-2 tyrosine phosphatase activity in endothelial cell migration however remains unclear. To this end I studied the role of the PECAM-1 ITIM in migration-related SHP-2 recruitment and activation, and the dephosphorylation of potential target phosphoproteins relevant to migration.

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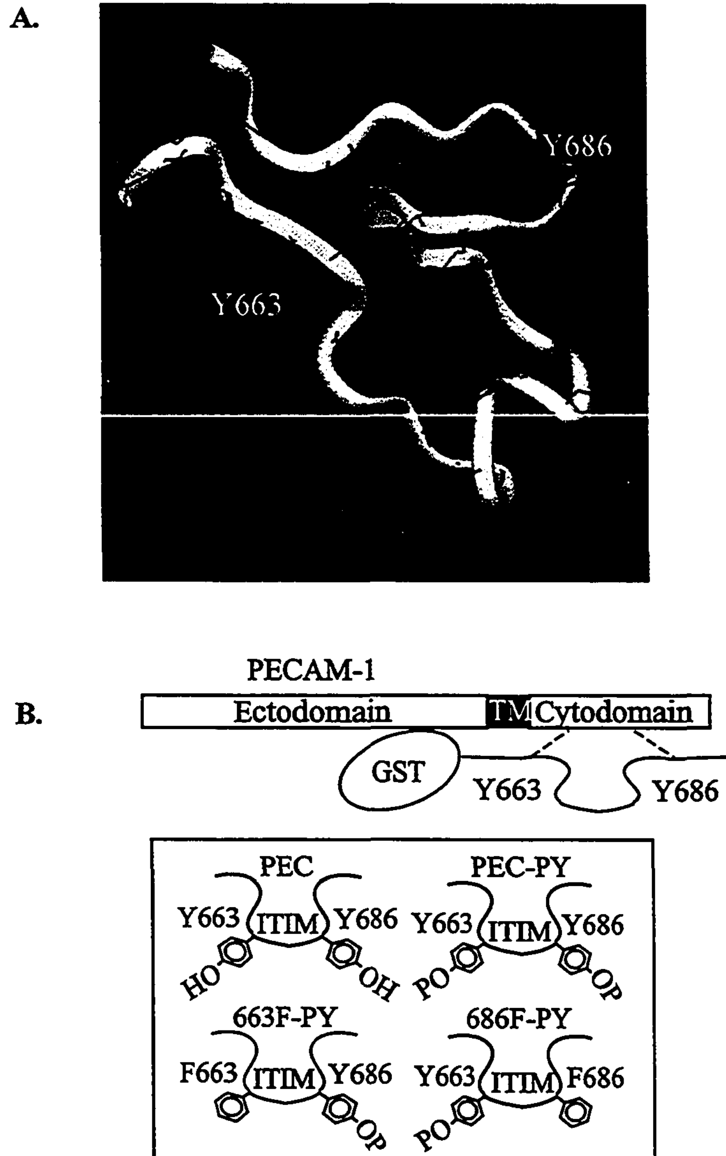

Figure 25: A model of the PECAM-1 ITIM.

A. A model of the PECAM ITIM reveals a loop in the cytoplasmic domain which makes tyrosines 663 and 686 readily available for phosphorylation (Kim C.S. *et al*, Lab Invest. 78:583, 1998).

B. The PECAM-1 cytoplasmic domain was as a GST fusion protein; the ITIM tyrosines were individually mutated to allow for selective phosphorylation.

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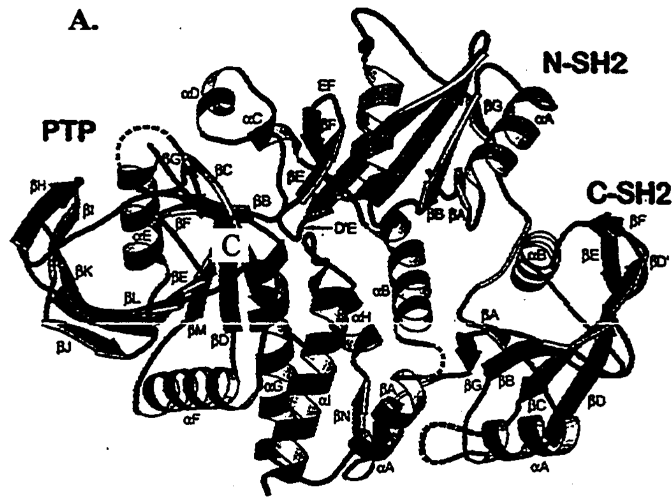

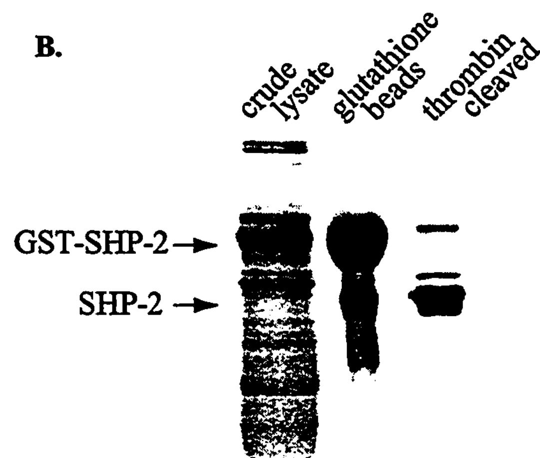

Figure 26: Recombinant SHP-2 production.

A. Model derived from crystal structure of SHP-2 (Hof P. *et al*, Cell 92:441 (1998).

N-SH2 domain moves out of catalytic cleft upon PY engagement. (C = catalytic cysteine)

B. GST-SHP-2 was captured on glutathione beads, then thrombin-cleaved to remove GST.

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Results

SHP-2 is preferentially bound by PECAM-1 phosphotyrosine 686

The PECAM-1 cytoplasmic domain was expressed as a fusion protein, with glutathione-S-transferase at its N-terminal (Lu et al., 1997) allowing ease of purification using glutathione agarose beads. GST-PECAM-1 containing site-directed mutations at tyrosines 663 and 686 of the ITIM was produced in bacteria expressing a kinase capable of phosphorylating available tyrosines **(Figure 25b)**. Western blotting with phosphospecific antibodies **(Figure 27a)** (Lu et al., 1996) confirmed site-specific tyrosine phosphorylation. Recombinant SHP-2 (rSHP-2) was cleaved from its GST tag at an N-terminal thrombin cleavage site **(Figure 26b)**. Pulldown assays using GST-PECAM-1 assessed the contribution of the respective ITIM phosphotyrosines to SHP-2 recruitment **(Figure 27b)**. PECAM-1 phosphorylated only at tyrosine 686 pulled down the same amount of SHP-2 as dually phosphorylated PECAM-1; lack of phosphorylation at 686 however significantly decreased SHP-2 binding. Thus both ITIM tyrosines are capable of binding SHP-2, but loss of phosphorylation at tyrosine 686 would likely lead to a significant decrease in PECAM-1-associated SHP-2.

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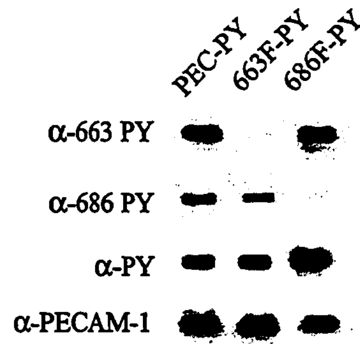
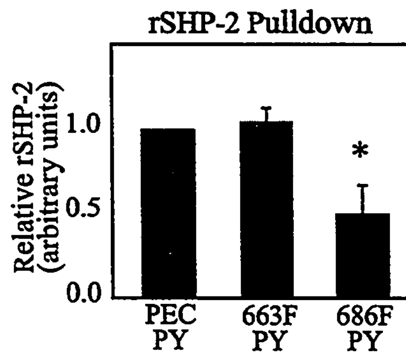
A. Phospho GST-PECAM-1:



B.



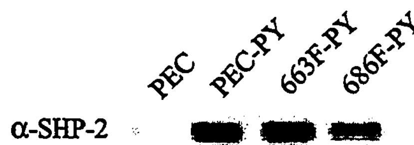


Figure 27: Contribution of ITIM tyrosines to SHP-2 binding.

A. GST-PECAM-1 phosphorylation was confirmed with phosphospecific antibodies.

B. Pulldown assay using phosphorylated GST-PECAM-1 as bait. 663F-PY pulls down as much SHP-2 as PEC-PY, but 686F-PY pulls down significantly less. ($n = 5$, $*p < 0.005$ vs PEC-PY).

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PECAM-1 phosphotyrosine 663 preferentially activates SHP-2

Engagement of the SH2 domains of SHP-2 serves not only to recruit the phosphatase but to activate it (Barford and Neel, 1998; Dechert et al., 1994). All reports of PECAM-1-mediated SHP-2 activation thus far have used phosphorylated peptides (Pluskey et al., 1995). However, the secondary and tertiary structure of the PECAM-1 cytoplasmic domain may have profound effects on SHP-2 binding and activation. I measured SHP-2 activity by monitoring dephosphorylation of the synthetic substrate pNPP (*p* nitrophenyl phosphate). Recombinant SHP-2 (rSHP-2) had a baseline activity of 15 nM pNP /minute, while rSHP-2 mutated in its catalytic acid (Flint et al., 1997) was inactive as expected (**Figure 28a**). The responsiveness of rSHP-2 to engagement of its SH2 domains was tested using ITIM phosphopeptides. Phosphopeptide 663 produced a dose-dependent increase in SHP-2 activity, while the same peptide in nonphosphorylated form produced no increase in phosphatase activity over baseline (**Figure 28b**).

The relative role of the two ITIM phosphotyrosines was then tested using phosphopeptides: while phosphopeptide 663 activated rSHP-2 in a dose-dependent manner, neither nonphosphorylated 663 nor phosphopeptide 686 did so (**Figure 29a**). Activation was then tested in the context of the full PECAM-1 cytoplasmic domain (**Figure 29b**). PECAM-1 phosphorylated at 663 alone activated SHP-2 as well as dually phosphorylated PECAM-1, whereas PECAM-1 phosphorylated at 686 caused very little activation over baseline. Thus the two individual phosphotyrosines of the PECAM-1 ITIM play individual and complementary roles in modulation of SHP-2 activity, with phosphotyrosine (PY) 686 recruiting and PY 663 activating the phosphatase.

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Figure 28: Assessment of recombinant SHP-2 phosphatase activity.

Phosphatase activity was measured using p-nitrophenyl phosphate (pNPP) as substrate.

A. rSHP-2 dephosphorylates pNP at 15 nM/min at baseline; a loss of function mutant (D425A) is inactive ($n = 5$, $*p < 0.005$).

B. Stimulation of SHP-2 activity by phosphorylated (663-PY) vs nonphosphorylated (663-Y) ITIM phosphopeptide.

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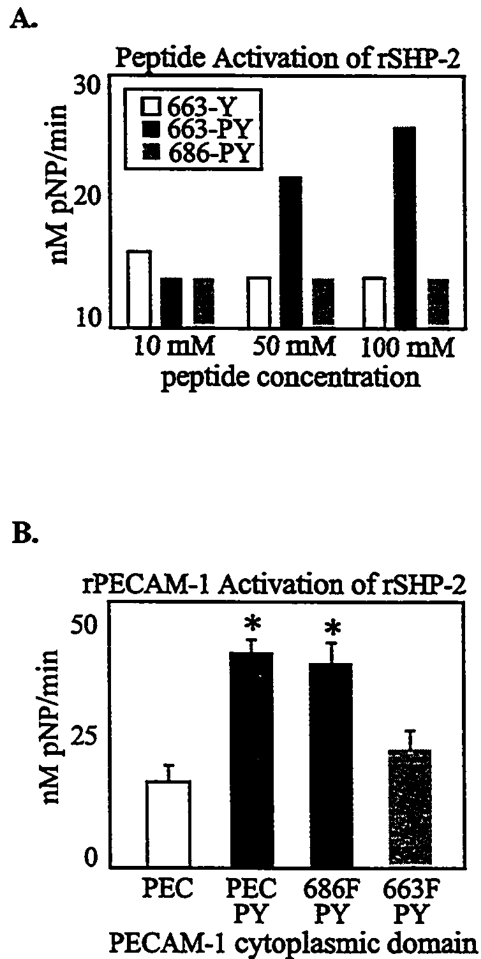

Figure 29: SHP-2 is preferentially activated by PY 663.

A. Phosphopeptides 663-PY but not 663-Y or 686-PY stimulate rSHP-2 activity in a dose dependent manner.

B. Stimulation of rSHP2 activity by rPECAM-1. PECAM-1 phosphorylated at both ITIM tyrosines (PEC-PY) or at 663 alone (686F-PY) but not 686 alone (663F-PY) significantly activated SHP-2 ($n = 6$, $*p < 0.005$).

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The PECAM-1 ITIM is selectively dephosphorylated during migration

PECAM-1 localizes to cell-cell contacts in confluent endothelial cells (Albelda et al., 1990); homophilically-engaged PECAM-1 is tyrosine-phosphorylated and able to bind SHP-2 (Bird et al., 1999). Accordingly PECAM-1 and SHP-2 co-localized to cell-cell borders of confluent human umbilical vein endothelial cells, whereas migrating cells displayed diffuse PECAM-1 and SHP-2 staining (**Figure 30**). Previous work from our laboratory has evaluated the relative phosphorylation of each of the PECAM-1 ITIM phosphotyrosines during migration (Gratzinger et al., 2003a). To assess PECAM-1 PY during migration, confluent bovine aortic endothelial cells (BAECs) were wounded by mechanical scraping. The remaining concentric circles of migrating endothelium were evaluated at 24 hours post wounding; by 72 hours, they had reachieved confluence. Immunoprecipitation revealed decreased total PECAM-1 PY, and consequently SHP-2 association, in migrating BAECs 24 hours post-wounding; at 72 hours, the newly confluent BAECs reachieved baseline PECAM-1 PY (**Figure 31a**). Analysis with phosphospecific antibodies revealed that relative dephosphorylation at tyrosine 686 (74%) was much greater than that at tyrosine 663 (21%) (**Figure 31b**). Thus, dephosphorylation of the PECAM-1 ITIM during migration occurs preferentially at the site that recombinant protein pulldown experiments implicate in recruiting SHP-2.

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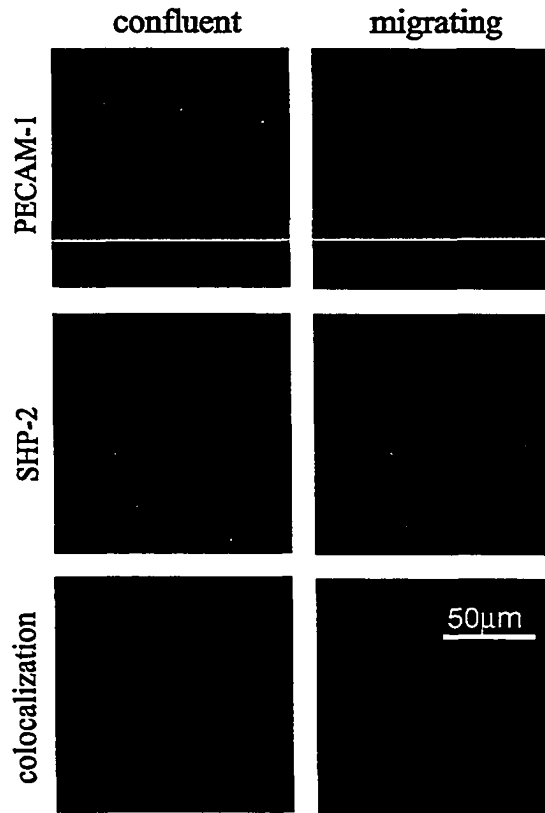

Figure 30: Colocalization of SHP-2 and PECAM-1.

Immunofluorescence reveals cell-cell border localization of both PECAM-1 (upper) and SHP-2 (middle) in confluent human umbilical vein endothelial cells; colocalization is shown at bottom. In migrating cells, PECAM-1 and SHP-2 are both diffusely localized.

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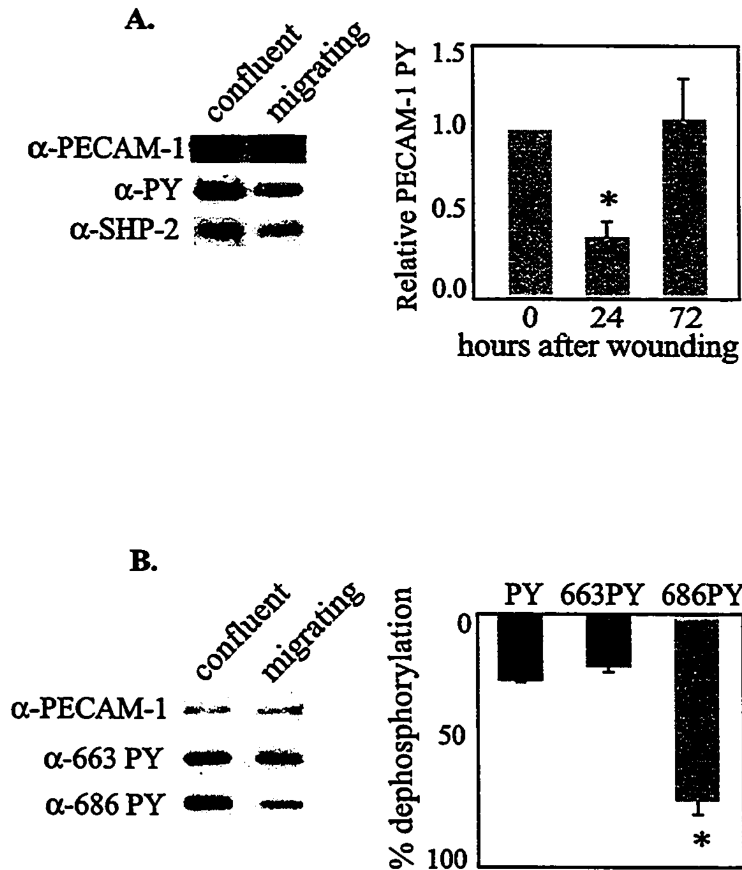

Figure 31: Preferential dephosphorylation at Y686 during migration.

A. Immunoprecipitation of PECAM-1 reveals decreased PY and SHP-2 association in migrating BAECs. Quantitation reveals a > 50% decrease in PECAM-1 PY at 24 hours wound healing, with baseline PY restored at confluence (72 hr) ($n = 3$, *$p < 0.05$ vs 0 hr).

B. Blotting with phosphospecific antibodies to the PECAM-1 ITIM reveals greater dephosphorylation at Y686 than at 663 ($n = 2$, *$p < 0.05$ vs total PY).

(Work of M. Barreuther (D. Gratzinger, M. Barreuther, J.A. Madri,unpublished data).

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PECAM-1 expression localizes SHP-2 to a Triton-insoluble fraction

Immortalized lung endothelial cells derived from the PECAM-1 knockout mouse (PecamKO) and reconstituted with full-length PECAM-1 (PecamRC) form contact-inhibited confluent monolayers and maintain expression of endothelial markers such as VE-cadherin (Graesser et al., 2002; Wong et al., 2000). To evaluate the role of PECAM-1 in localizing SHP-2 to potential target phosphoproteins, I assessed the relative recruitment of SHP-2 to a Triton X-100 insoluble fraction. Less than half as much SHP-2 was found in the Triton-insoluble pellet in PecamKO as PecamRC endothelial cells; Focal Adhesion Kinase (FAK) was by comparison found in comparable amounts regardless of endothelial PECAM-1 expression status (**Figure 32a**). The Triton-insoluble pellet represents cytoskeleton-associated proteins, but lipid raft components are also Triton-insoluble. Sucrose density gradient fractionation of the low-density, caveolin-positive lipid raft fraction (Sowa et al., 2001) however failed to demonstrate any significant partitioning of SHP-2 to rafts in either PecamKO or PecamRC endothelial cells (**Figure 32b**); SHP-2 instead localized to the higher density fractions along with beta actin.

The fact that PECAM-1 dependent SHP-2 partitioning to a Triton-insoluble fraction appears cytoskeletal rather than raft-associated despite the large fraction of PECAM-1 that fractionates in a low-density caveolin positive fraction (**Figure 19**) requires further clarification. Several possibilities exist: one, that PECAM-1-associated SHP-2 becomes dissociated at some point during the raft preparation process; two, that SHP-2 largely associates with that fraction of PECAM-1 that is not localized to lipid

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rafts; three, that SHP-2 is simultaneously raft and cytoskeletally associated, but fractionates with the cytoskeleton thanks to the properties of the separation technique; and four, that the change in SHP-2 localization is due not only to direct co-localization of SHP-2 with tyrosyl-phosphorylated PECAM-1, but to consequent changes in tyrosine phosphorylation of other SHP-2 target proteins which likewise provide sites for SHP-2 binding contribute to the relocalization of SHP-2. A combination of alternate biochemical methods of raft separation and immunostaining of intact cells should help to clarify these issues.

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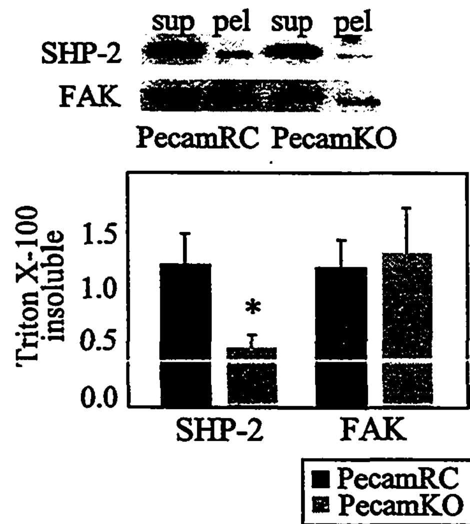

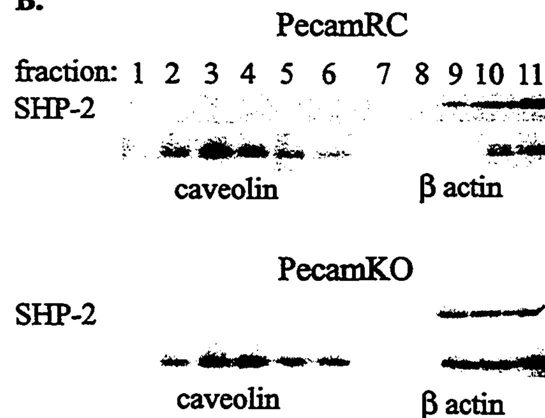

Figure 32: PECAM-1 modulates subcellular distribution of SHP-2.

Levels of SHP-2 and FAK in the Triton-insoluble pellet were normalized to actin.

A. PecamKO cells have significantly lower levels of SHP-2 but not FAK in the Triton X-100 insoluble fraction (n = 6, *$p < 0.05$ vs PecamRC).

B. Sucrose density gradient fractionation separates caveolin-postive lipid rafts (fractions 3-4) from nonraft proteins in β actin-positive fractions 9-11; SHP-2 is not present in rafts in PecamRC or PecamKO endothelial cells.

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SHP-2 binds alternate target proteins in the absence of PECAM-1

In the absence of PECAM-1, SHP-2 is inefficiently localized to a Triton-insoluble fraction (likely cytoskeletal), and consequently likely has access to an alternate set of target phosphoproteins. To test this hypothesis I immunoprecipitated SHP-2 from PecamKO and PecamRC endothelial cells and evaluated for co-precipitating tyrosyl phosphorylated proteins. SHP-2 from PecamRC and PecamKO endothelial cells consistently co-precipitated different phosphoproteins. The major bands were of apparent molecular weight ~125kD and 140kD, respectively, in PecamRC and PecamKO cells. An additional band was appreciated at ~ 100kD and 110kD, respectively. Candidate blotting experiments for a number of signaling, adhesion and adaptor proteins of these approximate sizes failed to identify the coprecipitating phosphoproteins; in particular, neither beta catenin nor VE-cadherin were present in any discernible quantitity. Further analysis will likely require direct analysis of the bands through in-gel tryptic digests and peptide mass identification.

To determine whether these phosphoproteins are potential targets of SHP-2 phosphatase activity, SHP-2 was immunoprecipitated in the presence of orthovanadate to inhibit tyrosine phosphatase activity. Orthovanadate was then washed out and SHP-2 allowed to dephosphorylate any co-precipitating phosphoproteins (**Figure 33b**). SHP-2 co-precipitated with different major phosphoproteins from PecamKO and PecamRC cells, and these phosphoproteins were substrates for the tyrosine phosphatase activity of SHP-2. Thus PECAM-1 is instrumental in localizing SHP-2 to a Triton-insoluble fraction in endothelial cells, and in directing SHP-2 to specific target phosphoproteins.

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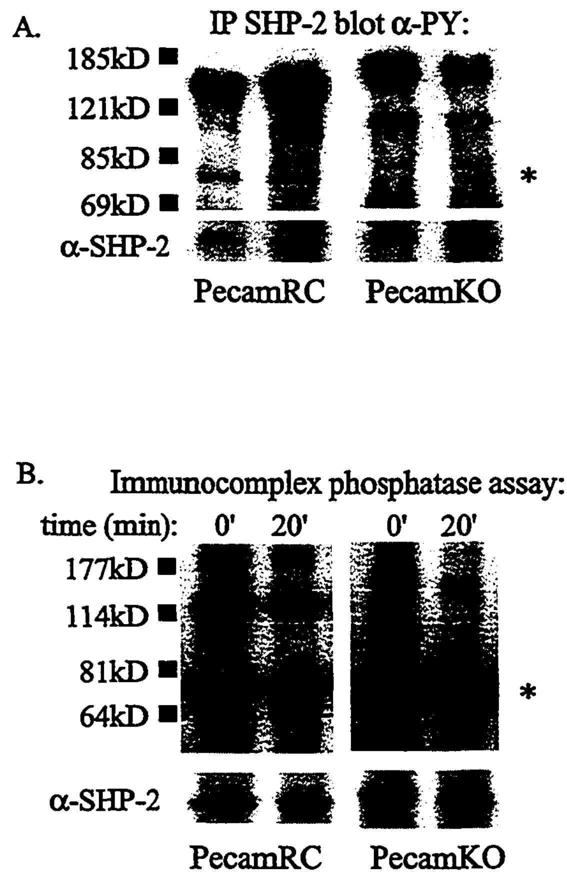

Figure 33: PECAM-1 modulates phosphoprotein association of SHP-2.

A. SHP-2 coprecipitates major phosphoproteins of apparent MW ~ 125kD and 140kD in PecamRC and PecamKO endothelial cells, respectively.

B. Immunocomplex phosphatase assay: SHP-2 was IPed, dephosphorylation was allowed to proceed for 0 or 20 min and coprecipitates were immunoblotted for PY. (*SHP-2)

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PECAM-1 ITIM signaling slows endothelial migration

To assess the role of PECAM-1 ITIM tyrosine phosphorylation in endothelial wound healing migration, PecamRC endothelial cells were transiently transfected with either wildtype PECAM-1 (PEC) or with PECAM-1 mutated in its ITIM domain (PEC 2F). Interestingly, PEC transfection slowed and PEC 2F transfection increased wound healing migration (**Figure 34a**). Thus the functional PECAM-1 ITIM domain, and its associated SHP-2, acts in a dose-dependent manner to retard migration. The ITIM-deficient PECAM-1 construct appears to act as a dominant negative, increasing wound healing migration in PECAM-expressing endothelial cells. It is not the case that a global increase in SHP-2 activity due to PECAM-ITIM-mediated activation retards migration. In fact, overexpression of wildtype SHP-2 increases wound healing migration, whereas a loss of function SHP-2 mutant (C459S) slows migration (**Figure 34b**). SHP-2 mutated in its C-terminal SH2 domain to prevent phosphotyrosine engagement, [R138K, (Pluskey et al., 1995)], proved to be equally effective in increasing endothelial migration as was wildtype SHP-2. Thus the positive role of SHP-2 in increasing endothelial wound healing is unlikely to be regulated by ITIM-bearing proteins such as PECAM-1. The role of PECAM-1 ITIM-associated SHP-2 activity in wound healing migration on the other hand appears to be that of a negative modulator.

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ITIM signaling modulates beta catenin and FAK phosphorylation

Decreased ITIM phosphorylation and thus lower PECAM-1-mediated SHP-2 tyrosine phosphatase activation should lead to decreased dephosphorylation of SHP-2 targets. Adherens junction component beta catenin dissociates from the actin cytoskeleton when tyrosine phosphorylated, as in response to shear stress (Ukropec et al., 2002). Immunoprecipitation revealed significantly increased beta catenin tyrosine phosphorylation in PEC 2F transfected endothelial cells (**Figure 35a**). Focal adhesion kinase (FAK) is a target of SHP-2 (Yu et al., 1998); FAK kinase activity in turn is required for endothelial cell migration (Gilmore and Romer, 1996). Western blotting with phosphospecific antibodies for the src-activating autophosphorylated tyrosine 397 of FAK (Eide et al., 1995) revealed increased FAK PY 397 in PEC 2F transfected endothelial cells (**Figure 35b**), pointing to a potential role for PECAM-1 in modulating focal contact turnover.

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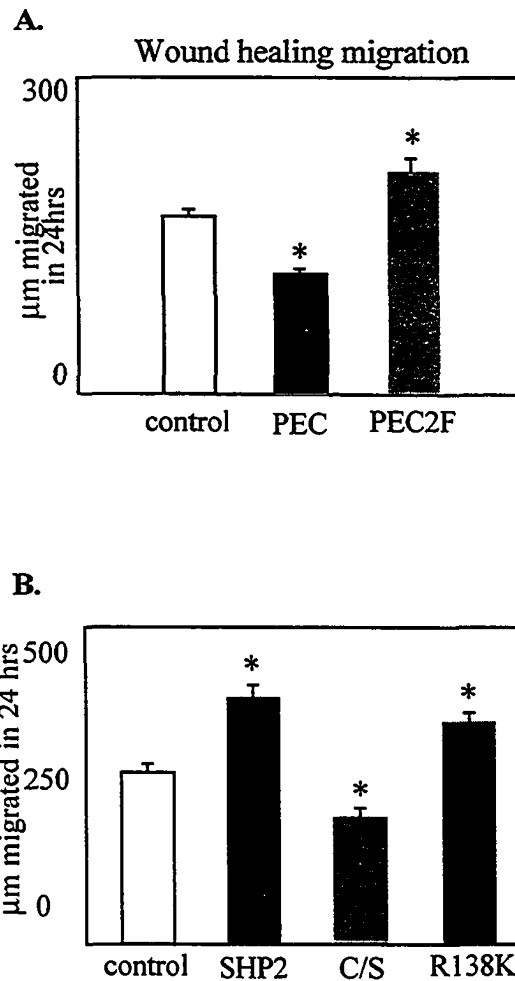

Figure 34: Role of Pecam ITIM domain in wound healing migration.

A. PecamRC cells were transiently transfected with wildtype (PEC) or ITIM defective (PEC 2F) PECAM-1. (n = 8, *p < 0.01).

B. PecamRC cells were transiently transfected with wildtype (SHP2), loss of function (C/S), or C-SH2 domain binding defective (R138K) SHP-2. (n = 4, p < 0.05)

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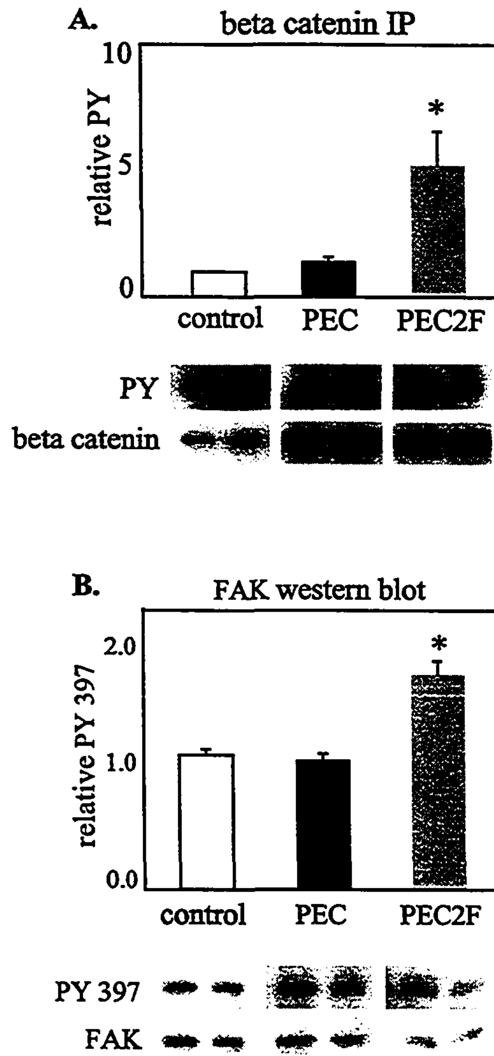

Figure 35: Targets for ITIM-mediated dephosphorylation.

A. Immunoprecipitation of beta catenin reveals significantly increased PY with PEC 2F transfection (n = 4, $^*p < 0.05$).

B. Western blotting reveals increased FAK PY 397 in PEC2F-transfected cells (n = 3, $^*p < 0.005$).

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Discussion

The role of PECAM-1 in coordinated endothelial cell behaviors appears paradoxical. As a whole, PECAM-1 plays a positive role in migration and angiogenesis (Cao et al., 2002; DeLisser et al., 1997). Yet expression of ITIM-defective PECAM-1 increases migration in bovine aortic endothelial cells (Kim et al., 1998), implying a negative regulatory role for PECAM-1 signaling in coordinated motility. Restoration of wound healing migration to PecamKO endothelial cells by PECAM-1 is independent of signaling via the ITIM domain (Gratzinger et al., 2003b). In PecamRC endothelial cells, on the other hand, expression of ITIM-functional PECAM-1 slows and ITIM-defective PECAM-1 speeds wound healing migration. Thus PECAM-1 is required at baseline to support migration, while ITIM-mediated signaling provides a damp or brake to modulate that migration.

In migrating endothelial cells, loss of cell-cell contacts releases PECAM-1 from the homophilic engagement that supports tyrosine phosphorylation and recruitment of SHP-2 (Bird et al., 1999). Accordingly, we observed a diffuse relocalization of both PECAM-1 and SHP-2 and a decrease in overall tyrosine phosphorylation and SHP-2 association with immunoprecipitated PECAM-1 in migrating endothelial cells. Using phosphospecific antibodies we determined that dephosphorylation was overwhelmingly at the second ITIM tyrosine, Y686, rather than Y663.

I assessed the effect of this dephosphorylation event on SHP-2 binding and activation *in vitro* using recombinant SHP-2 and PECAM-1 cytoplasmic domain. Lack of phosphorylation at tyrosine 686 significantly decreased SHP-2 binding, whereas

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phosphotyrosine 663 was dispensable for recruitment. In contrast, phosphotyrosine 686 did not contribute to SHP-2 activation, whereas PECAM-1 phosphorylated at tyrosine 663 activated SHP-2 as well as did fully phosphorylated PECAM-1. Engagement of the N-SH2 domain of SHP-2 allosterically disinhibits the phosphatase active site (Barford and Neel, 1998). Interestingly, Biacore experiments using PECAM-1 ITIM phosphopeptides have indicated that it is phosphotyrosine 663 that engages the N-SH2 domain (Jackson et al., 1997), consistent with a model of activation of PECAM-1-bound SHP-2 by phosphotyrosine 663.

Given the interaction of SHP-2 with phosphorylated PECAM-1 at cell-cell borders, it is plausible that SHP-2 itself may be responsible for the dephosphorylation of adjacent PECAM-1 molecules. The remaining ITIM phosphotyrosine 663 could help to backpropagate the dephosphorylation signal at the wound edge by sustaining SHP-2 activity toward adjacent phosphotyrosine 686. Since phosphorylation at tyrosine 686 is required to sustain PECAM-1 mediated homophilic cell-cell adhesion (Famiglietti et al., 1997), dephosphorylation at tyrosine 686 in one endothelial cell could be communicated to adjoining cells via decreased PECAM-1 engagement (inside out signaling) and consequent loss of phosphorylation at tyrosine 686 in the neighboring cell (outside in signaling).

In addition to any potential effects of SHP-2 on PECAM-1 phosphorylation and homophilic adhesion, engaged, tyrosyl-phosphorylated PECAM-1 should direct SHP-2 to target phosphoproteins involved in maintaining a stable, confluent as opposed to a loose, migrating endothelial monolayer. Using detergent-insolubility and sucrose-gradient fractionation techniques I determined that SHP-2 is inefficiently targeted to a Triton-

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insoluble, likely cytoskeletal fraction in PECAM-1 deficient endothelial cells. Furthermore, SHP-2 associates with and is capable of dephosphorylating an alternate set of phosphoproteins in the absence of PECAM-1.

I then transiently transfected endothelial cells with ITIM-defective PECAM-1 (PEC 2F) to interfere with their dephosphorylation. Beta catenin links adherens junction component VE-cadherin to the actin cytoskeleton via alpha catenin; tyrosine phosphorylation of beta catenin releases this linkage at the level of the beta catenin/alpha catenin interaction (Ukropec et al., 2002). PECAM-1 binds tyrosyl-phosphorylated beta catenin (Ilan et al., 1999); ITIM-regulated SHP-2 activity could thus modulate phosphorylation of membrane-associated beta catenin. I found that PEC 2F transfection increases overall beta catenin tyrosine phosphorylation without affecting VE cadherin association, potentially loosening adherens junction-actin cytoskeletal interactions and favoring migration.

Focal adhesion kinase (FAK) activity contributes to endothelial cell migration (Gilmore and Romer, 1996) and FAK is a target for SHP-2 phosphatase activity (Yu et al., 1998). SHP-2 but not FAK partitioning to the actin cytoskeletal fraction is decreased in the absence of PECAM-1; thus PECAM-1 might modulate SHP-2- mediated dephosphorylation of FAK. Indeed PEC 2F transfection increased phosphorylation of FAK at src-activating tyrosine 397 (Eide et al., 1995); thus phosphorylated PECAM-1 in confluent endothelial cells may serve to downregulate FAK activity in the absence of a migratory stimulus.

Figure 36 presents a model of PECAM-1 mediated SHP-2 activation at confluence and during endothelial migration. In confluent endothelial cells (**Figure 36a**),

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PECAM-1 is homophilically engaged at cell-cell borders and consequently is tyrosine phosphorylated on its ITIM. SHP-2 is engaged via both tyrosines 686 and 663, which respectively bind and activate the tyrosine phosphatase. SHP-2 targets proteins at the cell-cell junction and within the Triton-insoluble fraction, including beta catenin and FAK. Decreased tyrosine phosphorylation of beta catenin at adherens junctions would favor alpha catenin association and coupling to the actin cytoskeleton. Decreased FAK phosphorylation at tyrosine 397 would lower FAK activity and association of src kinase activity, decreasing focal contact turnover necessary for migration.

In wounded, migrating endothelial monolayers (**Figure 36b**), PECAM-1 is not homophilically engaged and becomes dephosphorylated, particularly at tyrosine 686. SHP-2 becomes dissociated from PECAM-1, and beta catenin and FAK are no longer targeted for dephosphorylation. Increased beta catenin tyrosine phosphorylation promotes weakening of adherens junctions and their dissociation from the cytoskeleton. Increased FAK activity promotes formation of focal complexes at the leading edge of the migrating endothelial cell. Selective dephosphorylation of the PECAM-1 ITIM domain appears to play a key regulatory role in coordinated endothelial migration.

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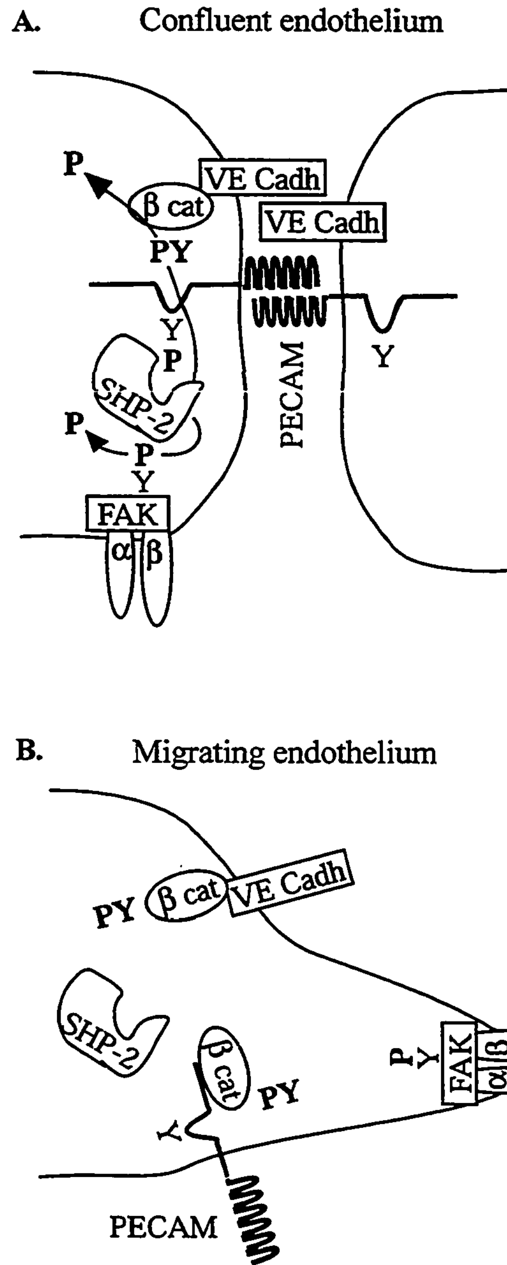

Figure 36: Role of PECAM-1 ITIM in endothelial migration.

Modulation of adherens junction and focal adhesion dynamics via PECAM-1 ITIM mediated targetting and activation of SHP-2. See text for discussion.

(ß cat = beta catenin, VE cadh = VE cadherin, Y = tyrosine, P = phosphate)

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Chapter 4: Answers and Questions

Answers

PECAM-1 plays at least two distinct roles in modulating endothelial cell migration: in one role, PECAM-1 helps to coordinate Galpha-protein mediated signaling to promote Rho-mediated wound healing migration. In the other, the phosphorylated PECAM-1 ITIM domain retards wound healing by modulating SHP-2 localization and activation. Endothelial expression of PECAM-1 modulates localization of specific Galpha protein subunits to caveolin-positive lipid raft subdomains, and hence the localized activation state of downstream signaling pathways. Local suppression of RhoGTP signaling (Arthur and Burridge, 2001) and activation of FAK, src and ERK kinase activity (Pintucci et al., 2002; Rosenfeldt et al., 2001) promotes directed extension formation as a component of a coordinated endothelial migratory response to mechanical wounding. Global suppression of Rho signaling and activation of Galphai2-mediated signaling pathways, on the other hand, promotes exuberant formation of lamellipodial extensions and nondirected single cell motility at the expense of coordinated migration.

In addition to and distinct from the overall positive role of PECAM-1 in coordinating endothelial wound healing, PECAM-1 ITIM-mediated signaling negatively modulates the wound healing response. Failure of developmental stage specific dephosphorylation of PECAM-1 ITIM tyrosine 686 in glucose-treated yolk sac vasculature, for example, (Pinter et al., 1999) would be expected to interfere with coordinated endothelial migration. It may thus partly explain the consequent faulty

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vasculogenesis that results in ectatic vessels, with failure to progress from the primary capillary plexus stage to a serially branching mature vasculature. The PECAM-1 ITIM is maximally phosphorylated in confluent as opposed to migrating endothelial cells (Lu et al., 1996). In this situation SHP-2 is activated at cell-cell junctions in a cytoskeletally associated fraction, maintaining low baseline levels of tyrosine phosphorylation of adherens junction and focal adhesion components. Stimuli such as mechanical wounding (M. Barreuther, unpublished data) and integrin engagement (Lu et al., 1996) promote dephosphorylation of the PECAM-1 ITIM, particularly at the second ITIM tyrosine which predominantly promotes SHP-2 binding. Increased phosphorylation of beta catenin and FAK then promotes loosening of cell-cell adhesion and extension formation at the leading edge of the wound. PECAM-1 is then both a necessary component and a modulator of coordinated endothelial migration.

ITIM signaling questions

Further analysis of PECAM-1 ITIM mediated signaling is complicated by the multiple roles played by SHP-2 in proliferative and migratory responses to growth factor receptor signaling (Feng, 1999). Manipulation of overall cellular tyrosine phosphatase activity or even SHP-2 activity is therefore not informative in dissecting this pathway. A construct encoding a fusion protein consisting of the extracellular domain of PECAM-1 fused directly to the constitutively active tyrosine phosphatase domain of SHP-2 (Noda et al., 2001) has been made for the purpose of addressing this question. This may yield candidate SHP-2 substrate phosphoproteins, although it is unclear that a construct lacking

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the entire cytoplasmic domain would be targeted to the same membrane subdomains as full length PECAM-1; additionally, proteins such as beta catenin that associate with the PECAM-1 cytoplasmic domain may not be accessible to this construct. A construct comprising full-length PECAM-1 fused to a substrate-trapping mutant of the SHP-2 tyrosine phosphatase domain (Flint et al., 1997) may be useful to identify direct targets by co-immunoprecipitation.

The role of the PECAM-1 ITIM in modulating VE-cadherin associated catenin phosphorylation and the integrity of vascular adherens junctions remains to be elucidated. Is alpha catenin lost from beta catenin-VE cadherin complexes when PECAM-1 ITIM signaling is inhibited? Is plakoglobin also a substrate for PECAM-1 ITIM-mediated dephosphorylation? Plakoglobin links VE-cadherin to vimentin, a component of the intermediate filament cytoskeleton; in wounded endothelial monolayers, it is the first component lost from the dissolving adherens junctions in mechanically wounded endothelium (Lampugnani et al., 1995). Tyrosine phosphorylation of plakoglobin in histamine-stimulated endothelium is accompanied by loss of coupling of VE cadherin to vimentin and thus intermediate filaments (Shasby et al., 2002). Not only may PECAM-1 ITIM mediated signaling modulate adherens junction stability; PECAM-1 itself binds both beta catenin and plakoglobin (Ilan et al., 2000), and may itself be coupled to the actin and/or intermediate filament cytoskeleton. The potential modulation of PECAM-1 and/or VE-cadherin-vimentin coupling via the phosphorylated ITIM domain remains to be explored.

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Galpha/Rho signaling questions

The evaluation of PECAM-1 modulation of heterotrimeric G protein and Rho signaling pathway activities presented above has been performed using confluent endothelial cells. These data evaluate signaling while cells are in a steady state, rather than in reaction to some physiologic stimulus. In reality migration involves transient, spatially appropriate activation of signaling pathways, such as the activation of ERK signaling in response to FGF-2 at the migrating front of wound-healing endothelial cells (Pintucci et al., 2002) and the transient suppression of Rho signaling by p190RhoGAP at the leading edge of a migrating cell upon engagement of alpha5beta1 integrins (Arthur and Burridge, 2001). Raft localization of signaling molecules, including heterotrimeric G protein alpha subunits, might also vary temporally both over the course of migration and in response to specific chemotactic stimuli. It will be worthwhile to explore the kinetics of signaling pathway activation in PECAM-1 deficient as compared to PECAM-1 expressing endothelial cells.

Much work remains to be done to elucidate the precise mechanisms of PECAM-1-mediated coordination of Galpha signaling. Is the observed decrease in RhoGTP levels in PECAM-1 deficient endothelium due only to increased p190RhoGAP tyrosine phosphorylation secondary to promiscuous activation of src via Galphai2, or is it also mediated by decreased activation of PDZRhoGEF by Galpha13 (Fukuhara et al., 1999)? Does PECAM-1 modulate raft recruitment of Galpha subunits directly, or through effects on Galpha interacting proteins? Both cadherins and catenins have been found to localize to caveolin positive rafts (Galbiati et al., 2000). Active, GTP-bound Galpha13 competes

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with beta catenin for VE cadherin binding (Meigs et al., 2001); competition by PECAM-1 for beta catenin association could free VE cadherin to associate with activated Galpha13, promoting the exchange of GDP for GTP and activating Rho signaling. Caveolin-1 association inhibits Galpha subunit GTP loading (Li et al., 1995) and consequently of downstream signaling. Does PECAM-1 modulate not only targeting to lipid rafts, but also caveolin-1 association, of Galpha subunits?

The role of PECAM-1 in properly transmitting S1P mediated signaling information may hold clues to the known phenotypes of the PECAM-1 knockout mouse. Is the defect in angiogenesis during the chronic phase of foreign body inflammation secondary to deficient S1P mediated signaling? *In vitro* tube formation assays, as well as *in vivo* assays of angiogenesis using pellets impregnated with S1P alone or in combination with other growth factors, may help to address this question.

The possible role of PECAM-1 mediated Rho signaling in transmitting the inhibitory of effect of high glucose on single cell motility of transforming endocardial cushion cells has already been discussed. Further clarification is needed of the signaling pathways involved in the endocardial cushion system. Experiments to confirm the protective role of Rho inhibition are under way in this system. Further work will explore the role of VEGF-mediated Rac activation in mediating migration of transforming endocardial cells over the collagen I gel (see **Figure 15b**). Treatment with N-acetylcysteine to block Rac mediated production of H_2O_2 should disinhibit low molecular weight PTPase. The dephosphorylation of p190RhoGAP would be expected block local inhibition of Rho signaling, and should therefore block endocardial cell motility even in the presence of supplemental VEGF.

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S1P increases transendothelial resistance, a measure of vascular junctional integrity, in a pertussis-inhibitable, Rho-dependent manner (Garcia et al., 2001). PECAM-1 null endothelium exhibits prolonged permeability in response to histamine, and transmigration of T cells is enhanced across PECAM-1 null endothelium. This vascular defect may in part contribute to the susceptibility of PECAM-1 null mice to invasion of their central nervous system by leukocytes as a part of the pathogenesis of a mouse model of multiple sclerosis (Graesser et al., 2002). Is this due in part to the decreased responsiveness of the PECAM-1 deficient vasculature to the serum-derived sphingolipids that promote the reestablishment of tight vascular junctions? *In vitro* testing of S1P-mediated increases of transendothelial resistance of PECAM-1 deficient versus wildtype vasculature could address this question.

Similarly, the bleeding time defect of PECAM-1 null mice is attributable to the vasculature rather than to bone marrow derived components (Mahooti et al., 2000). High concentrations of S1P are released from platelets during clotting, but successful stanching of bleeding involves more than just platelet activation and thrombosis. The subjacent endothelium must also contract to reveal the underlying thrombogenic collagen surface in response to platelet-released factors such as thrombin (Cotran et al., 1994). Thrombin mediates endothelial cell contraction through RhoGTP-mediated activation of p160ROCK, myosin light chain kinase, and consequently actin-myosin mediated contractility (Essler et al., 1998). In the context of inflammation, histamine likewise promotes a p160ROCK-mediated increase in endothelial permeability (Wojciak-Stothard et al., 2001). Preliminary studies indicate that both thrombin and histamine activate Rho

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to a significantly lower extent in PECAM-1 null as opposed to PECAM-1 reconstituted endothelial cells (data not shown).

The thrombin receptor belongs to the seven-transmembrane heterotrimeric G protein linked family of protease activated receptors (PARs) and promotes endothelial cell migration and angiogenesis through pathways involving Rho signaling downstream of Galpha13 (Maragoudakis et al., 2002; Richard et al., 2001). Histamine receptor subtype H1, which promotes vascular permeability, is also a seven transmembrane receptor, although unlike the thrombin receptor it is ligand activated and primarily signals through Galphaq/11 (Leurs et al., 1995). Thus the role of PECAM-1 in modulating S1P mediated wound healing migration may extend to modulation of signaling via Galpha subunits downstream of multiple seven transmembrane receptors. Importantly, CXCRs, seven-transmembrane receptors for cysteine-x-cysteine motif containing chemokines including SDF-1 (CXCR4) (Salvucci et al., 2002) and interleukin-8 related cytokines (CXCR1/2) (Belperio et al., 2000), play an important role in angiogenesis in the settings of injury, inflammation and neoplasia. Further pursuit of the signaling pathways and (patho)physiologic sequelae of PECAM-1 expression should prove fruitful for years to come.

In vitro migration assays: how should they be interpreted?

Nondirected single cell motility, or chemokinesis, represents an increase in cell movement in response to serum or cell derived growth factors; angiogenic growth factors

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such as VEGF and S1P induce not only an increase in this baseline cell movement, but directional motility with respect to a concentration gradient (chemotaxis if the gradient is in the liquid phase, haptotaxis if it is fixed to cell surfaces or to extracellular matrix). Most developmental or physiologic cell movements *in vivo* occur in a directional manner in response to some chemotactic or haptotactic cue, with a balance between the degree of increase in baseline cell motility and the degree to which that motility is strictly directional. At one end of the spectrum of *in vivo* correlates to chemokinesis as opposed to chemotaxis, for example, lies hepatocyte growth factor (HGF). HGF induces scattering motility *in vitro* through activation of the Met tyrosine kinase receptor and promotes the dissemination and invasive behavior of malignant carcinoma cells in response to autocrine signaling (Danilkovitch-Miagkova and Zbar, 2002). At the opposite end of the random versus directed single cell motility spectrum, neuronal cell migration *in vivo* is exquisitely directionally guided through a combination of chemotactic and haptotactic cues which tightly control Rho and Rac activation and hence directional motility (Park et al., 2002b).

Coordinated wound healing migration necessarily involves directional movement, as the wounding induces release of chemotactic factors at the leading edge and uncovers haptotactic factors previously laid down by the endothelial monolayer. For example, injury induces endothelial release of fibroblast growth factor-2 (FGF-2), which contributes to the endothelial wound healing response *in vitro* (Pintucci et al., 2002; Santiago et al., 1999). *In vivo*, denuded basement membrane promotes platelet aggregation and release of multiple chemotactic factors including S1P, which also promotes endothelial wound healing migration (Lee et al., 2000a). Finally, exposed

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extracellular matrix components such as fibronectin themselves provide strong haptotactic cues to endothelial wound healing and angiogenesis (Kim et al., 2000). PECAM-1 deficient endothelial cells perform poorly in the wound healing assay *in vitro* and *in vivo* demonstrate poor angiogenesis during the chronic phase of foreign body inflammation (Solowiej et al., 2002).

Although the single cell motility of PECAM-1 deficient endothelial cells is dramatically increased with respect to PECAM-1 expressing cells, I would predict that these cells would respond weakly if at all to a directional signal in assays of single cell chemotaxis or haptotaxis. Indeed, I believe that the very property that allows these cells to form multiple extensions and initiate motility in the absence of a directional signal, their low level of RhoGTP signaling at baseline, would perforce lower their sensitivity to inputs that modulate directionality by transiently decreasing Rho signaling to allow for extension formation at the leading edge (see Fig 15b). Thus, for example, a Transwell™ single cell motility assay of chemotaxis toward VEGF might reveal the same or even a greater number of transmigrated PecamKO than PecamRC cells due to the chemokinetic advantage of PecamKO cells; however the increase in transmigration with the addition of VEGF to the bottom well would be expected to be dramatically greater in the case of the PecamRC cells.

Does PECAM-1 signaling modulation have any real-world applicability?

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A thorough understanding of the mechanics of PECAM-mediated modulation of heterotrimeric G-protein mediated signaling in endothelial cells may yield viable tools not only for the benchtop researcher but for the modulation of epidemiologically important aspects of cardiovascular pathology. For example, PECAM-1 knockout mice have significant defects in reestablishment of cell-cell junctional integrity following a stimulus that increases permeability. Histamine induces a transient increase in endothelial permeability; the duration of increased permeability is several times longer in brain and dermal vasculature of PECAM-1 knockout mice and in PecamKO endothelial cells *in vitro* (Graesser et al., 2002). This failure to reestablish cell-cell junctions following what should be a transient increase in permeability may likewise underlie the increased transmigration of T lymphocytes (Graesser et al., 2002) as well as polymorphonuclear neutrophils (Solowiej et al., 2002) across PECAM-1 negative endothelia. Further experiments will be required to determine how much of this defect is due to a failure to dephosphorylate beta catenin, and thus to reassemble stable actin cytoskeleton-linked adherens junctions, due to a lack of PECAM-1 ITIM-mediated SHP-2 activity at cell-cell borders. Stable transfectants of PecamKO endothelial cells expressing ITIM-defective, full-length PECAM-1 should prove informative in this regard. Therapeutic modulation of PECAM-1 ITIM phosphorylation may be achievable with carefully developed anti-PECAM-1 antibodies or recombinant portions of the PECAM-1 ectodomain.

Increased endothelial permeability contributes to the pathogenesis of a variety major causes of morbidity and mortality such as atherosclerosis, chronic inflammation, diabetic retinopathy, and sepsis. Heterotrimeric G protein mediated signaling and

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modulation of Rho activation through Galpha12/13 and Galphaq/11 (Sah et al., 2000) is prominently involved in modulating endothelial permeability (van Nieuw Amerongen and van Hinsbergh, 2001). The histamine receptors for example are G-protein coupled seven transmembrane receptors (Leurs et al., 1995); the major receptor subtype involved in modulating vascular permeability, H1, is linked to Galphaq/11. Thrombin signals through Galpha12/13 to mediate Rho signaling and increased vascular permeability as well as angiogenesis (Richard et al., 2001). Lipophosphatidic acid (LPA), a product of low density lipoprotein (LDL) oxidation present in atherosclerotic plaques (Siess et al., 1999), likewise stimulates endothelial cell permeability through heterotrimeric G protein mediated modulation of Rho signaling. Sphingosine-1-phosphate, on the other hand, promotes vascular maturation and decreased permeability through Edg-receptor dependent signaling, including Rho activation (Garcia et al., 2001). It seems likely that precise modulation of the timing, duration and localization of Rho signaling by heterotrimeric G proteins is required to maintain a stable rather than a dysfunctional, leaky vasculature. Since PECAM-1 appears to be capable of modulating the localization of specific Galpha protein subunits to membrane microdomains, an analysis of the particular region of the PECAM-1 cytoplasmic domain responsible for this localization could prove to be quite valuable. Should an oligopeptide be found that is capable of influencing the localization and thus the signaling properties of specific Galpha subunits, its delivery to dysfunctional vascular beds may prove to be useful in modulating the responsiveness of endothelia to permeability-inducing or atherogenic stimuli.

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Chapter 5: Materials and Methods

Antibodies

Rabbit polyclonal antibody to murine PECAM-1 extracellular domain (Sleet), human PECAM-1 cytoplasmic domain (BooBoo), and to PECAM-1 ITIM phosphotyrosines 663 or 686 (Lu et al., 1996; Pinter et al., 1997) were generated in our laboratory and have been previously described. Monoclonal antibodies to extracellular signal regulated kinase 2 (ERK2), phosphotyrosine, and SHP-2 and polyclonal antibody to caveolin and paxillin phosphotyrosine 118 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to beta catenin, Rac1, Rho and focal adhesion kinase (FAK) and polyclonal antibodies to heterotrimeric G protein subunits Galphai2 and Galpha13 were purchased from Transduction Laboratories (San Diego, CA). Monoclonal antibodies to RhoGAP (Guanosine triphosphate Phosphatase Activating Protein) p190 and polyclonal antibodies to FAK phosphotyrosine 397 and RasGAP p120 were purchased from Upstate Biotechnologies (Lake Placid, NY). Anti-phosphothreonine/tyrosine ERK polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibody to beta actin was purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal hamster antibody against murine PECAM-1, and monoclonal rat antibody against murine vascular endothelial cadherin, for fluorescence activated cell sorting (FACS) analysis were purchased from PharMingen (San Diego, California, USA).

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Reagents

Rhodamine-phalloidin and methyl beta cyclodextrin was purchased from Sigma-Aldrich (St. Louis, MO). *C. botulinum* exoenzyme C3 (exo C3), pertussis toxin (PTX), sphingosine-1-phosphate (S1P) and Y27632 were purchased from Calbiochem-Novabiochem (San Diego, CA). Complete Protease Inhibitor™ (CPI) was purchased from Roche Diagnostics (Mannheim, Germany). Polypeptides, both phosphorylated and nonphosphorylated, were synthesized by the Howard Hughes Medical Institute-Keck Foundation Lab at Yale University. Sequences were: for the PECAM-1 ITIM tyrosine 663 sequence, PLN SDV QY (phospho) T EVQ VSS; and for the tyrosine 686 sequence, KKD TET VY (phospho) S EVR KAV.

Constructs

Full-length human PECAM-1 in the mammalian expression vector pAPEX-1 (Alexion, New Haven, CT) and its Y663F mutant have been described (Lu et al., 1996). An additional mutation at Y686F was made using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions to produce an ITIM-defective construct. A myc-tagged truncated construct in pAPEX-1 lacks most of the ectodomain (Kim et al., 1998). The PECAM-1 cytoplasmic domain in a glutathione-S-transferase (GST) fusion protein expression construct (pGEX2T, Pharmacia, Piscataway NJ) (Lu et al., 1997) was also mutated at Y686F and at Y663F.

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pGEX2T-SHP-2 construct was a gift of H. Ohnishi et al.(Ohnishi et al., 1996); a phosphatase-dead mutant (D425A) (Flint et al., 1997) was made using site-directed mutagenesis.

The Rho binding domain of Rhotekin in pGEX2T (Pharmacia) was a gift of X.D. Ren and M.A. Schwartz (Ren and Schwartz, 2000). A GST-fusion protein expression construct of the Rac binding region of PAK, pGEX4T-1 PAK70-106, was a gift of F. Giancotti (Mettouchi et al., 2001).

Myc-tagged SHP-2 and SHP-2 loss of function mutant C459S in pIRES-EGFP (Clontech, Palo Alto, CA) were a gift of M. Kontaridis and A. Bennett. An SHP-2 mutant incapable of engaging its C-terminal SH2 domain (R138K) (Pluskey et al., 1995) was made by site-directed mutagenesis. pCDNA3-VCAM mammalian expression construct was made by subcloning the full-length cDNA purchased from Biogen (Cambridge, MA).

Oligonucleotides

PECAM-1 antisense oligonucleotides: 5' TCC TTC CAG GGA TGT GAT C 3' for human PECAM-1 and control scrambled oligonucleotide 5' TTC TAC CTC GCG CGA TTT AC 3' were gift of T. Condon at Isis™ Pharmaceuticals (Carlsbad, CA).

Mutagenic primers were: for PECAM-1 Y686F, 5' GGA CAC AGA GAC AGT GTC AGT GAA GTC CGG 3' and its inverse; for PECAM-1 Y663F, 5' CCT CTG AAC TCA GAC GTG CAG TTC ACG GAA GTT CAA GTG TCC 3' and its inverse; for SHP-2 D425A, 5' GGA CCT GGC CAG CCC ATG GCG TGC C 3' and its inverse; for

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SHP-2 R138K, 5' CAT GGT AGT TTT CTT GTA AAA GAG AGC CAG AGC CAC CCT GG 3' and its inverse.

Cell culture

Endothelioma cell line luEnd.PECAM-1.1 (PecamKO) was established by retroviral transduction of primary endothelial cell culture with the polyoma virus middle T-oncogene (Wagner and Risau, 1994; Wong et al., 2000). PecamKO cells retrovirally transduced with full-length murine PECAM-1 cDNA to generate a PecamRC (reconstituted) cell line (Graesser et al., 2002; Wong et al., 2000). Cells were cultured in luEnd media [Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES pH 7.4, 1% L-glutamine, 1% nonessential amino acids, 1% pyruvate, 10,000 U/ml penicillin/ streptomycin, and 10^5 M 2-mercaptoethanol (GIBCO BRL)] and incubated at 37°C in 8% CO_2. Selection of PECAM-1 expression on PecamRC cells was maintained with 1 μg/ml puromycin.

Human umbilical vein endothelial cells (HUVEC) were purchased from the Boyer Center for Molecular Medicine Cell Culture Core (Yale Medical School) and were cultured on gelatin in M199 medium (Life Technologies) supplemented with 20% heat-inactivated FBS, 50 mg/ml endothelial cell growth supplement, 50 mg/ml heparin, 10 mM HEPES, 2 mM L-glutamine and antibiotics. Bovine aortic endothelial cells (BAECs) were cultured as previously described (Madri et al., 1988). Hemangioendothelioma (Eoma) cells were obtained from Robert Auerbach (University of

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Wisconsin, Madison, WI) and were grown in complete DMEM supplemented with 10% FBS and antibiotics (Obeso et al., 1990).

Immunostaining

Cells were incubated overnight on 8-chamber glass culture slides (Falcon, Becton-Dickinson) that had been coated with fibronectin. Cells were fixed in Streck's Tissue Fixative (STF, Streck Laboratories, La Vista, NE), permeabilized with 0.5% Triton X-100 in TBS, and stained with rhodamine-phalloidin 1:200, anti-phosphopaxillin 1:500, anti-PECAM-1 1:100 or anti-SHP-2 1:100 followed by fluorescein or rhodamine-conjugated secondary (Jackson Immunoresearch Labs, West Grove, Pennsylvania) at 1:200. After coverslipping with Antifade Mounting Media (Molecular Probes, Eugene, OR) cells were photographed using a Carl Zeiss Research microscope (Göttingen, Germany) and SPOT digital camera (Diagnostic Instruments Inc, Sterling Heights, Michigan).

Transient transfections

Subconfluent PecamKO or PecamRC endothelial cells were transfected using LipofectAMINE2000, Life Technologies (Grand Island, NY) according to the manufacturer's instructions. Briefly, cells were split 1:3 into 100 mm tissue culture plastic dishes and cultured overnight. When they had reached 70-80% confluence they

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were transfected using 1 µg pIRES EGFP + 9 µg test DNA and 30 µl transfection reagent. Expression was assessed using green fluorescence at 24 hours.

For antisense oligonucleotide transfections, passage three HUVEC at 70-80% confluence were transfected using Lipofectin (Life Technologies) according to the manufacturer's instructions. PECAM-1 expression was assessed by western blotting at 24 or 48 hours by western blot.

Nondirected motility assay

8 µm pore size Transwells® (Corning Inc. Corning, NY) were coated overnight with 12.5 µg/ml fibronectin (Madri et al., 1988) or type I collagen (Haas et al., 1998) and blocked with 5% BSA as described. 100 µL of media was added to the top well and 500 µL to the bottom well. Endothelial cells were briefly trypsinized, washed twice in endothelial media, and 100 µL of a 10^6 cell/ml single cell suspension was added to the top well. Lung endothelial cell transmigrations were done in luEnd media. HUVEC transmigrations were done in HUVEC media lacking endothelial cell growth supplement and with FBS decreased to 2%. After 2.5 hours of incubation at 37°C in 8% CO_2 the cells were fixed in STF, and stained with crystal violet. Cells on top of the filter were removed with a cotton swab and cells on the bottom surface were quantitated as number of cells transmigrated per high power field (20x objective, corresponding to an area of approximately $5mm^2$).

Wound healing assay

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60 mm Falcon® petri dishes (Becton Dickinson, Franklin Lakes NJ) were coated with fibronectin as above. Butanol-extracted fibronectin and BSA were used for experiments involving S1P. Confluent PecamKO and PecamRC monolayers grown on fibronectin-coated plates were scraped with a 15-well minigel comb leaving concentric rings of cells, washed with PBS, and incubated 24 hours. HUVEC wound healing migrations were on gelatin-coated plates. For S1P experiments, CS-FBS was prepared by incubating 50 mL FBS with 5 g activated charcoal (Sigma) overnight at 4°C (Lee et al., 1998). The CS-FBS was centrifuged (10 min at 1000*g*) and filtered through a 0.22 µm sterile Nalgene® filter (Sybron Corp, Rochester NY). Distance migrated was quantitated by taking pictures at 0 and 24 hours with a Nikon Coolpix995 Digital Camera (Nikon Corp., Tokyo Japan) on an Olympus IM light microscope (Melville, NY) and measuring distance of wound edge from a mark on the bottom of the plate.

Quantitation of extension formation

10^4 cells/well were plated in a fibronectin-coated 24-well plate. Cells were fixed in STF and stained with crystal violet, photographed as above, and quantitated using the public domain NIH Image program (National Institutes of Health) to determine perimeter and cell area. The theoretical radius (r) had the cell been a perfect circle was determined according the formula $r_{perimeter}$ = perimeter/2π and $r_{area} = \sqrt{(area/\pi)}$, and the ratio $r_{perimeter}$ to r_{area} was used to determine deviation from circularity.

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Western blotting

Confluent monolayers of PecamRC and PecamKO cells were washed twice in ice-cold PBS containing 1 mM orthovanadate, and scraped into lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and CPI). Wound-healing BAECs were lysed in 200 µl [50 mM Tris, pH 7.4, 100 mM NaCl, 0.5 % Triton X-100, 0.5 % sodium deoxycholate, a protease cocktail (CPI, Boehringer-Mannheim) and 0.2 mM orthovanadate]. Lysates were centrifuged at 14,000*g* at 4 °C for 10 minutes, and supernatant protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) as directed. 20 µg protein were boiled with 4x sample buffer (240 mM Tris pH 6.8, 40% glycerol, 8% SDS, 0.002% bromophenol blue, 0.002% ß-mercaptoethanol).

Samples were separated by SDS PAGE, transferred to an Immobilon P membrane, (Millipore, Bedford, MA), probed with anti-ERK 1:10,000, anti-phosphoERK 1:1000, anti-FAK 1:10,000 and anti-FAK PY397 1:2000, followed by horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG, (Promega, Madison, WI) and developed using Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA). Blots were scanned on an Arcus II scanner (Agfa, Mortsel, Belgium) and quantitated using BioMax 1D software (Kodak, Rochester, NY).

Immunoprecipitation

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200 μg protein were pre-cleared with protein A/G-Sepharose (Santa Cruz); the cleared supernatant was incubated with the appropriate antibody for one to two hours turning end-over-end at 4°C, then precipitated with A/G-Sepharose. Beads were washed three times in lysis buffer and boiled in 4x sample buffer. Samples were separated by SDS-PAGE, transferred to an Immobilon P membrane, probed with anti beta catenin 1:10,000, anti-p190 RhoGAP 1:5000, anti-p120 RasGAP 1:5000, anti-SHP-2 or anti-phosphotyrosine 1:10,000, and developed and quantitated as above.

Triton X-100 fractionation

Triton X-100-soluble and insoluble fractions were prepared as described in (Rajasekaran et al., 2001). Briefly, cells were scraped into 200 μl lysis buffer (50 mM NaCl, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose, 1 mM PMSF, 100 U/ml DNAse) for 10 min at 4°C, then centrifuged at 4°C for 30 min at 14,000 rpm. Pellet and supernatant fractions were boiled in 4x sample buffer prior to separation by SDS-PAGE, immunoblotting, and probing with anti-beta actin 1:10,000, anti-FAK 1:10,000, or anti-SHP-2.

Raft fractionation

Sucrose density gradient lipid raft fractionation was carried out in a detergent-free sodium bicarbonate buffer essentially as described by (Song et al., 1996). Briefly, confluent endothelial cells were scraped into lysis buffer (500 mM sodium

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bicarbonate) and sonicated. 85% sucrose in MBB (25 mM MES, pH 6.5, 150 mM NaCl, and 250 mM sodium bicarbonate) was added to bring the sample to 42.5% sucrose. The two mL sample was placed in an ultracentrifuge tube on ice for 4 hours, then layered with 8 mL of 30% sucrose in MBB followed by 2 mL 5% sucrose in MBB were layered to make a discontinuous gradient.

The sample was centrifuged at 39,000 rpm for 18 hr at 4°C in an SW41 rotor (Beckman Instruments, Palo Alto, CA), at which time an opalescent band representing the caveolin-positive raft fraction was visible at the 30%/5% sucrose interface. One mL fractions were carefully pipetted, and 20 µL samples boiled in 4x sample buffer for analysis, separated by SDS-PAGE and transferred to an Immobilon P filter. Blots were probed with anti-PECAM-1, anti-SHP-2, antiphosphotyrosine, anti-caveolin, or anti-beta actin at 1:10,000 or anti-Galphai2 or anti-Galpha13 at 1:250 and developed and quantitated as above.

Recombinant protein production

GST-PECAM-1 cytoplasmic domain was expressed in protease deficient BL21 bacteria (Pharmacia) induced with 0.5 mM isopropyl-ß-D-thiogalactoside (Sigma) for 3 hours at 37°C. Bacteria were lysed with Bacterial Protein Extraction Reagent (Pierce) and Complete Protease Inhibitor (CPI) (Boehringer Mannheim). After spinning 30 min at 14,000*g* at 4°C, the bacterial lysate supernatant was incubated with a 50% slurry of glutathione agarose beads (Sigma) to pull out the GST fusion protein, and washed 5 times in TBS + 1 mM ethylene diamene tetraacetic acid to eliminate bacterial proteins.

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Tyrosine phosphorylated GST-PECAM-1 was produced as directed in TKX1 bacteria (Stratagene) and phosphorylation confirmed by western blotting with phosphospecific antibodies to ITIM phosphotyrosines 663 and 686 at 1:5000 as described.

Concentration was determined using a standard curve of bovine serum albumin (Pierce) after SDS-polyacrylamide gel electropheresis (PAGE) and Coomassie staining. GST-SHP-2 was produced essentially as described (Dechert et al., 1994), but in BL21 bacteria (Pharmacia) and at 28°C. Thrombin cleavage of recombinant SHP-2 from its GST tag was carried out as described (Dechert et al., 1994); concentrations were determined as with Bio-Rad Protein Assay (Hercules, CA).

Recombinant SHP-2 pulldowns

10 μg of glutathione-agarose-bound GST-PECAM-1 was combined with 5 μg recombinant SHP-2 in 500 μl Pulldown Buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.5 % Tween-20, 10 mM β-mercaptoethanol) and rotated for 30 min at 4°C. The glutathione agarose beads with bound GST-PECAM-1 and any associated SHP-2 were washed in cold Pulldown Buffer. Samples were separated by SDS PAGE, western blotted as described, probed with anti-SHP-2 1:10,000 or anti-PECAM-1 1:10,000, and developed and quantitated as described.

RhoGTP and RacGTP pulldown assays

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GST-Rho binding domain was expressed and purified using glutathione-agarose beads (Sigma-Aldrich) as described (Ren and Schwartz, 2000). GST-p21 Activated Kinase (PAK) Rac binding domain was expressed and purified as described (Royal et al., 2000). Freshly confluent PecamRC or PecamKO cells grown on fibronectin-coated 100 mm plates were lysed, 20 μL lysate set aside for normalization, and RhoGTP or RacGTP pulled down using glutathione-agarose-bound GST-Rho binding domain or GST-PAK Rac binding domain as described (Ren and Schwartz, 2000; Royal et al., 2000). Samples were separated by SDS PAGE, transferred to an Immobilon P membrane, probed with anti-Rho 1:100 or anti-Rac 1:100 and developed and quantitated as above.

Recombinant SHP-2 phosphatase assays

To assess activation of recombinant SHP-2 by GST-PECAM-1, 3 μg bead-bound GST-PECAM-1 and 2 μg recombinant SHP-2 was suspended in 45 μl Phosphatase Buffer (50 mM sodium acetate, pH 5.3, 50 mM NaCl, 10 mM dithiothreitol) in 96-well plates (Falcon); at t = 0' 5 μl 500 mM *p*-nitrophenyl phosphate (pNPP) were added and the mixture shaken 10 min (adapted from (Ohnishi et al., 1996)). A Wallac Victor Multilabel Counter attached to a PC laptop was used to record absorbance at 405 nm.

Immunoprecipitated SHP-2 phosphatase activity

Phosphatase activity of immunoprecipitated SHP-2 was assessed essentially as described (Tang et al., 2000). Briefly, cells were treated with pervanadate prior to lysis

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and SHP-2 was immunoprecipitated in the presence of orthovanadate on ice. Orthovanadate was washed out to disinhibit phosphatase activity and dephosphorylation allowed to proceed for 0 or 20 minutes at 25°C prior to quenching with 10mM orthovanadate. Tyrosine phosphorylation of SHP-2-associated proteins was evaluated by western blotting.

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