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Role Of Gravin In Endothelial Wound Healing

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ROLE OF GRAVIN IN ENDOTHELIAL WOUND HEALING

by

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This thesis, submitted by Bhaskar Roy in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done, and hereby approved.

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ABBREVIATIONS

AKAP..... a kinase anchoring protein

AMP.....adenosine monophosphate

ANOVA.....analysis of variance

AS.....adenylyl cyclase

ATP.....adenosine triphosphate

β_2 AR .....beta-2 adrenergic receptor

BCA.....bicinchoninic acid

cAMP.....adenosine-5',3'cyclic- monophosphate

cGMP.....guanosine-5',3'cyclic- monophosphate

CBD.....cAMP-binding domain

DAPI.....4',6'-diamino-2-phenylindole dihydrochloride hydrate

ELISA.....enzyme-linked immunosorbent assay

EPAC.....exchange protein activated by cAMP

FBS.....fetal bovine serum

GPCR.....G protein coupled receptors

G-protein.....GTP- binding proteins

$G_{\alpha s}$ .....stimulatory guanine nucleotide binding protein

$G_{\alpha i}$ .....inhibitory guanine nucleotide binding protein

GPCR.....G-protein-coupled receptor

HRP.....horse radish peroxidase

HUVEC.....human umbilical vein endothelial cell

PBS.....phosphate buffer saline

PMSF.....phenylmethylsulfonyl fluoride

PKA.....cyclic AMP dependent protein kinase

PKC.....protein kinase C

PDE.....phosphodiesterase

SD.....standard deviation

SDS-PAGE.....sodium dodecyl sulfate-polyacrylamide gel electrophoresis

siRNA.....small interfering ribonucleic acid

SSeCKS.....*Src* suppressed C kinase substrate

TRITC.....tetra methyl rhodamine isothiocyanate

VE-cadherin.....vascular endothelial cadherin

VEGF.....vascular endothelial growth factor

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ABSTRACT

Gravin (AKAP12), a 300 kDa AKAP, is widely distributed in cells and binds to several signaling molecules including PKA-RII subunits, PKCα, PDE4, β_2 adrenoreceptor and actin cytoskeleton. Initial characterization of gravin has revealed that this protein is undetectable in endothelia *in vivo* but expressed at the periphery of cultured endothelial cell (EC). Although the precise role of gravin in human ECs is not yet known, the cortical distribution of gravin in EC suggests that it may be involved in cAMP regulation by targeting PKA and PDE4 to plasma membrane. Several studies indicate that localization of PKA activity by A-kinase anchoring proteins (AKAPs) to the leading edge and other cytoskeletal regulators is an important factor in cell migration. SSeCKS, the rodent orthologue of gravin is reported to be involved in cell migration and regulation of actin cytoskeleton in cancer cell lines. Further, Western blot analysis, in the current study reveals that gravin upregulation in EC is associated with an active state, when cells are at a low cell density culture condition that promotes migration and proliferation, whereas downregulation occurs when cells became confluent and quiescent, indicating that gravin may have a functional role in ECs during their proliferative and migratory stage. Therefore, based on these findings, we hypothesized that gravin may play a role in EC migration.

To test this hypothesis, following experiments were conducted. First, the effect of gravin knockdown on EC migration was determined in a scratch wound and a 96-well based cell migration assay using antisense oligonucleotide and siRNA treatments. The

effect of antisense oligonucleotide and siRNA treatments on gravin expression was assessed by Western blotting and cell based ELISA. Finally, the reorganization of cortical actin band was quantitatively analyzed in antisense oligonucleotide treated cells to determine a role for gravin in actin cytoskeleton remodeling.

A significantly reduced distance, that wound edge moved after 18-20hr, was observed in both antisense oligonucleotide and siRNA treated cells in a scratch wound model. Consistent with this, the number of cells present in the cell free zone after 42-44hr was also reduced significantly in the cells treated with antisense oligonucleotide and siRNA treatments in a 96-well cell migration assay. Finally, antisense oligonucleotide treatment also reduced the length of cortical actin band normalized with the free edge (edge not touching adjacent cells) of cells at the wound edge, which further revealed that gravin was also involved in cortical actin remodeling in migrating EC.

This study proposes a possible mechanism for gravin mediated EC migration which involves the formation of a gravin-PKA-PDE4 functional complex, facilitating a compartmentalized regulation of cAMP/PKA dynamics that induces cortical actin remodeling and EC migration. In summary, the present study provides a new insight into the control of gravin expression in cultured EC and the role of gravin in EC migration and wound healing.

CHAPTER 1

INTRODUCTION

The endothelium is a single cell layer lining blood vessels that establishes a semi-permeable barrier between blood and surrounding tissue. During wound closure, endothelial cells migrate from pre-existing vessels, proliferate and form new capillary networks (Davis and Senger, 2005; Lamalice et al., 2007). Cell migration, which has been extensively studied over many decades (Lamalice et al., 2007; Lauffenburger and Horwitz, 1996; Ridley et al., 2003), can be conceptualized as a cyclic process that is categorized into four steps -1. morphological polarization and membrane protrusions, 2. formation and stabilization of attachment to extracellular matrix (ECM), 3. translocation of the cell body, and 4. detachment and retraction of the trailing edge. Acquisition of morphological polarization (i.e. clear distinction between cell front or leading edge and cell rear or trailing edge) is the initial step in cell migration which leads to the formation of protrusive membrane processes. Two types of processes are formed: broad lamelliopodia and spike like filopodia (Pollard and Borisy, 2003; Welch and Mullins, 2002). The constant remodeling of the actin cytoskeleton into filopodia, lamelliopodia and stress fibers is essential for endothelial cell (EC) migration. GFP and FRET-based imaging technologies have revealed a list of proteins that are being regulated during all cytoskeletal reorganization events including nucleation, assembly, elongation, severing, capping and cross linking (Lauffenburger and Horwitz, 1996; Pollard and Borisy, 2003; Ridley et al., 2003; Weijer, 2003). Studies have shown that the Rho family of small

guanosine triphosphate (GTP) binding proteins (GTPases), Rho, Rac and Cdc42 (Foster et al., 1996; Hall, 2005) promote the formation of cytoskeletal structures in protruding lamellipodia (Kraynov et al., 2000). However, given the complexity of cell movement, it is well known that other protein molecules are involved in cell migration and cytoskeletal regulation and coordinate the over all process. Among those proteins, cAMP dependent protein kinase (PKA) is tremendously important in the control of cell migration and actin cytoskeletal reorganization.

I. cyclic AMP –dependent protein kinase (PKA)

A growing literature has described the role of PKA in cell migration. PKA is ubiquitously expressed and targets most cellular structures including membrane, cytoplasm, mitochondria, nucleus and microfilaments (Howe, 2004; Pidoux and Tasken, 2010; Shabb, 2001; Skalhegg and Tasken, 2000). PKA, which was first discovered by Krebs and his group (Walsh et al., 1968), is a heterotrimeric complex that consists of two regulatory and two catalytic subunits (Krebs and Beavo, 1979). In mammals, PKA has four regulatory (RIα, RIβ, RIIα and RIIβ) and three catalytic subunits (Cα, Cβ and Cγ) each of which is encoded by a unique gene (Doskeland et al., 1993).Thus, PKA holoenzyme exists in two types of heterotetrameric forms: type I (RIα and RIβ) and type II (RIIα and RIIβ). The type I PKA is predominantly cytoplasmic and more sensitive to cAMP with an activation constant (K_{act}) of 50-100 nM of cAMP while almost 75% of type II PKA isoforms associate with specific cell structures and have a K_{act} of 200-400nM of cAMP (Cadd and McKnight, 1989; Dostmann and Taylor, 1991; Gamm et al., 1996). The catalytic (C) subunits of PKA exhibit similar substrate specificity and kinetic features (Edelmann et al., 1987; Taylor et al., 1992), whereas the regulatory or (R) subunits

determine the characteristics of the holoenzyme. The RI and RII subunits are known as modular proteins and consist of an N-terminal dimerization domain, an autophosphorylation site acting as the principal contact site for the catalytic subunits and two cAMP binding sites (Corbin et al., 1978; Doskeland, 1978). Binding of two cAMP molecules to each of the regulatory subunits results in conformational changes in these subunits and terminates the PKA autoinhibitory contact, allowing the catalytic subunits to dissociate and catalyze the phosphorylation of substrate proteins (Taylor et al., 1990) (Gibbs et al., 1992; Wang et al., 1991)

Over the last three decades, PKA has been designated as one of the principal regulators of migration, adhesion, and cytoskeletal organization in various cell types. PKA mediated phosphorylation of cytoskeletal structures was shown to increase at the protrusive end of cells during growth factor induced migration (Howe et al., 2005; Deming et al., 2008; Rivard et al., 2009). PKA was also reported to have an enhancing effect on breast and squamous carcinoma cell migration (O'Connor and Mercurio, 2001) and fibroblast cell migration (Edin et al., 2001). In contrast, other groups demonstrated that PKA activation inhibited $\alpha_v\beta_3$-dependent EC migration on vitronectin (Kim et al., 2000) and EGF induced fibroblasts migration (Shiraha et al., 1999). Consistent with this, it was also observed that inhibition of $\alpha_v\beta_3$ integrin dependent PKA activation reduced EC migration (Dormond and Ruegg, 2003). Besides migration, PKA also plays an important role in regulation of cell adhesion. Several groups have shown that PKA both promotes and inhibits integrin mediated cell-cell and cell-ECM adhesion (Howe et al., 2002; Howe and Juliano, 2000; O'Connor and Mercurio, 2001; Whittard and Akiyama, 2001). Others have also reported that PKA regulates members of the Rho family of small GTPases,

such as Rac and cdc42, and actin cytoskeletal organization during integrin mediated cell adhesion and migration (Dormond et al., 2002; Feoktistov et al., 2000; O'Connor and Mercurio, 2001). Dormond et al. (2002) demonstrated that $\alpha_v\beta_3$-mediated EC adhesion and spreading was mediated by PKA dependent Rac activation. Studies by O'Connor and Mercurio (2001) showed that PKA mediated Rac1 activation promoted the growth factor induced β_1 clustering during the adhesion and migration of carcinoma cells. Whittard and Akiyama (2001) demonstrated that PKA activation promoted the assembly of F-actin polymerization during cell adhesion. In contrast, other groups showed that PKA activation had inhibitory effects on other members of small GTPases, such as Rho and P21 activated kinase. Inhibition of PKA caused an enhanced translocation of Rho A to the membrane ruffles in colon carcinoma cell lines (O'Connor et al., 2000). P21 activated kinase1 (PKA1), a major target for Rac and cdc42 in regulation of actin dynamics, was also inhibited by PKA activation in fibroblast cell lines (Howe and Juliano, 2000).

Taken together, studies from different groups indicate that PKA has several targets associated with cell adhesion, migration and cytoskeletal assembly and can both facilitate and inhibit cell migration depending upon the cell types and experimental conditions. The positive and negative effects and large number of targets further suggest that the PKA dependent signaling pathway is highly complex at the cellular level and must have a mechanism to link specific PKA activity to specific functions. Studies have indicated that the recruitment of PKA to a specific subcellular location is mediated through an interaction with A-kinase anchoring proteins (AKAPs) (Carlisle Michel and Scott, 2002; Logue and Scott, 2010; Welch et al., 2010). From this, a paradigm has evolved in which PKA scaffolding by AKAPs plays a critical role in regulating PKA dependent signaling

cascades and may contribute to the diverse functions of PKA in cell migration.

II. A-Kinase Anchoring Protein (AKAP)

The term 'A-Kinase Anchoring Proteins (AKAPs)' was coined by Rubin and his group (Hirsch et al., 1992), to refer to structurally diverse but functionally similar PKA scaffolding proteins. The microtubule associated protein MAP2 was the first AKAP shown to have a direct interaction with the RII subunit of PKA (Theurkauf and Vallee, 1982). The AKAP family consists of more than 50 members (Beene and Scott, 2007) and the number is still growing. The work of several groups has led to the concept that AKAPs target PKA to distinct subcellular domains where they facilitate PKA phosphorylation of specific substrates (Brandon et al., 2003); (Ruehr et al., 2003). Each AKAP contains an amphipathic helix of 14 to 18 residues that is involved in binding to the N-termini of PKA-RII dimer (Carr et al., 1991; Newlon et al., 1999; Newlon et al., 1997). The majority of AKAPs serve as scaffolds for the RII subunits of PKA whereas a few are dual-specificity AKAPs (e.g. D-AKAPs, Ezrin and AKAP220) which interact with RI dimer with lower affinity (Herberg et al., 2000).

AKAPs also contain a unique subcellular targeting domain that restricts its locations to specific cell structures or organelles (Dell'Acqua and Scott, 1997; Rubin, 1994; Tasken and Aandahl, 2004). For example, AKAP450 localizes at centrosomes and the Golgi apparatus and is involved in cell cycle progression and membrane trafficking (Takasaki et al., 1999). AKAP350 is also targeted centrosomes to regulate the centrosomal cytoskeletal system (Chen et al., 1997; Lin et al., 1995). AKAP79/150 localizes to the plasma membrane and associates with glutamate receptors (Colledge et al., 2000). AKAP18, another membrane associated AKAP is involved in regulation of L-

type Ca^{2+} channels in pancreatic β-cells (Fraser et al., 1998). AKAP95 localizes within the nucleus and is associated with chromatin condensation during mitosis (Steen et al., 2000). In summary, it can be seen that the wide distribution of AKAPs and their ability to scaffold PKA at different subcellular structures makes them potential candidates for a wide range of signaling events associated with PKA dependent signaling cascade.

III. AKAPs organize signaling complexes at different subcellular domains

Besides binding to PKA, most of the AKAPs have a unique ability to interact with several binding partners simultaneously to form a signaling complex (Scott and Pawson, 2009; Tasken and Aandahl, 2004). The roles of AKAPs in organizing multivalent signaling have been highlighted in detail in several review articles (Carlisle Michel and Scott, 2002; Logue and Scott, 2010; Smith et al., 2010; Welch et al., 2010). AKAP-Lbc for example, anchors PKCη and protein kinase D (PKD) in a way that facilitates PKD activation (Carnegie et al., 2004; Diviani, 2007). In addition to that, AKAP-Lbc contains tandem Dbl homology-pleckstrin homology (DH-PH) domains which function as a guanine exchange factor for Rho (Diviani et al., 2001). The activity of AKAP-Lbc's Rho guanine nucleotide exchange factor (GEF) is stimulated by the heterotrimeric G protein Gα 12 and terminated by homo oligomerization of two AKAP-Lbc proteins. It has been shown that the activation of AKAP-Lbc induced remodeling of actin cytoskeleton to increase the stress fiber formation (Jin et al., 2004). The anchored complex of AKAP-Lbc with its binding partners like PKC, PKCη, PKD creates a point of convergence between cAMP and Rho signaling and plays a critical role in spatial distribution of PKA at the leading edge of migrating cells (Paulucci-Holthauzen et al.,2009). Several other AKAPs like, AKAP79/150 and mAKAP have unique binding partners and form signaling

complexes to facilitate various events in cells as well. AKAP150 (murine homologue of AKAP79) is an anchoring protein found in hippocampal neurons and scaffolds PKA, PKC and protein phosphatase2B (PP2B) at membrane to glutamate receptors through its binding with synapse associated protein 97 (Coghlan et al.,1995; Klauck et al.,1996 ; Colledge et al.,2000). This complex ensures a precise PKA phosphorylation of the receptors to facilitate flow of ions through the channels (Coghlan et al.,1995; Klauck et al.,1996 ; Colledge et al.,2000). Likewise, in heart, the muscle-specific anchoring protein mAKAP binds to PKA, PDE4D3 and extracellular signal regulated kinase-5 (ERK5) and organizes different combination of protein complex to involve in intracellular cAMP regulation (Dodge-Kafka et al.,2005; Dodge-Kafka et al.,2006). From this, it can be concluded that most AKAPs can interact with different combinations of enzymes, substrates and signaling molecules to function as activators, effectors, initiators or terminators of signaling cascades as well as serve as a focal point where two or more signaling events converge.

IV. cAMP –synthesis and hydrolysis

The synthesis and hydrolysis of cAMP is tightly controlled in cells. cAMP is synthesized from 5'-ATP by several adenylyl cyclase (AC) (EC 4.6.1.1) isoforms (Sutherland, 1970), the majority of which are embedded in the cell surface plasma membrane (Cooper, 2003). In 1989, the first mammalian AC was cloned and purified from bovine brain (Chen, 2000). Since then, eight more isoforms of AC have been identified in mammals which are encoded by ten AC genes. All nine isoforms of mammalian AC share a primary structure consisting of two transmembrane domains (M1 and M2) and two catalytic domains (C1 and C2) (Krupinski et al., 1989). The C1 and C2

domains, which are further subdivided into C1a, C1b, C2a, and C2b, contain conserved regions and several regulatory sites for nucleotides (Liu et al., 1997; Tesmer et al., 1997), metal ion binding (Tang and Gilman, 1995) and binding to G-protein subunits (Tesmer et al., 1997; Yan et al., 1997; Zimmermann et al., 1998). The catalytic unit of cAMP is provided by C1a and C2b heterodimerization (Tang and Gilman, 1995). Several cAMP elevating compounds [i.e. isopreterenol, prostacyclin, prostaglandin E_2 and β adrenergic agonist of G_s protein coupled receptors (GPCRs)] induce their respective transmembrane receptors to release the $G_{\alpha s}$ protein which binds to C1a and C2a subunits of AC to activate rapid production of cAMP (Zimmermann et al., 1998).

The phosphodiesterase enzymes (PDE) catalyze cAMP hydrolysis and are regarded as key components of cAMP signaling as well (Beavo, 1995; Beavo and Brunton, 2002; Bender and Beavo, 2006; Conti et al., 2003; Houslay and Adams, 2003). Eleven distinct members of PDE (PDE1-PDE11) and more than 50 distinct isoforms have been identified out of which PDE4, PDE7 and PDE8 selectively hydrolyze cAMP and PDE1, PDE2, PDE3, PDE10, PDE11 families of enzyme can hydrolyze both cAMP and cGMP (Maurice et al., 2003). Vascular ECs differentially express four PDE isoforms: PDE2, PDE3, PDE4 and PDE5 (Netherton and Maurice, 2005).

V. c-AMP controls many cellular events

cAMP, a secondary messenger, regulates numerous cellular functions. PKA, which serves an important role in a myriad of cellular processes and has a wide range of substrates, is directly stimulated by cAMP (Langan, 1968; Lohmann et al., 1980; Steinberg et al., 1975). PDE, especially PDE3 and PDE4 are the main contributors of cAMP hydrolyzing activity in cells and regulate a wide range of cellular activities. PDE4

selective inhibitors such as rolipram have been reported to affect pathways associated with cell migration. Also, inhibition of PDE4 suppresses the formation of actin adhesion microspike structures and inhibits the migration of rat embryo fibroblast cells (Fleming et al., 2004). Selective inhibition of PDE4 also reduces VEGF induced migration of several cultured endothelial cells (Netherton and Maurice, 2005) . Selective inhibition of PDE4 has also been shown to inhibit PDGF induced vascular smooth muscle cell migration (Palmer et al., 1998). It has been reported that migration and proliferation of HUVEC is impaired by rolipram dependant inhibition of PDE4 (Favot et al., 2003). In summary, these studies suggest that intracellular cAMP level regulates several cellular events associated with cell migration.

VI. Signaling complex of cAMP mediated by AKAPs, AC and PDE

Several studies have shown that many AKAPs bind both PKA and PDEs to form a signaling complex that regulates cAMP flux at discrete subcellular locations (Baillie et al., 2005; Dodge et al., 2001; Rich et al., 2007; Willoughby et al., 2006; Xin et al., 2008). This mechanism of AKAP mediated compartmentalization of cAMP was demonstrated in the muscle selective AKAP (mAKAP) in cardiomyocytes by Dodge et al (Dodge et al., 2001). mAKAP assembles PKA and PDE4D3 at the nuclear membrane and facilitates PKA dependant PDE4D3 phosphorylation of Ser54 which in turns enhances cAMP hydrolysis (Sette and Conti, 1996). The extracellular signal-regulated kinases (ERKs), a binding partner of the mAKAP complex, phosphorylates PDE4D3 on Ser579 to deactivate PDE activity and counterbalances the effect of increased cAMP hydrolysis (Hoffmann et al., 1999).

The real time measurement of cAMP by several groups using electrophysiological

studies and fluorescence resonance energy transfer (FRET) and genetically modified biosensors has further revealed that AKAP mediated cAMP regulation is compartmentalized within several microdomains in cells (Jurevicius and Fischmeister, 1996; Rochais et al., 2006; Zaccolo and Pozzan, 2002). These studies demonstrated that local stimulation by β_2- agonists of adrenergic receptors caused a selective increase in cAMP dynamics in the area of membrane exposed to the stimulus suggesting that $G_{\alpha s}$ mediated activation of AC occurred in a restricted area. Further analysis showed that knocking down PDE, which was anchored AKAPs, dissipated the intracellular cAMP gradient, confirming that PDEs anchored to AKAPs facilitated the hydrolysis of cAMP in distinct areas (Willoughby et al., 2006; Xin et al., 2008). In summary, these studies have demonstrated that many AKAPs maintain a functional complex of AKAP-PKA-PDE and contribute to the spatial compartmentalization of cAMP signals.

VII. Gravin /SSeCKS (AKAP12)

Gravin (also known as AKAP12 or AKAP250), a member of the multivalent AKAP family, was originally identified in a human umbilical vein endothelial cell expression library with serum from a patient with myasthensia gravis (Gordon et al., 1992). Subsequently, gravin was also identified in a fetal brain expression library and was shown to be an AKAP (Nauert et al., 1996). Like many AKAPs, gravin has been reported to bind PKA RII subunit *in vitro* (Grove and Bruchey, 2001; Nauert et al., 1996; Piontek and Brandt, 2003) through the interaction with an amphipathic helical domain near C-terminal region between residues 1541 and 1554 (Nauert et al., 1996). It occurs as three major isoforms (designated as alpha, beta and gamma) which share 95% amino acid sequence homology and differ at their N-terminal sequence (Streb et al., 2004).

Expression of this protein was detected by immunoblot either as a doublet protein at 300 and 287 kDa or a 250 kDa minor isoform (considered to be proteolytic fragment of gravin) (Gelman, 2002; Grove et al., 1994). Src suppressed C Kinase Substrate (SSeCKS), is the rodent orthologue of gravin, sharing areas of similarity with the N-terminal 1000 residues and C-terminal 25-40 residues of gravin (Lin et al., 1995). Gravin is expressed in wide variety of tissues including fibroblasts, components of the central and peripheral nervous system, adrenal medulla and cells associated with renal glomerulus (Grove et al., 1994). The gene for gravin is located at a single chromosomal site 6q24-25.2 (Xia et al., 2001). Confocal and immunoelectron microscopy have revealed that gravin is localized at the cell margin in some cells (Grove and Bruchey, 2001; Piontek and Brandt, 2003). Several binding partners of gravin have been identified. Gravin has been shown to bind PKC (Chapline et al., 1996; Erlichman et al., 1999; Grove and Bruchey, 2001; Nauert et al., 1996) and is involved in PKC mediated redistribution of PKA in cultured cells (Yan et al., 2009). In addition, gravin controls putative binding domains that interact with SH3 domains of Src (Gelman, 2002), protein phosphatase 2B (Shih et al., 1999), β-adrenergic receptors (Shih et al., 1999; Tao and Malbon, 2008; Tao et al., 2003), Ca^{2+}/calmodulin (Tao et al., 2006), cyclin D (Lin et al., 2000) and PDE4D (Willoughby et al., 2006).

Gravin interacts with β-AR and PDE4D in regulation of cAMP dynamics. Gravin has been shown to be involved in resensitization and recycling of desensitized β-AR (Tao and Malbon, 2008; Tao et al., 2007), whereas Cooper and his group demonstrated that PDE4D anchored to the membrane by gravin is involved in termination of subplasmalemmal cAMP (Willoughby et al., 2006). Knockdown experiments confirmed

that gravin was involved in the spatiotemporal regulation of cAMP flux beneath the plasma membrane (Willoughby et al., 2006).

Further, functional aspects of gravin/SSeCKS have been studied by several other groups and three different roles have been described. These are tumor suppression, control of cell cycle and regulation of cytoskeleton. SSeCKS has been reported to be down-regulated in v-Jun transformed murine fibroblasts (Cohen et al., 2001) and the re-expression has been reported to suppress v-Src induced morphological transformation (Lin and Gelman, 1997). Moreover, SSeCKS was reported to bind CyclinD and form a complex that plays a role in G1 to S progression through the cell cycle (Lin and Gelman, 2002), while Gelman et al (2000) reported that SSeCKS plays a role in cell migration during embryogenesis (Gelman et al., 2000). Gelman and his group have also demonstrated that translocation of SSeCKS from the plasma membrane to a perinuclear region after treatment with phorbol ester coincided with the loss of actin stress fibers in Rat-6 cells (Lin and Gelman, 1997; Lin et al., 1996). Further study by Xia and Gelman showed that FAK-dependent tyrosine phosphorylation of SSeCKS modulated its binding to the actin cytoskeleton, suggesting that SSeCKS plays a role in mitogen induced cytoskeletal organization (Xia and Gelman, 2002).

VIII. Statement of Problems

Since gravin was discovered in 1992, a number of studies have been published on its tissue expression, subcellular distribution and physiological functions. Gravin/SSeCKS has not been detected in vessels *in vivo* but is expressed in cultured endothelial cells. However, the role of gravin in cultured endothelial cells is not yet clear. Immunofluorescence and immunoelectron microscopy have revealed that gravin

expression is concentrated at the cell periphery (Grove et al., 1994; Grove and Bruchey, 2001) and the peripheral distribution of gravin suggests that it might be associated with cortical cytoskeleton. Consistent with these findings, SSeCKS, the rodent orthologue of human gravin has been reported to associate with actin stress fibers in cultured murine cells (Lin and Gelman, 1997; Lin et al., 1996). At present the role of gravin in vascular wound healing is unknown. However, based on these studies, it can be predicted that gravin may be involved in EC migration and cytoskeletal reorganization through PKA dependent pathways.

Therefore, in the present studies it is hypothesized that gravin plays a role in endothelial cell wound healing by promoting cell migration in cultured HUVEC. It is also hypothesized that gravin promotes cell migration by changing the distribution of cortical actin in the membrane ruffles of migrating cells. To test these hypotheses, gravin expression in human umbilical vein endothelial cells (HUVEC) in their active and quiescent stage was determined by Western Blotting. Further, the effect of gravin knockdown on cell migration was determined using a scratch wound model and a 96-well plate based cell migration assay in cultured HUVEC. Finally, the effect of gravin knockdown on actin cytoskeletal distribution was determined using epifluorescence microscopy and image analysis.

CHAPTER II

METHODS AND MATERIALS

The present study investigates the hypothesis that gravin plays a role in endothelial cell migration. To investigate this, the following experiments were performed. In first experiment, gravin expression in active and quiescent ECs was determined using Western blotting. In the second experiment, the effect of gravin knockdown on cell migration was analyzed using an *in vitro* scratch wound assay and a 96-well plate based cell migration assay. Finally, the effect of gravin knockdown on actin cytoskeletal reorganization was investigated. The methodological approaches and material used to accomplish this are outlined below.

I. General Methods

a. Reagents

i. Oligonucleotides

The antisense and missense oligonucleotides used in this study were purchased from Oligos etc., (Wilsonville,OR) and consisted of phosphorothioate 20-mers that were either complementary in sequence to a region in 3'end UTR of the full length human gravin transcript (designated as antisense oligonucleotide) or had a scrambled sequence (designated as missense oligonucleotide) The sequences of the antisense and missense oligonucleotides were as below.

antisense oligonucleotide: 5'-CAGTCTCAGCAGCAGCATTC-3',

missense oligonucleotide: 5'-CAGTCTCAGGACCAGCATCT-3'

All bases in the oligonucleotides were phosphorothioated to increase resistance to nuclease reactions while maintaining the capacity to hybridize target sequences and induce RNase H activity. A 200μM stock of both antisense oligonucleotide and missense oligonucleotide in RNase free water (Invitrogen Life Technologies) was stored as 10μl aliquots at -80°C and used to make working dilutions.

ii. siRNA

Two different siRNAs (designated as siRNA1 and siRNA2) against gravin and a non-targeting control or universal negative siRNA (siRNA Control, Cat. No. SIC001) were purchased from Sigma-Aldrich (St. Louis, MO). Sequences of the three siRNAs were as follows:

siRNA1	5’-CGAAACAGCUGUUACCGUA-3’
	5’-UACGGUAACAGCUGUUUCG-3’
siRNA2	5’-GUAGAAGGUUCCACUGUAA-3’
	5’-UUACAGUGGAACCUUCUAC-3’
siRNA Control	Proprietary sequence

A 100μM stock of each siRNA in RNAse free water was stored as 10μl aliquots at -80°C and diluted to make the working concentrations.

iii. Other reagents

Unless specified, all the chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). MilliQ reagent grade water was used in this entire study.

b. Cell Culture

HUVEC were purchased from Lifeline Cell Technology (Frederick, MD; Cat. No. FC0003) and cultured in VascuLife basal media (Fredrick, MD; Cat. No LL-0002) supplemented with VascuLife EnGS LifeFactors (Fredrick, MD; Cat. No. LS-1019) containing FBS(2%), EnGS (0.2%), rhEGF (0.5ng/ml), ascorbic acid (50μg/ml), L-glutamine(10Mm), hydrocortisone hemisuccinate (1μg/ml) and heparin sulfate(0.75U/ml). HUVEC were grown in culture with or without VEGF (5ng/ml) lacking antimicrobial agents and phenol red. HUVEC were grown in $25cm^2$ or $75cm^2$ tissue culture treated flasks (Corning, NY) at 37°C with 5% CO_2 atmosphere in a waterjacketed CO_2 incubator (Forma Scientific Inc. OH, model 3546). The media was replaced with fresh growth media three times per week and cells were split 1:20 upon reaching confluence. When splitting and harvesting HUVEC, the cells were detached using 0.25% trypsin-EDTA (Lifeline Cell Technology, MD). Only HUVEC at passages 3-6 were used in experiments in this study.

c. Western Blotting

To perform Western blotting, the protein samples were first separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared by mixing the cell lysate with sample buffer [0.5M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol and a pinch of bromophenol blue] while on ice and then incubating the mixture at 100°C for 5min. 10μg total protein of each sample was loaded into the wells of 5% (v/v) SDS-polyacrylamide gels and run for 3.5hr at 30mA using an electrophoresis gel system (mini protean II, BioRad, CA) and an EC 250-90 electrophoresis power supply system (E-C Apparatus Co., FL). Following SDS-PAGE, the gels were incubated with freshly prepared transfer buffer [25mM Tris, 92 mM

Glycine, 20% methanol, pH 8] for 15 min and then sample proteins were transferred to nitrocellulose membranes (Bio-Rad, CA) for 1hr at 350mA using a mini Trans-Blot (Bio-Rad,CA) according to the manufacture's protocol. After that, the membranes were blocked overnight in blocking buffer [0.2% (w/v) purified casein (I-block, Tropix, MA, Cat No. T-2015)] and probed the next day with a 1:5000 dilution of rabbit polyclonal anti-gravin antibody (Rb7753, from Dr. Bryon Grove) or a 1:1000 dilution (stock concentration 1.78 mg/ml) of monoclonal alpha actinin antibody (Abcam, MA, Cat No. ab18061) in blocking buffer at room temperature for 2hr. Following incubation, the membranes were washed three times (5 min/wash) with washing buffer [0.1% (v/v) Tween-20 in PBS] and incubated with a 1:100,000 dilution of alkaline phosphatase conjugated goat anti rabbit IgG (Sigma-Aldrich, MO, Cat No. A3812) or a 1:5000 dilution of alkaline phosphatase conjugated goat anti mouse IgG (Sigma-Aldrich, MO, Cat No. A-3562) at room temperature for 2hr respectively. Following this incubation, the membranes were washed three times (5min/wash) with washing buffer and then briefly incubated with 1x assay buffer [20mM Tris (pH 9.8), 1mM $MgCl_2$] followed by 0.25mM chemiluminescent substrate CDP-Star (Applied Biosystem, MA, Cat. No. T2218) for 5min at room temperature using manufacturer's protocol. The chemiluminescent signal was detected using Fujifilm Super RX film.

d. Cell based ELISA

A cell based ELISA was performed to detect the level of gravin in cells treated with either antisense oligonucleotide or siRNA. HUVEC were grown to sub-confluence (20,000 cells/cm^2) in each well of a tissue culture treated 96-well plate (Corning, NY) for 24hr and transfected either by an antisense oligonucleotide and controls or siRNA and

controls as described later in Chapter II, Material and Methods, Section General methods, Sub-section e. Transfection of cultured cells. Following transfection, cells were incubated for 18-20hr and were then fixed with 3.7% (w/v) paraformaldehyde (Polyscience Inc, PA) in PBS [150mM NaCl, 4mM Na/K phosphate and 5mM KCl (pH 7.3)] for 15min and washed twice with 0.1% BSA in PBS. Cells were then permeablized with digitonin (60μg/ml) for 10min and blocked with 10% donor horse serum (Atlanta Biological, GA) in 0.1% BSA in PBS for 30min. Following the blocking, cells were incubated for 1hr at 37°C with 1:1000 dilution of primary monoclonal gravin antibody in 3% donor horse serum (0.1% BSA in PBS). A duplicate set of antisense oligonucleotide or siRNA treated wells, along with their controls, was incubated with non immune antimouse IgG (Jackson Immunoresearch Laboratories Inc., PA, Cat No. 015-000-003) at a dilution (1:8000) that was titrated to match with the gravin antibody titer. These wells served as control for any non specific antibody interactions. Each treatment and their corresponding controls were conducted in triplicate for gravin and control IgG in these assays.

Following the incubation, cells were washed with 0.1% BSA in PBS for three times and incubated with a 1:1000 dilution of HRP conjugated donkey antimouse antibody (Jackson Immunoresearch Laboratories Inc, PA, Cat. No. 715-035-150) for 1hr at 37°C. Wells treated with secondary antibodies only and no primary antibody served as controls. Finally, cells were washed three times with 0.1% BSA in PBS and developed with Sigma FAST ^{TM} OPD tablet (Sigma, Cat. No. P9187-50SET) for 5min according to the manufacturer's instructions. The OD was measured at 490 nm using a 96-well plate reader (Spectra Max Plus, Molecular Device, CA). The ELISA signal was then normalized against total protein in each well. Total protein (μg) was measured using BCA

protein assay kit (Pierce, IL; Cat. No. 23227). Briefly cells in the 96-well plates were washed three times with 1X Tris-buffered saline with 0.1% Tween-20 (TBST, pH 7.4) and incubated with 200µl freshly prepared BCA reagent for an hour at 60°C. At the end of the incubation, absorbance was measured at 540 nm and from a standard curve of protein concentration prepared by dissolving BSA in lysis buffer containing leupeptin (10µg/ml), total protein in each well was quantified.

e. Transfection of cultured cells

In all the experiments involving transfection, HUVEC were either treated with 0.05µM antisense oligonucleotide or 0.25µM siRNA (siRNA1 and siRNA2) and their concentration matched controls (missense oligonucleotide and siRNA control). Treatments without oligonucleotide or siRNA served as additional controls for both experiments. Oligofectamine reagent (Invitrogen Life Technologies, NY, Cat. No. 12252-011) and OPTI-MEM media with no phenol red (Invitrogen Life Technologies, NY, Cat. No. 11058-021) were used as transfection reagent and medium for the transfection.

i. Transfection with antisense oligonucleotide

Briefly, 50µl Oligofectamine was diluted with 106µl OPTI-MEM and incubated for 10min at room temperature. The diluted antisense oligonucleotide/missense oligonucleotide was prepared by mixing 1µl of antisense or missense oligonucleotide (200µM stock solution) in 700µl of prewarmed OPTI-MEM and incubating for 20 min at room temperature. Then 25µl of diluted oligofectamine solution was mixed with 175µl of prediluted antisense oligonucleotide/missense oligonucleotide to obtain the oligofectamine-oligonucleotide solution. Cells were then washed twice with prewarmed OPTI-MEM and transfected with a mixture of 200µl oligofectamine-oligonucleotide

solution and 800µl prewarmed OPTI-MEM. After that, cells were incubated at 37°C for 4hr with 5% CO_2 atmosphere. Finally, at the end of the incubation period, the transfection solution was replaced with prewarmed VascuLife EnGS cell culture medium supplemented with VEGF (5ng/ml) and 0.05µM antisense or missense oligonucleotide.

ii. Transfection with siRNA

Briefly, 54µl of oligofectamine was diluted with 216µl of OPTI-MEM and incubated for 10min at room temperature. The diluted siRNA1/siRNA2/siRNA control solutions were prepared by mixing 3µl siRNA1/siRNA2/siRNA control (100µM stock solution) in 192µl prewarmed OPTI-MEM and incubating for 20 min at room temperature. Then, 45µl of diluted Oligofectamine solution was mixed with 180µl of the diluted siRNA1/siRNA2/siRNAcontrol solutions to obtain the oligofectamine-siRNA solution. Cells were washed and transfected with a mixture of 225µl siRNA-oligofectamine solution and 900µl prewarmed OPTI-MEM and were incubated at 37°C for 4hr with 5% CO_2 atmosphere. At the end of the incubation period, the transfection solution was replaced with prewarmed VascuLife EnGS cell culture medium supplemented with VEGF (5ng/ml).

Experimental protocols

a. Effect of cell density on gravin expression

HUVEC were seeded into tissue culture treated 25cm^2 culture flasks (Corning, MA) at five cell densities ($1.2x10^5$ cells/cm^2, $0.6x10^5$ cells/cm^2, $0.2x10^5$ cells/cm^2, $0.1x10^5$ cells/cm^2 and $0.05x10^5$ cells/cm^2) and incubated for 48hr. Following incubation, cells were photographed and detached with 0.25% trypsin-EDTA (Cellgro,VA). An small aliquot of cells from each flask was then counted using a hemocytometer while the rest of

the sample was lysed by washing the cells twice with PBS (pH 7.2) to remove residual trypsin-EDTA and then resuspended in ice cold 20-50µl extraction buffer containing (20µM Tris base, 150mM NaCl, 10mM disodium EDTA,10mM benzamidine HCl, 1% Triton X-100, 0.05% Tween-20, 1mM phenylmethylsulfonyl fluoride, 100µg/ml leupeptin pH 7.4) and incubated on ice for 10min. Following this, the extract was centrifuged for 10min at 16,000x g at 4°C and supernatant was collected. The protein concentration of the cell extracts was determined using a BCA protein assay kit (Pierce, IL) and the remainder of the sample was analyzed for gravin expression using western blotting.

b. Effect of gravin knockdown on cell migration

i. Scratch wound assay

HUVEC were plated on tissue culture treated 8-well plates (Thermo Scientific Nunc,NY) with $7x10^5$ cells per well and grown to confluence for 48hr. A single wound was created at the center of the confluent monolayer by gently scraping with a sterile teflon stick with a 2mm wide tip. The residual cell debris was removed with a single wash of OPTI-MEM and then transfected either with antisense oligonucleotide or siRNA as described earlier in Chapter II Material and Methods, section General methods, sub-section e. Transfection of cultured cells.

In the scratch wound assay, cells were allowed to incubate for 18-20hr following antisense oligonucleotide or siRNA mediated transfection. Phase contrast images of the wounded area were collected using a 4X phase contrast objective on a Nikon Diaphot inverted microscope equipped with a SPOT2 digital camera (Diagnostic Instruments, Inc., MI). Cells in the same field were captured at two different time points based on the

reference lines drawn on the bottom of the plates. These reference lines defined a grid that marked the wounded region of the monolayer. Five alternating microscopic fields covering the wound region of the monolayer were photographed immediately after the transfection (0hr) and 18-20hr after transfection.

To analyze the changes in the wound over 48hr time period, two images of the same field, captured at two different time points (0hr and 18-20hr) were overlaid on each other using Adobe PhotoshopCS3 software. The area between the wound edges was measured at the two different time points using ImageJ software (ImageJ 1.44k downloaded from NIH image home page http://rsb.info.nih.gov/ij/), converted from pixels to μm^2 and then mean distance between wound edges was derived by dividing the area by the length of the wound length of the image field. The distance the wound edges moved was calculated by subtracting mean wound with at 0hr and 18-20hr. Experiments were repeated for at least 3 times.

ii. 96 well plate migration assay

HUVEC were seeded ($1x10^4$ cells per well) into wells on 96-well tissue culture microplate (Grenier CELL STAR black, NC, Cat. No. T-3026-16) that contained a cell seeding stopper designed to cover a circular region in the center of each well. Immediately after removal of the cell stoppers following 24hr incubation, a circular cell free exclusion zone of 1.2mm diameter was created in center of each well. Cells in each well were rinsed once with OPTI-MEM to clean the residual non adherent cells and then transfected with either the antisense oligonucleotide and controls or the siRNA and controls as described earlier in Chapter II Material and Methods, Section General methods, Sub-section e. Transfection of cultured cells. Following transfection, the

transfection medium was replaced with VascuLife EnGS cell culture medium supplemented with VEGF (5ng/ml) and the cells were incubated for 44-46hr after which, the number of the cells present in the cell free zone was quantified. Phase contrast images of the cells in each well were collected using a 4X phase contrast objective on a Nikon Diaphot inverted microscope equipped with a SPOT2 digital camera (Diagnostic Instruments, Inc.). The treatments and corresponding controls were represented by four separate wells in each experiment. Experiments were repeated at least 3 times. The number of HUVEC present in the cell free zone after 46hr was quantified with the ImageJ software. To quantify number of HUVEC present in the cell free zone, a line was drawn at the boundary between the edge of the monolayer and the cell free zone over the images captured at 0hr. Two images of the exact same field captured at two different time points were overlaid using Adobe Photoshop software based on the reference lines drawn on the bottom of the plate. The number of cells that migrated into the cell free zone from the surrounding monolayer and the area of the cell free zone at 0hr were quantified for each well and then the number of cells in each well was normalized with the corresponding area of the cell free zone (mm^2). All experiments were repeated at least 3 times on separate occasions.

c. Effect of gravin knockdown on actin organization

i. Experimental design

In this experiment, confluent HUVEC monolayers grown on 25mm diameter glass coverslip (Fisher Scientific, PA, Cat. No. 12-545-102) coated with 2% gelatin were scratched by a Teflon stick across the center of the coverslip. The resulting wound was approximate 15mm in length. The cells were then transfected with antisense

oligonucleotide (0.05μM) and missense oligonucleotide (0.05μM), or no oligonucleotide as described earlier (Chapter II Material and Methods, Section General methods, Sub-section e. Transfection of cultured cells) and incubated with VascuLife EnGS cell culture medium supplemented with VEGF (5ng/ml) for 18-20hr. The cells were then fixed with 3.7% (w/v) paraformaldehyde in PBS for 15min, washed twice with 0.1% BSA in PBS, permeablized with digitonin (60μg/ml) for 10min and washed a final time with 0.1% BSA in PBS. Cells were then incubated with a 1:200 dilution of TRITC labeled phalloidin (Sigma, MO, Cat. No. P-1951) from a stock solution (200μM in DMSO) for 1hr at room temperature. Following incubation, the cells were rinsed three times with 0.1% BSA in PBS and mounted on glass slides using ProLong Gold antifade reagent with DAPI (Invitrogen, OR, Cat. No. P36935). The cells were photographed using a Nikon TE300 inverted epifluorescence microscope using a 63X oil objective lens. Approximately 30-40 images were captured along the length of the wound with an overlap of approximately 50 pixels between two adjacent images. From an arbitrary starting point, on the left or right side of the wound, every other image was captured in a zone spanning about 5mm out of the approximate 15mm total wound length. Experiments were repeated three times.

ii. Image Analysis

To analyze the effect of gravin knockdown on actin cytoskeleton reorganization, images of the wounded monolayer were analyzed using ImageJ software. In each image, only the cells in the very first layer adjacent to the wound edge were selected for quantitative analysis. In those cells, the actin filaments were stained brightly by TRITC-phalloidin, and visualized as a band at the cortical region. The length of these actin bands

and the free perimeter (edge not touching neighboring cells) of the cells were quantified using ImageJ software. The mean length (μm) of the cortical actin band was normalized with the mean length (μm) of free edge of cells and results were represented as a ratio of two lengths. All experiments were repeated at least 3 times on separate occasions.

III. Statistical Analysis

Statistical analysis was performed using SigmaStat software (version 3.5, Systat Software Inc.) for parametric comparison and GraphPad Prism software (version 6) was used for non parametric analysis. Comparison of multiple treatment groups was performed using a one way ANOVA followed by Holm-Sidak test for parametric com parisons. In the case of data sets that failed to the test of normality, the non parametric Kruskal-Wallis one way analysis of variance on ranks was used to compare groups followed by Dunn's test for making multiple comparisons. Data were expressed as the mean ±SD (parametric comparison) or median with 25 and 75 percentile (non parametric comparison). Significant difference was indicated by $P<0.05$.

CHAPTER III

RESULTS

I. Effect of Cell Density on Gravin Expression

To determine the effect of cell density on gravin expression, the level of gravin in cell extracts from HUVEC cultured at different cell densities was assessed using SDS-PAGE and Western blotting.

Western blot analysis demonstrated that there was an effect of cell density on endogenous gravin expression in cultured HUVEC. The intensity of the gravin bands, which were visible as a doublet at approximately 300kDa, was lowest in cell extracts from cultures at the highest density and highest in the cell extracts from the lowest density cultures (Figure 1). The cell extracts from three intermediate densities also showed a trend of cell density dependent gravin expression in which the level of gravin expression progressively decreased as cell densities increased. To confirm that the observed pattern of gravin expression was not due to unequal loading of protein samples in the gel, alpha-actinin (molecular weight 105kDa) was selected as a suitable loading control which showed an even and equal pattern of band intensity at different cell densities (Figure 1, panel C). Taken together, these observations demonstrated that the expression of gravin level in cultured condition is cell density dependent.

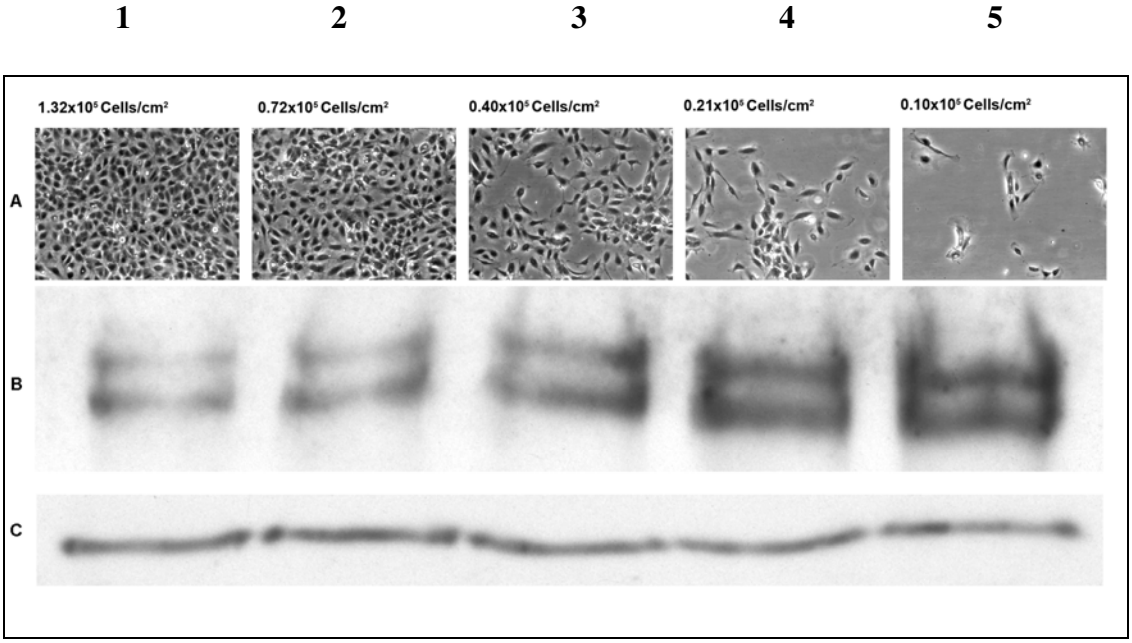


Figure 1. Phase contrast micrographs and Western blot demonstrating cell density dependent gravin expression in cultured HUVEC. (A) Phase contrast micrographs of HUVEC seeded at different cell densities ($1.2x10^5$ cells/cm^2, $0.6x10^5$ cells/cm^2, $0.2x10^5$ cells/cm^2, $0.1x10^5$ cells/cm^2 and $0.05x10^5$ cells/cm^2) after 48hr of incubation. (B) Western blot demonstrating the correlation between the level of gravin expression and cell density. Cells, at their lowest density had the greatest gravin expression and the expression was decreased as cell density increased. (C) Western blot of alpha actinin demonstrating the equal amount of protein in each well.

II. Effect of antisense oligonucleotide and siRNA treatment on gravin expression

To determine the effect of gravin knockdown in cultured HUVEC, the level of gravin expression was analyzed in cells treated with either an antisense oligonucleotide or siRNA using Western blotting and an enzyme-linked immunosorbent assay (ELISA).

a. Western blot analysis of HUVEC extracts

Cells were seeded at 20,000 cells/cm^2 in tissue culture treated flasks and incubated for 24hr. These cells were then transfected either with antisense and control oligonucleotides or siRNA and siRNA controls and then extracted 24hr post transfection. Blots of cell extracts demonstrated that the intensity of the gravin doublet at approximately 300kDa was decreased significantly in the extracts obtained from both antisense oligonucleotide and siRNA treated cells compared to the controls (Figure 2a). Furthermore, siRNA1 had a greater knockdown effect on gravin expression than the siRNA2 (Figure 2b). There was no visible difference in the intensity of gravin bands between the controls for the antisense oligonucleotide and siRNA treatments. The alpha-actinin band used as a loading control confirmed that an equal amount of total protein was loaded in each of the wells of gel. (Figure 2a and 2b, panel B).

Figure 2a. Western blot demonstrating the effect of antisense oligonucleotide treatment on the level of gravin expression in cultured HUVEC. The level of gravin expression was decreased in the cell extracts with antisense oligonucleotide treatment compared to the controls whereas no difference in gravin expression was observed between the two controls (missense oligonucleotide and no oligonucleotide) (Panel A). The alpha actinin band demonstrated that the protein loading in each well was equal (panel B).

Figure 2b. Western blot demonstrating the effect of siRNA treatment on the level of gravin expression in cultured HUVEC. Gravin expression was decreased in the cell extracts treated with either siRNA1 or siRNA2 compared to controls (siRNA control and no siRNA) (Panel A). The treatment with siRNA1 caused a greater reduction in gravin expression when compared with siRNA2 (Panel A). The alpha actinin band demonstrated that an equal amount of protein was loaded in each well (panel B).

Figure 2a

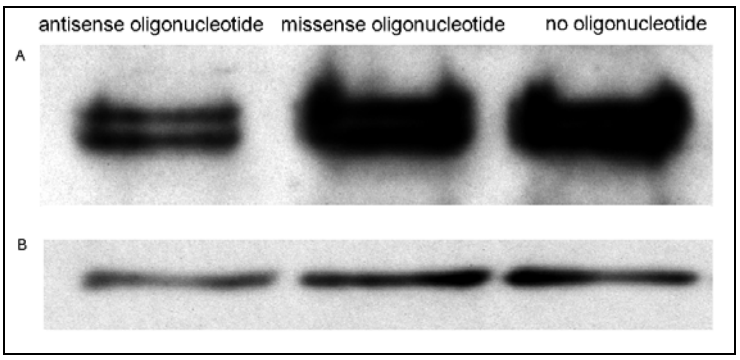
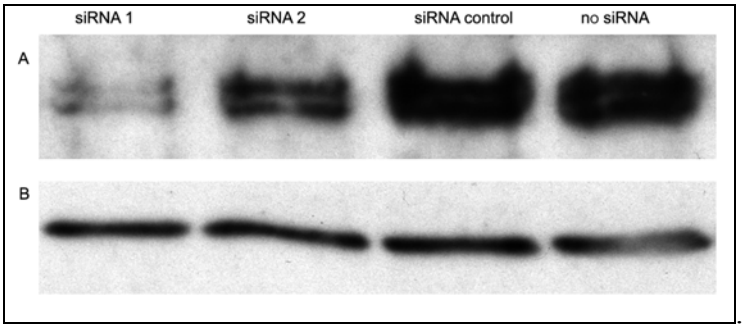


Figure 2b



b. Analysis by cell based ELISA

In addition to the Western blotting analysis, a cell based ELISA was used to measure the effect of antisense oligonucleotide and siRNA treatment on gravin levels. The results of this assay confirmed the findings from the Western blotting study. Both data from three individual experiments and the pooled data demonstrated that treatment of HUVEC with an antisense oligonucleotide caused a significant ($P < 0.05$) reduction in the gravin signal compared to treatment with a missense oligonucleotide or no oligonucleotide (Table 1, Figure 3). In three independent experiments the gravin signal was reduced by approximately 22.72%, 14.70% and 35% as compared to the controls.

Similarly, the cell based ELISA also demonstrated a significant reduction in gravin signal in siRNA1 and siRNA2 treated cells compared with siRNA control and no siRNA (Table 2, Figure 4). The siRNA1 treatment showed approximately 20%, 28 % and 21% reduction in gravin signal as compared to the two controls in three individual experiments (Table 2, Figure 4). The approximate reduction in gravin signal was 14%, 12% and 12% with siRNA2 treatment as compared to the two controls in these experiments (Table 2, Figure 4). Thus, siRNA1 showed a greater reduction in gravin signal than siRNA2.

Table 1. Mean±SD values of the normalized ELISA signal demonstrating the effect of antisense oligonucleotide treatment on gravin expression in cultured HUVEC. Results from individual experiments showed that gravin level was significantly reduced in antisense oligonucleotide treated cells compared to the controls. Asterisk (*) indicates a statistical difference ($p<0.05$) between antisense oligonucleotide and the two controls (missense oligonucleotide and no oligonucleotide).

Experiment #	ELISA signal/total protein (Mean±SD)			Statistical analysis	
	antisense	missense	no oligos	significance	test performed
exp 1	0.51±0.04	0.66±0.02	0.66±0.02	*	one way ANOVA, Holm-Sidak
exp 2	0.29±0.01	0.34±0.00	0.34±0.01	*	one way ANOVA, Holm-Sidak
exp 3	0.44±0.10	0.68±0.11	0.66±0.02	*	one way ANOVA, Holm-Sidak

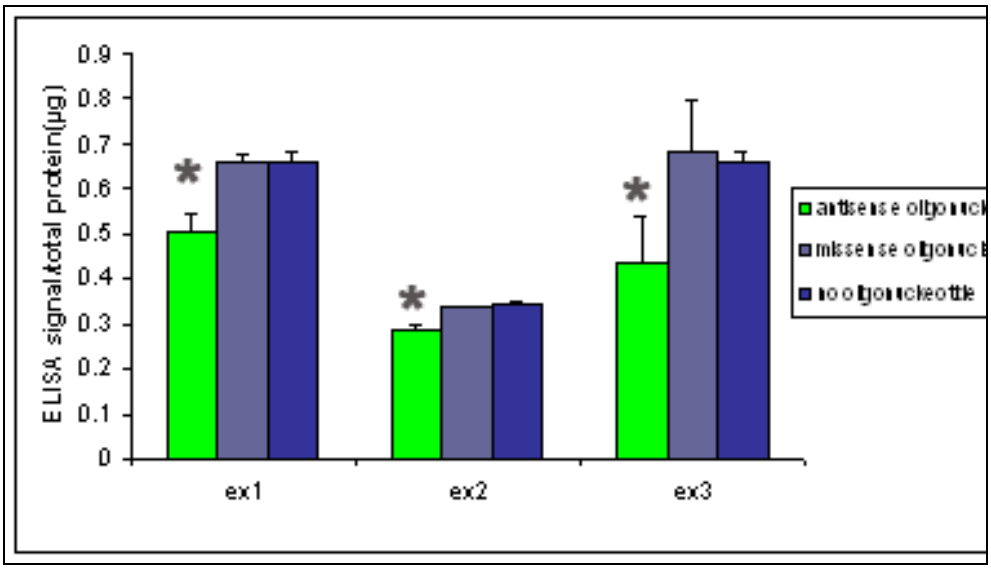


Figure 3. Histogram (mean±SD) representing the normalized cell based ELISA signal confirming the results obtained from Western blot analysis for antisense oligonucleotide treatment. The quantification of ELISA signal was normalized with total protein. Treatment with antisense oligonucleotide showed a significant reduction in ELISA signal in individual experiments but not in the pooled data. (*) indicates a statistical difference ($p<0.05$) between antisense and the two controls (missense and no oligonucleotide).

Table 2. Mean±SD values of the normalized ELISA signal demonstrating the effect of siRNA treatment on gravin expression in cultured HUVEC. Results from the individual experiments and pooled data showed that gravin levels were significantly reduced in siRNA treated cells compared to the controls. Results also indicated that siRNA1 treatment had a grater effect on gravin knockdown than siRNA2 treatment. Asterisk (*) indicates a statistical difference ($p<0.05$) between siRNA2 treatment and the two controls (control siRNA and no siRNA), (#) indicates a statistical difference ($p<0.05$) between siRNA1 treatment and the two controls.

Experiment #	ELISA signal/total protein (Mean±SD)				Statistical analysis	
	siRNA1	siRNA2	siRNA contr	no siRNA	significanc	test performed
exp 1	0.28±0.01	0.30±0.01	0.35±0.01	0.35±0.00	* #	one way ANOVA, Holm-Sidak
exp 2	0.23±0.02	0.28±0.01	0.32±0.01	0.32±0.01	* #	one way ANOVA, Holm-Sidak
exp 3	0.26±0.01	0.29±0.00	0.33±0.01	0.33±0.01	* #	one way ANOVA, Holm-Sidak
Pooled data	0.26±0.03	0.29±0.01	0.33±0.02	0.33±0.01	* # **	one way ANOVA, Tukey

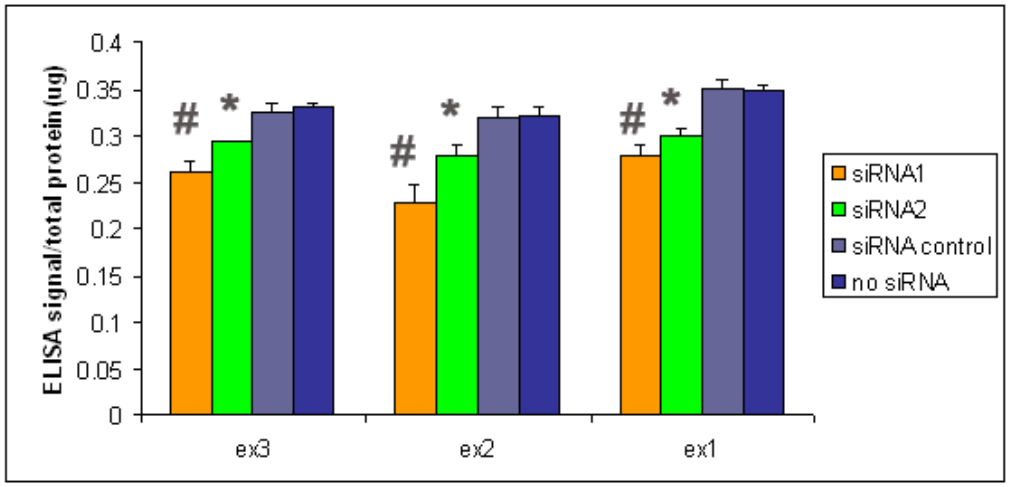


Figure 4. Histogram (mean±SD) representing the normalized cell based ELISA signal confirming the results obtained from Western blot analysis for siRNA treatment. The quantification of the ELISA signal was normalized with total protein. Both siRNA1 and siRNA2 treatments showed a significant ($p < 0.05$) reduction in ELISA signal. (#) indicates a statistical difference ($p<0.05$) between siRNA1 treatment and the two controls, (*) indicates a statistical difference ($p<0.05$) between siRNA2 treatment and the two controls (control siRNA and no siRNA).

III. Effect of gravin knockdown on wound closure

To test the hypothesis that gravin plays a role in EC wound healing, the effect of gravin knockdown on wound closure was determined using antisense oligonucleotide or siRNA treatment in cultured HUVEC. HUVEC grown to confluence in tissue culture treated 8-well plates were mechanically wounded by scratching the monolayer with a teflon stick and then transfected with antisense oligonucleotide to knockdown gravin. The width of wound measured immediately post transfection and 48hr later was then used to determine the extent of wound closure in each of the treatments.

To determine the working concentration of antisense oligonucleotide, two concentrations (0.1μM and 0.05μM) were tested in scratch wound assay. 0.1μM concentration reduced wound closure, but showed a significant difference between missense oligonucleotide and no oligonucleotide controls. On the other hand, the lower concentration (0.05μM) of antisense oligonucleotide showed a reduced wound closure, but no difference between the missense oligonucleotide and no oligonucleotide treatments. Similarly, two different concentrations (0.5μM and 0.25μM) were tested for siRNA1 and siRNA2 in the scratch wound model and the lower concentration (0.25μM) of siRNA1 and siRNA2 was found to be effective in impairing wound closure with no difference between siRNA control and no siRNA. Therefore, 0.05μM antisense oligonucleotide and 0.25μM siRNA1/siRNA2 were used in all the experiments involving antisense oligonucleotide and siRNA mediated gravin knockdown. Analysis of four individual experiments demonstrated varying degrees of difference between antisense oligonucleotide and the controls (Table 3, Figure 5 and Figure 6). In three experiments, antisense oligonucleotide treatment significantly reduced the distance the wound edge

moved with approximate values of 49%, 32%, 30% compared with controls (Table 3, Figure 5 and Figure 6). The fourth experiment did not show any significant difference in wound closure between the antisense oligonucleotide and the controls (Table 3, Figure 5 and Figure 6). However, the pooled data obtained from all experiments showed a significant difference between antisense oligonucleotide and the controls, where antisense oligonucleotide treatment showed approximately a 30% reduction in the distance the wound edge moved as demonstrated in Table 3, Figure 5 and Figure 6.

Treatment with both siRNA1 and siRNA2 had a similar effect on the wound closure as illustrated in Table 4, Figure 7 and Figure 8. Analysis of individual experiments revealed that siRNA2 treatment significantly reduced the distance the wound edge moved (Table 4, Figure 7 and Figure 8) compared with its controls. The siRNA2 treatment reduced the distance the wound edge moved by approximately 36%, 21%, and 47% in three independent experiments compared with controls. siRNA1 treatment also showed reduced wound closure but there was varying degrees of difference between treatment and controls (Table 4, Figure 7 and Figure 8) when data from individual experiments were analyzed. In one experiment, the siRNA1 showed a significant difference in wound closure compared with two controls where siRNA1 treatment showed an approximately 14% reduction in wound closure (Table 4, Figure 7 and Figure 8). In one of the three experiments, there was no significant difference between the siRNA1 treatment and the two controls (Table 4, Figure 7 and Figure 8). In another experiment, siRNA1 treatment showed a significant difference compared with siRNA control only and no significant difference was observed between siRNA1 treatment and no control (Table 4 and Figure 6).

Table 3. Mean±SD values of the distance the wound edge moved demonstrating the effect of antisense oligonucleotide treatment on the wound closure in cultured HUVEC. Results from individual experiments and pooled data showed that after 18 hr, cells treated with antisense oligonucleotide had a significant reduced distance (μm) the wound edge moved compared to the controls. Asterisk (*) indicates a statistical difference ($p<0.05$) between antisense oligonucleotide and two controls.

Experiment #	The distance the wound edge moved (μm) (Mean±SD)			Statistical analysis	
	antisense	missense	no oligos	significance	test performed
exp 1	259.21±44.29	515.43±89.06	554.26±70.18	*	one way ANOVA, Holm-Sidak
exp 2	447.17±60.37	656.85±98.21	641.07±66.85	*	one way ANOVA, Holm-Sidak
exp 3	461.62±136.45	507.51±120.56	517.99±139.07	no	One way ANOVA
exp 4	404.82±66.33	578.38±47.68	604.74±57.95	*	one way ANOVA, Holm-Sidak
Pooled data	393.21±113.07	564.54±104.99	579.51±95.32	*	one way ANOVA, Holm-Sidak

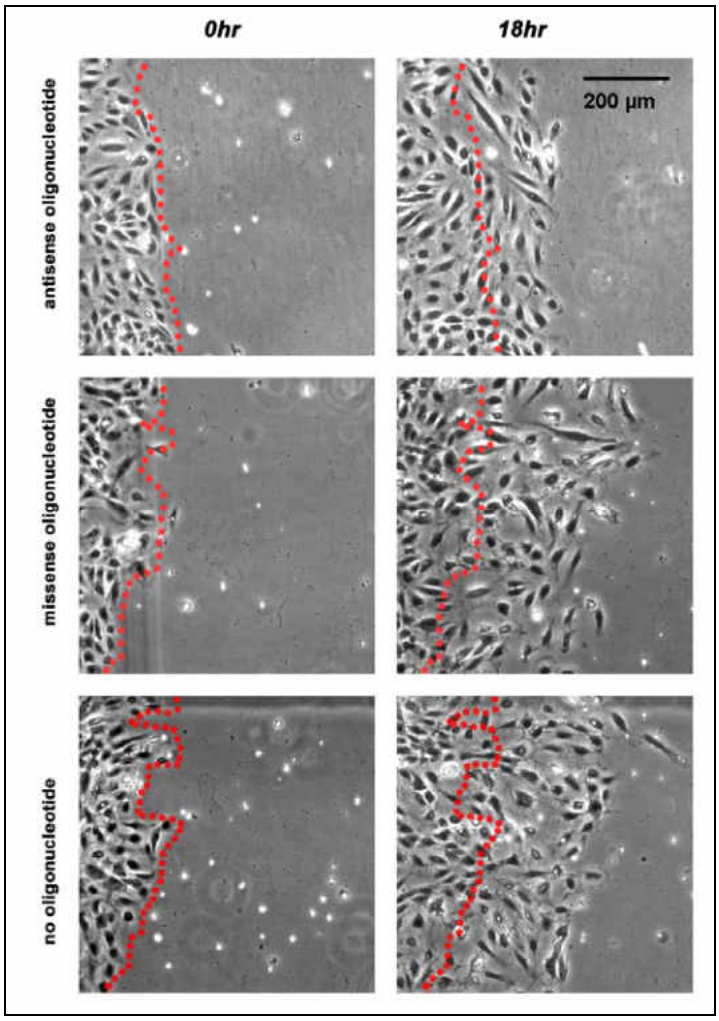


Figure 5. An *in vitro* scratch wound assay demonstrating an impaired wound closure in antisense oligonucleotide treated HUVEC after 18hr of transfection. Cells were photographed at 0hr and 18hr using phase contrast microscope at 4x magnification. Red dotted lines represent the edge of the scratch wound at 0hr. The distance that the wound edge moved was significantly reduced in antisense oligonucleotide treated cells compared to the controls (missense oligonucleotide and no oligonucleotide treated cells).

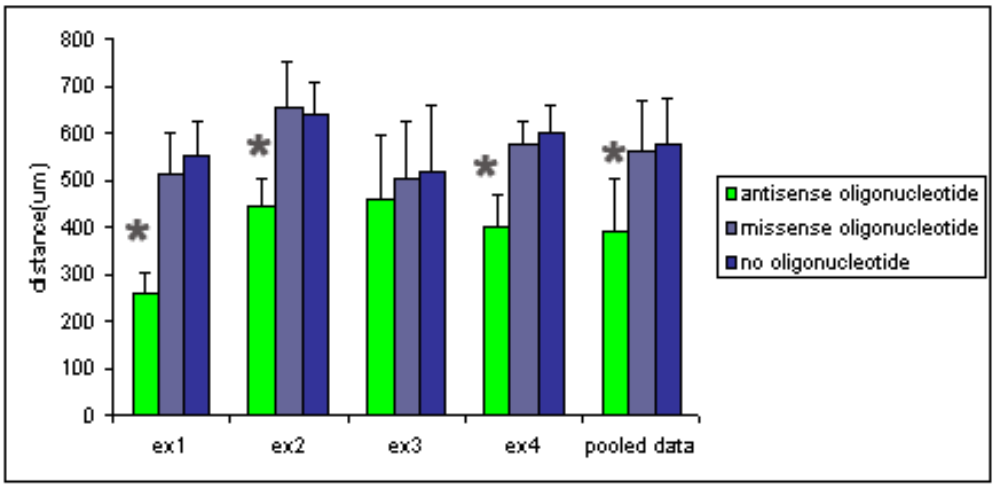


Figure 6. Histogram (mean ±SD) demonstrating the effect of antisense oligonucleotide treatment on wound closure in cultured HUVEC using a scratch wound model. The distance the wound edge moved was significantly reduced in antisense oligonucleotide treated cells compared to the controls in three out of four individual experiments and the pooled data. (*) indicates a statistical difference ($p<0.05$) between the antisense and the two controls.

Table 4. Mean±SD values of the distance the wound edge moved in wounded HUVEC monolayers treated with gravin siRNA. Results from individual experiments and pooled data showed after 18hr of transfection, cells treated with siRNA2 had a significant reduction in the distance the wound edge moved compared to the controls. Asterisk (*) indicates a statistical difference ($p<0.05$) between siRNA2 treatment and the two controls, (#) indicates a statistical difference ($p<0.05$) between siRNA1 treatment and the two controls.

Experiment #	The distance the wound edge moved (μm) (Mean±SD)				Statistical analysis	
	siRNA1	siRNA2	siRNA control	no siRNA	significance	test performed
exp 1	568.21±61.78	420.63±25.59	664.40±36.47	648.07±43.74	* #	one way ANOVA, Holm-Sidak
exp 2	519.66±37.41	450.53±33.72	569.53±51.72	588.00±47.52	*	one way ANOVA, Holm-Sidak
exp 3	503.64±42.67	336.08±66.69	635.31±49.02	585.64±35.61	* #	one way ANOVA, Holm-Sidak

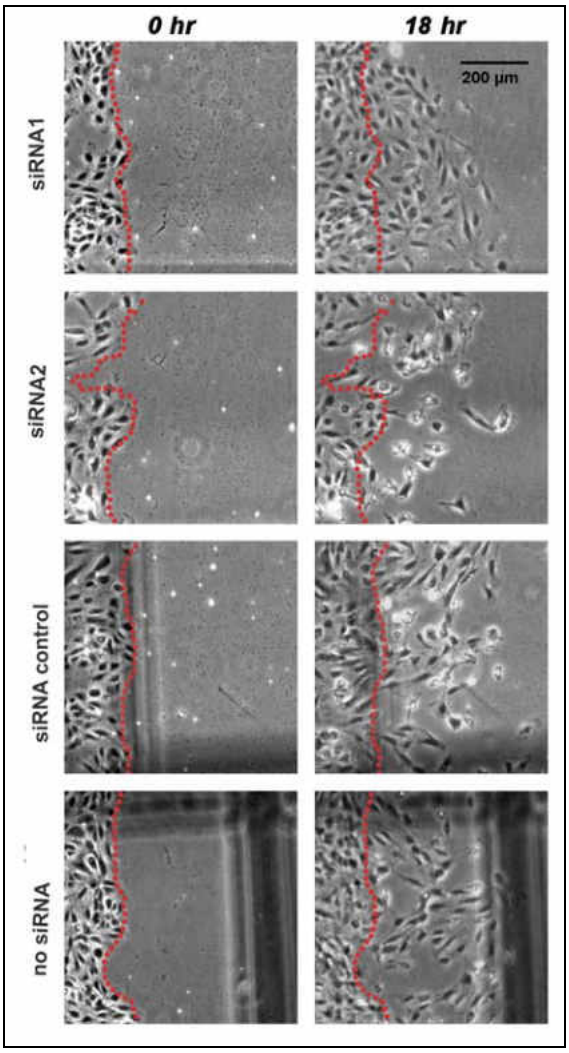


Figure 7. An *in vitro* scratch wound assay demonstrating an impaired wound closure in siRNA treated HUVEC after 18hr of transfection. Cells were photographed at 0hr and 18hr using a phase contrast microscope at 4X magnification. Red dotted lines represent the edge of the scratch wound at 0hr. Treatment with both siRNA1 and siRNA2 reduced the distance the wound edge moved. Moreover, siRNA2 treatment impaired wound closure to a greater extent than siRNA1 treatment showing more reduction in the distance the wound edge moved.

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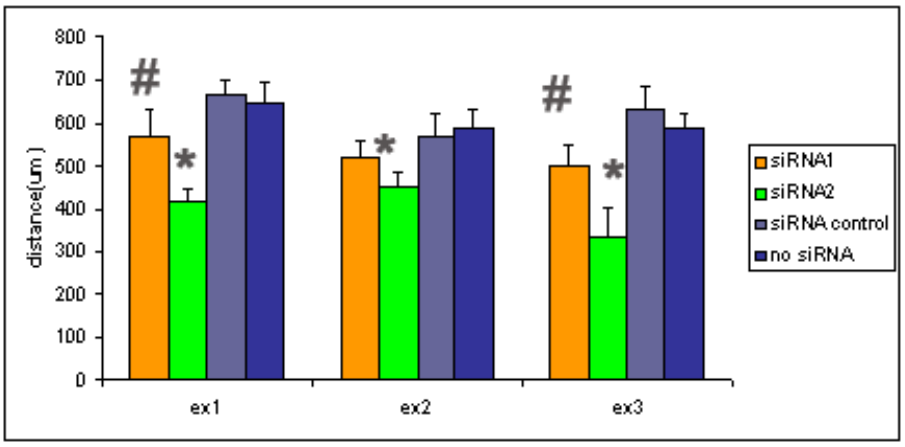

Figure 8. Histogram (mean±SD) demonstrating the effect of siRNA treatment on wound closure in cultured HUVEC using a scratch wound model. siRNA2 treatment reduced the distance the wound edge moved to a greater extent than siRNA1 treatment. (*) indicates a statistical difference ($p<0.05$) between siRNA2 treatment and the two controls (control siRNA and no siRNA), (#) indicates a statistical difference ($p<0.05$) between siRNA1 treatment and the two controls.

IV. Effect of gravin knockdown on cell migration

It is now well known that cell migration plays an important role in wound healing. The observation that gravin knockdown impairs EC wound healing *in vitro* suggests that gravin may play a role in cell migration. To further investigate this, the effect of antisense oligonucleotide and siRNA treatment on cell migration in a 96-well plate based cell migration assay was tested.

The results from 96-well cell migration assays are presented in Table 5, Figure 9 and Figure 10. The three experiments showed that antisense mediated gravin knockdown significantly reduced the number of cells present in the cell free zone 48hr after the removal of cell stoppers from the wells in the 96 well plate (Table 5, Figure 9 and Figure 10). In three experiments, antisense oligonucleotide treatment reduced the number of cells in the cell free zone by approximately 30%, 32% and 38% compared with the two controls (Table 5, Figure 9 and Figure 10).

siRNA1 and siRNA2 treatments also showed an effect in this assay. Analysis of data from three experiments showed that the siRNA2 mediated gravin knockdown significantly reduced the number of endothelial cells in the cell free zone by approximately 39%, 52% and 52% respectively compared with the controls (Table 5, Figure 11 and Figure 12). Similarly, data from individual experiments showed that siRNA1 treatment significantly reduced the number of cells present in the cell free zone compared with the controls by approximately 30%, and 41% in two experiments (Table 5, Figure 11 and Figure 12). There was no significant difference between the siRNA1 treatment and the controls in one of the experiments.

Table 5. Mean±SD values of number of cells present in the cell free zone demonstrating the effect of antisense oligonucleotide in 96-well based cell migration assay. Results from individual experiments and pooled data showed that after 44-46hr of transfection, antisense oligonucleotide treatment significantly reduced number of cells present in the cell free zone (cells/mm^2) compared to the control cells. Asterisk (*) indicates a statistical difference ($p<0.05$) between antisense oligonucleotide and the two controls.

Experiment #	Number of cells present in the cell free zone (cells/mm^2) (Mean±SD)			Statistical analysis	
	antisense	missense	no oligos	significance	test performed
exp 1	204.68±10.67	293.75±16.41	294.21±20.00	*	one way ANOVA, Holm-Sidak
exp 2	65.00±3.30	95.06±3.01	95.69±3.10	*	one way ANOVA, Holm-Sidak
exp 3	41.15±1.53	72.62±2.94	73.48±2.33	*	one way ANOVA, Holm-Sidak

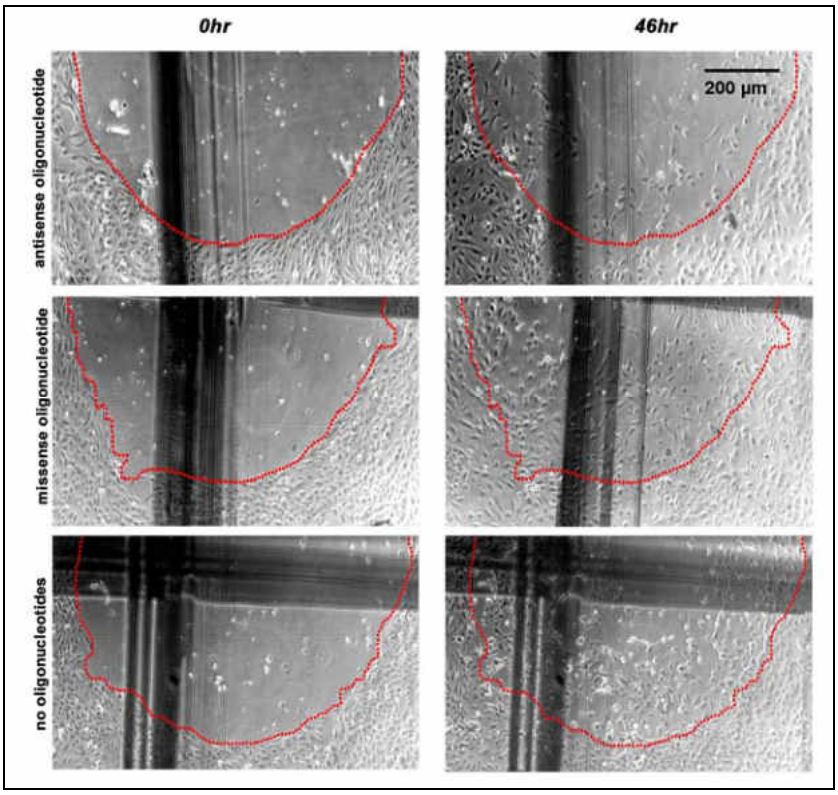

Figure 9. An *in vitro* cell migration assay demonstrating the effect of antisense mediated gravin knockdown on the number of cells present in the cell free zone. Antisense treatment reduced the number of cells present in the cell free zone after 46hr of transfection. Cell were photographed at 0hr and 46hr under phase contrast microscope at 4X magnification. Red dotted lines represent the edge of cell free zone at 0hr. The number of cells present in the cell free zone after 46hr of transfection was counted.

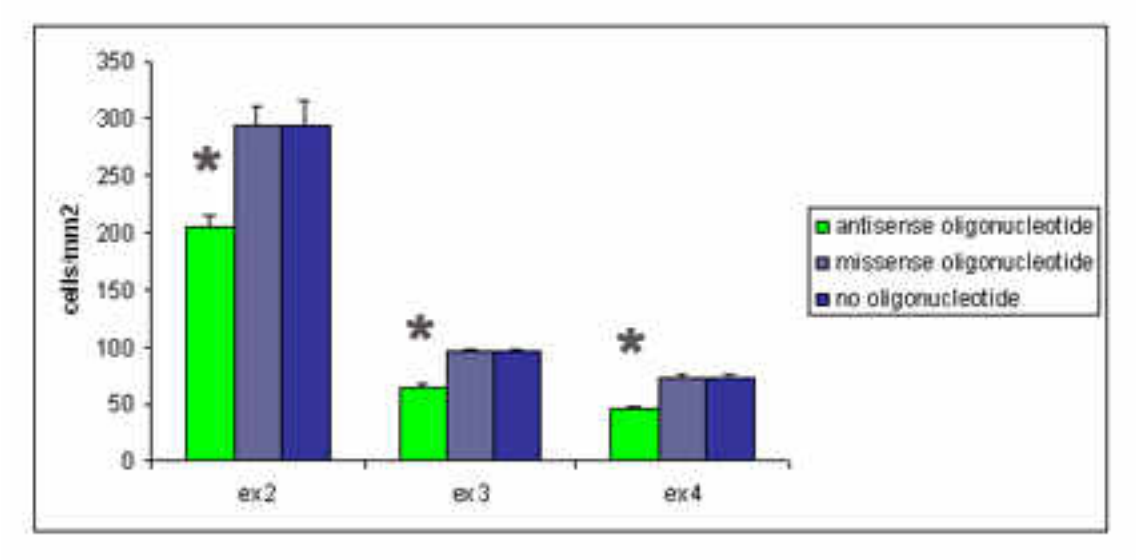

Figure 10. Histogram (mean±SD) demonstrating the effect of antisense oligonucleotide treatment on the number of cells present in the cell free zone in a 96-well based cell migration assay. Results from individual experiments and pooled data showed that after 44-46hr of transfection, antisense oligonucleotide treatment significantly reduced the number of cells present in the cell free zone (cells/mm^2) compared with the control cells. Asterisk (*) indicates a statistical difference ($p<0.05$) between antisense oligonucleotide and control treatments.

Table 6. Mean±SD values of number of cells present in the cell free zone demonstrating the effect of siRNA treatment in 96-well based cell migration assay. Results from the individual experiments and the pooled data showed that after 44-46hr of transfection, siRNA2 treatment significantly reduced the number of cells present in the cell free zone compared to the control cells. Asterisks (*) indicate a statistical difference ($p<0.05$) between siRNA2 and the two controls, (#) indicates a statistical difference ($p<0.05$) between siRNA1 and the two controls, (***) indicates a significant difference ($p<0.05$) between the two controls.

Experiment #	Number of cells present in the cell free zone (cells/mm^2) (Mean±SD)				Statistical analysis	
	siRNA1	siRNA2	siRNA control	no siRNA	significance	test performed
exp 1	77.70±6.40	68.92±5.36	111.64±6.29	109.31±5.53	* #	one way ANOVA, Holm-Sidak
exp 2	71.30±1.99	42.39±3.18	64.64±3.06	67.31±7.36	*	one way ANOVA, Holm-Sidak
exp 3	42.40±3.18	34.38±6.26	71.30±1.99	64.64±3.06	* # ***	one way ANOVA, Holm-Sidak

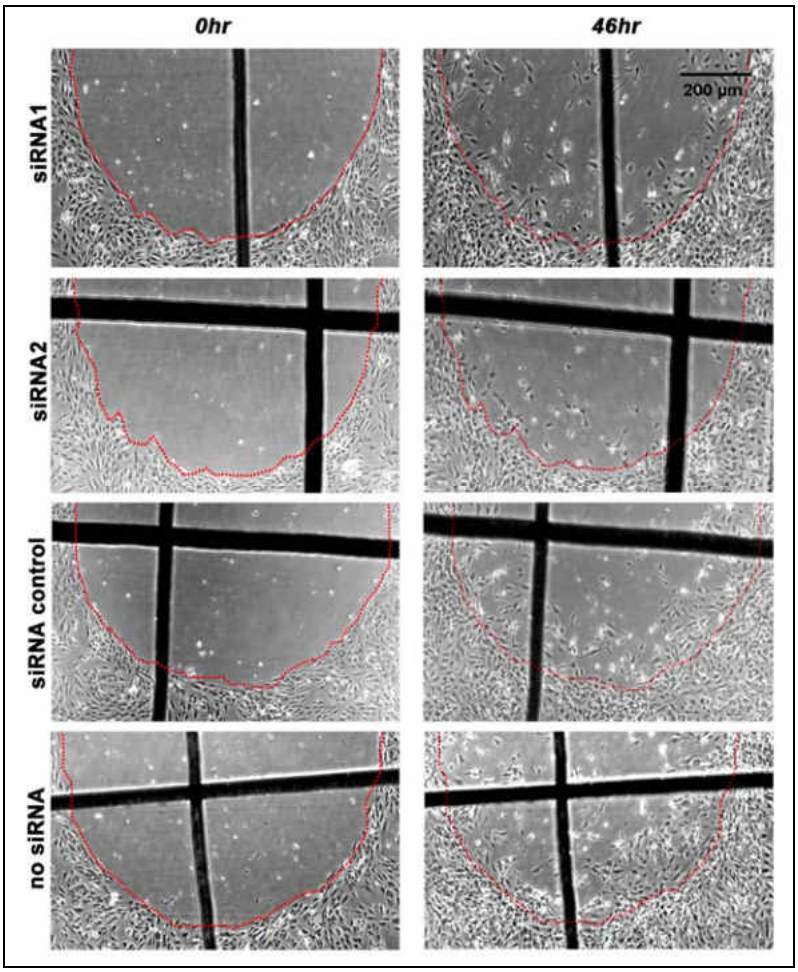

Figure 11. An *in vitro* cell migration assay demonstrating a reduction in number of cells present in the cell free zone in siRNA treated HUVEC after 46hr of transfection. Both siRNA1 and siRNA2 treatments reduced the number of cells present in the cell free zone. siRNA2 treatment resulted in fewer cells in the cell free zone compared to siRNA1 treatment. Cells were photographed at 0hr and 46hr using a phase contrast microscope at 4X magnification. Red dotted lines represent the edge of the cell free zone at 0hr. The number of cell present in the cell free zone after 46hr of transfection was counted.

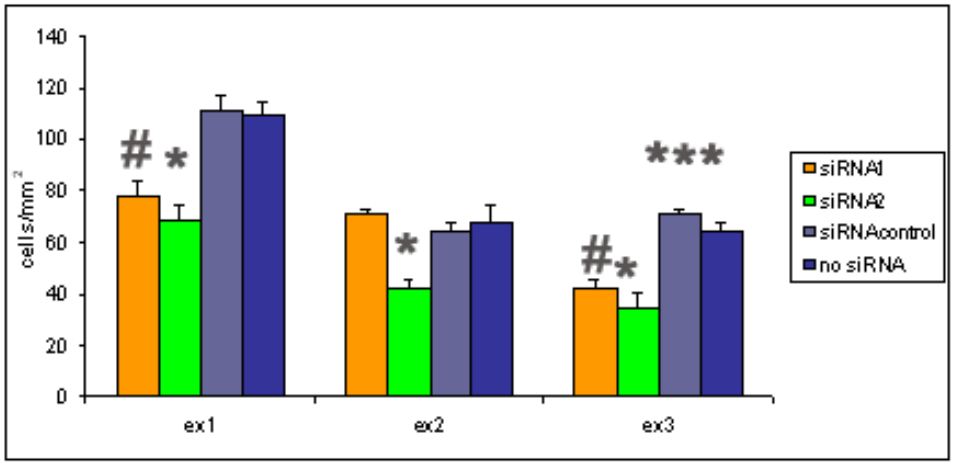

Figure 12. Histogram (mean±SD) demonstrating the effect of siRNA treatment on number of cells present in the cell free zone in 96-well based cell migration assay. Results from individual experiments and pooled data showed that after 44-46hr of transfection, siRNA2 treatment significantly reduced the number of cells present in the cell free zone compared to the control cells. Asterisk (*) indicates a statistical difference ($p<0.05$) between siRNA2 treatment and the two controls, (#) indicates a statistical difference ($p<0.05$) between siRNA1 treatment and the two controls, (***) indicates a significant difference ($p<0.05$) between the two controls

V. Effect of gravin knockdown on actin re-organization

Rearrangement of F-actin is considered to be an integral part of cell migration. Because gravin knockdown reduced the rate of wound closure in a scratch wound assay and reduced the number of cells present in the cell free zone in a cell migration assay, the effect of gravin knockdown, using antisense oligonucleotide treatment, on the actin cytoskeleton was also determined. When observed with a 63X oil objective lens, a thick and bright band of actin, stained with TRITC- labeled phalloidin, was found localized at the cell membrane adjacent to the membrane ruffles in cells at the wound edge in wounded monolayer after 18-20hr (Figure 13). The localization and distribution pattern of these actin bands suggested that they might have a structural and functional role in the formation of membrane protrusions associated with cell migration. In addition, these actin bands were clearly distinct from the longitudinal actin stress fibers which are typically associated with cell adhesion in cultured cells.

Analysis of the proportion of actin to free surface at the wound edge in cells treated with oligonucleotides revealed that treatment with antisense oligonucleotide significantly reduced the normalized length of actin bands compared to the control treatments. The three experiments showed that the antisense oligonucleotide treatment significantly reduced the normalized length of actin bands by approximately 43%, 38% and 53% compared with controls (Table 7, Figure 13 and 14).

Table 7. Median (25 percentile and 75 percentile) values for the normalized length of cortical actin bands demonstrating the effect of antisense oligonucleotide treatment on cortical actin length in cultured HUVEC. Results from individual experiments and pooled data showed that after 18-20hr of transfection, antisense oligonucleotide treated cells at the edge of the wounded monolayers, had a significant reduction in the normalized length of the cortical actin band compared to the controls. Asterisk (*) indicates a statistical difference ($p<0.05$) between antisense oligonucleotide and two control treatments.

xperiment	alized length of cortical actin band [Median(25percentile, 75percer			Statistical analysis		
	antisense	missense	no oligos	significan	test performed	
exp 1	0.28 (0.16, 0.34)	0.49(0.40, 0.58)	0.46(0.38, 0.55)	*	Kruskal-Wallis one way ANOVA, Dunn's	
exp 2	0.21(0.16, 0.30)	0.34(0.20, 0.53)	0.37(0.19, 0.53)	*	Kruskal-Wallis one way ANOVA, Dunn's	
exp 3	0.18(0.09,0.30)	0.39(0.28, 0.54)	0.40(0.25,0.51)	*	Kruskal-Wallis one way ANOVA, Dunn's	

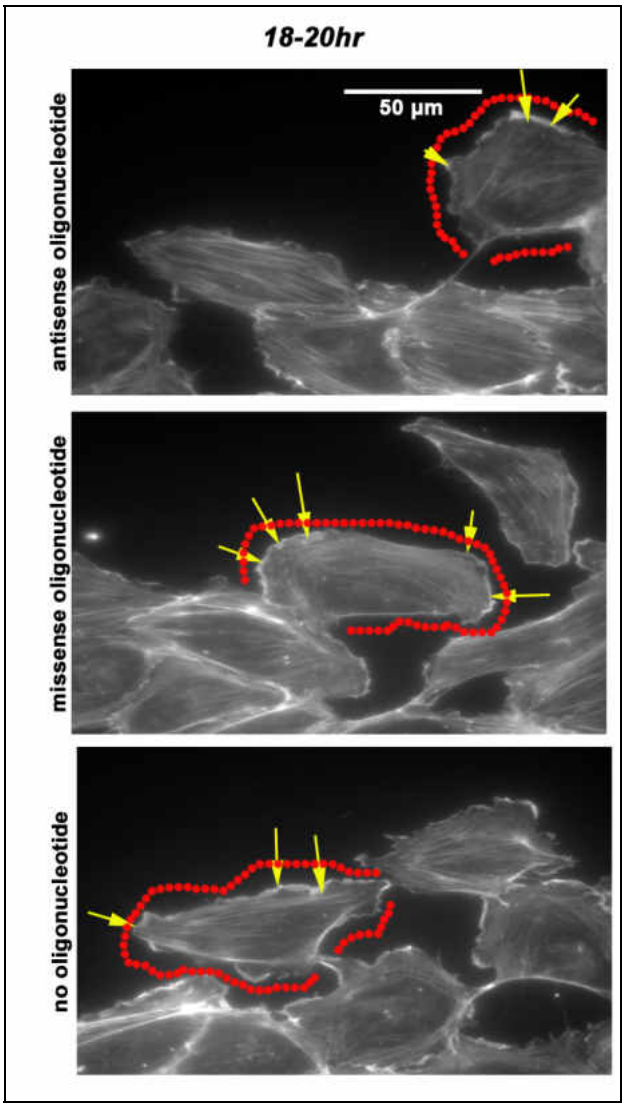

Figure 13. The effect of antisense oligonucleotide treatment on cytoskeletal organization in HUVEC. Actin band stained with TRITC –phalloidin and localized at the cell cortex were observed with epifluorescence microscope at 60X magnifications after 18-20hr of transfection. The length (µm) of cortical actin bands (arrow) were quantified and normalized with the length (µm) of free edge (dotted lines) of cells. Antisense treatment showed a reduction in the length of cortical actin band when normalized with the free edge of cells.

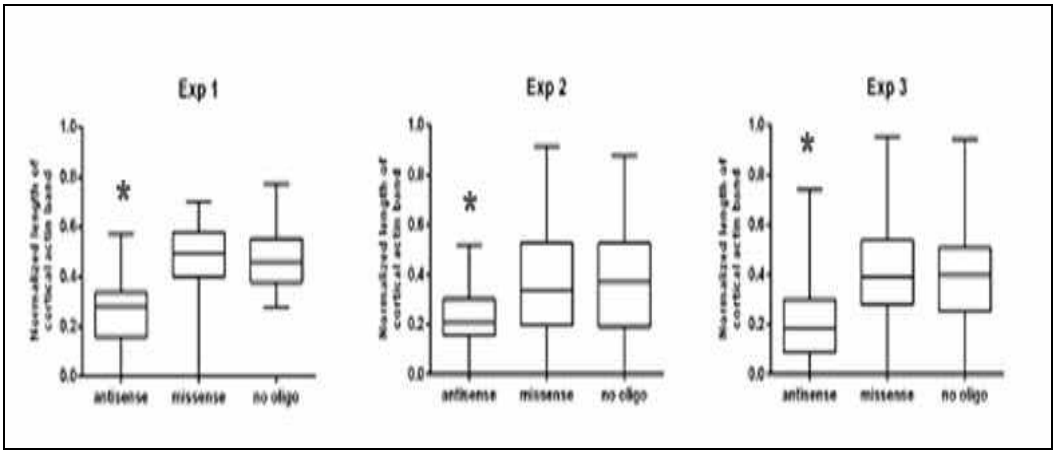

Figure 14. Box and Whisker plot (median with 25 percentile and 75 percentile) demonstrating the effect of antisense oligonucleotide treatment on cortical actin bands in HUVEC using a scratch wound model. Treatment with antisense oligonucleotide showed a significant reduction in the length of cortical actin band. Asterisk (*) indicates a statistical difference ($p<0.05$) between antisense oligonucleotide and control treatments

CHAPTER IV

DISCUSSION

Cell migration is an essential part of development, immune responses, tumorigenesis and wound healing. During the proliferative phase of wound healing, endothelial cells (EC) migrate into the damaged area under the influence of several chemotactic/angiogenic factors secreted by macrophages and platelets. The failure of cells to migrate or the migration of cells to inappropriate locations causes many disease processes such as dysfunction of wound healing. Appropriate and effective EC migration requires precise co-ordination of several cellular signaling pathways.

One important cell signaling protein is PKA, which regulates diverse cell functions associated with cell migration by modulating several of its down stream targets. AKAPs, which organize multivalent signaling complexes, mediate a spatio-temporal regulation of PKA dependent signaling by scaffolding and targeting PKA and other signaling molecules to different subcellular localizations. The functional role of AKAPs in several cellular processes including cell migration, adhesion and actin organization have been described. Gravin/SSeCKS (also known as AKAP12), a 300kDa protein and member of AKAP family, is expressed in several adherent cells in culture (Grove et al., 1994). It has been reported to be involved in controlling various cellular functions including cell adhesion (Gelman et al., 1998), cytoskeletal organization (Gelman et al., 1998; Lin et al., 1996), and cytokinesis (Choi et al., 2008; Hutchins et al., 2010). In addition, gravin/SSeCKS has been involved in other functions including functioning as a

tumor metastasis suppressor (Liu et al., 2011; Xia et al., 2001), regulating blood-brain barrier function (Lee et al., 2003; Su et al., 2006) and controlling progression through the cell cycle (Choi et al., 2008; Frankfort and Gelman, 1995; Lin et al., 2000). However, the role of gravin in cultured EC is not much known. The *in vivo* and *in vitro* expression of gravin was quite interesting to observe. Initial characterization revealed that gravin was undetectable in intact vascular EC *in vivo* but expressed in cultured EC (Grove et al., 1994; Grove and Bruchey, 2001). ECs in culture condition remain in an active state that promotes proliferation and migration compared to the blood vessels where they remain in a non-active stage with no migratory or proliferative behaviors Therefore, it is reasonable to believe that gravin expression is upregulated in ECs when the cells are in an active stage and it may very well be possible that upregulation of gravin expression is associated with the active behavior of cells, such as migration and proliferation. Consistent with this, result from scratch wound study has demonstrated that expression of gravin was upregulated in ECs at the wound edge of a confluent HUVEC (Grove, unpublished data) This further indicates that gravin expression is upregulated in the cells which are involved in migration and wound healing. Previous studies has shown that gravin was localized at the cortical region of EC suggesting that it may play a role in EC migration and wound healing by regulating cortical cytoskeleton and cell migration (Grove and Bruchey, 2001; Piontek and Brandt, 2003). Collectively, the evidences support a role for gravin in EC and therefore in the present study it is hypothesized that gravin plays a role in EC wound healing by regulating cell migration and cortical actin reorganization.

1. Gravin expression is regulated by cell density

This study first demonstrated that the level of gravin expression in cultured EC is regulated by cell density. Immunoblots of HUVEC lysates from different cell densities clearly demonstrated that cultured HUVECs, at their lowest density, had the greatest level of gravin expression. In contrast, cells, at their highest density showed the least level of gravin expression. Consistent with this, three intermediate cell densities showed a similar pattern of expression in which a decrease in gravin expression was associated with increasing cell densities. Given that HUVEC at low cell density are active, proliferative and migratory, suggests that this protein may be involved in cellular activities such as cell migration and wound healing. Consistent with this, unpublished data (Grove, unpublished data) from our lab also demonstrated that upon induction of a scratch wound, the active and migratory cells at the wound edge showed an upregulation of gravin expression indicating that gravin expression was associated with active behavior of cells. In addition to that previous findings by other group have supported the idea that gravin/SSeCKS may be involved in cell proliferation and migration. For example, Gelman and his group reported that gravin was involved in inhibition of G1 to S cell cycle progression in mouse embryo fibroblasts by regulating cyclinD1 (Frankfort and Gelman, 1995; Lin et al., 2000). This group also demonstrated that upregulation of SSeCKS inhibited cancer cell migration by PKA or PKC mediated pathway (Busch et al., 2008; Su et al., 2010).

The finding of the present study that the upregulation of gravin expression in active and low density culture and downregulation in high density confluent and quiescent culture further correlates to *in vivo* and *in vitro* expression of gravin (Grove et al., 1994; Gordon et al., 1992). The expression of gravin in EC remained quiescent during the non active state as observed in intact blood vessels (*in vivo*) which was represented by

an *in vitro* model of confluent monolayer. Based on the results of the present study, it can be speculated that gravin expression may be upregulated in ECs in the wounded blood vessels.

Although, the molecular events involved in cell density dependent gravin expression in cultured HUVEC are not well understood; one can postulate that several cell density dependent factors could be involved in regulation of gravin expression. Earlier it has been reported that the expression of SSeCKS, the rodent orthologue of gravin, is high in confluent culture and low in subconfluent culture of untransformed fibroblasts, prostate epithelial cells, messengial cells and vascular smooth muscle cells (Coats et al., 2000; Lin et al., 1995; Nelson et al., 1999; Xia et al., 2001). In contrast, the present study observed that high cell density suppressed gravin expression whereas low cell density increased gravin expression. In confluent culture, SSeCKS is reported to be in an underphosphorylated state ensuring a stable expression (Gelman et al., 2000) whereas subconfluent culture undergoes serine and tyrosine phosphorylation (Nelson and Gelman, 1997). It can be hypothesized that gravin transcripts in cultured HUVEC may have been underphosphorylated at low cell density which in turn stabilized the protein expression under cultured condition. On the other hand, a confluent cell density might have induced increased gravin phosphorylation causing a reduced protein expression. There might be a possibility that gravin expression, like SSeCKS, is regulated by a density dependent phosphorylation. However, further study is required to determine how gravin expression could be regulated by phosphorylation in cultured EC.

Alternatively, it can be possible that change in gravin expression at different cell densities in cultured HUVEC is partially caused by cell-cell adhesion. Vascular

endothelial cadherin (VE-cadherin), the protein involved in cell-cell adhesion at adherent junction (AJ) interaction (Dejana et al., 1999), may be involved in cell dependent gravin regulation through cell-cell contact. VE cadherins bind and activate TGF-β complex to induce Smad dependent transcription TGF-β (Rudini et al., 2008). It has been reported to be down regulated in confluent EC and over-expressed in low density culture (Merrilees and Sodek, 1992; Pepper et al., 1992; Pepper et al., 1993) and is believed to play a role in angiogenesis and vascular wound healing. The precise mechanism of TGF-β mediated gravin expression is yet to be discovered. In addition, several other proteins including growth factors like bFGF (Healy and Herman, 1992), heparin sulfate (Castellot et al., 1981) and secretion factors that control smooth muscle proliferation (Dodge et al., 1993) were also affected by cell density in cultured EC. How these proteins are related to gravin is not clearly known but it can be speculated that they could be potential regulators of cell density dependent gravin expression. Further studies are necessary to determine the factors and mechanisms associated with density dependent regulation of gravin.

To sum up, the cell density dependent gravin expression suggested that upregulated gravin expression was associated with EC migration and therefore hypothesized that gravin plays a role in cell migration and wound healing.

II. Gravin plays a role in migration and wound healing

Gravin knockdown experiment using a scratch wound assay revealed that both antisense oligonucleotide and siRNA treatment inhibited wound closure. Similarly, gravin knockdown experiment using a 96-well based cell migration assay demonstrated that both antisense oligonucleotide and siRNA treatments significantly reduced the number of cells appearing in the cell free zone after 48hr compared to controls. This study also

tested if cell migration during wound healing was coincided with cell proliferation.

It is noteworthy to mention that the effect of siRNA mediated gravin knockdown on wound healing and EC migration did not correspond to their level of gravin suppression. Western blot and cell based ELISA analysis demonstrated that siRNA1 mediated knockdown had a greater effect on suppressing the gravin expression in cultured HUVEC than siRNA2, whereas data obtained from scratch wound and cell migration assay consistently showed that siRNA2 treatment had a greater effect on wound closure and reduction of the cell number present in the cell free zone than siRNA1. The siRNA treatments were applied to HUVEC when the culture was maintained at low cell densities and in active stage. In contrast, both migration and wound healing assays were performed in a model system in which HUVEC were grown to confluence and majority of the cell population was contact inhibited and quiescent. Although, no studies were further conducted to address why greater knock-down of gravin with siRNA2 treatment had a lesser effect on wound closure and cell migration, it could be speculated that the gravin expression and its functional role behaved differently under these two different culture conditions. It has already been mentioned earlier in this chapter that several proteins play a role in angiogenesis and wound healing and show different levels of expression when cultured in confluent versus non confluent cell cultures. For instance, TGF-β, urokinase plasminogen activator, urokinase plasminogen receptors has been reported to be down regulated in confluent EC but over-expressed in low density culture (Merrilees and Sodek, 1992; Pepper et al., 1992; Pepper et al., 1993). In additions to that, accumulation of growth factors like bFGF (Healy and Herman, 1992), heparin sulfate (Castellot et al., 1981) and secretion factors that control smooth

muscle proliferation (Dodge et al., 1993) are also affected by cell density of cultured EC. These could very well be contributing factors in affecting the cell migration and wound healing.

It can also be possible that the down regulation of gravin below a threshold level might impact its functional role in a different manner from the one when gravin level is above the threshold. It is reasonable to believe that knockdown mediated by siRNA1 treatment might have eliminated gravin from the cellular system below a threshold level due to which cells might have adapted in a different way to compensate for it. In this case it could be possible that the function of other AKAPs or signal proteins in the cellular system might have been activated or attenuated as a consequence.

In summary, results obtained from the scratch wound and 96-well based cell migration assay together support that gravin plays a role in wound closure and cell migration. The findings of the present study are consistent with earlier work demonstrating that SSeCKs overexpression promoted motility in prostate cancer cells (Xia et al., 2001). Also, gravin localized at the cell periphery (Grove and Bruchey, 2001; Piontek and Brandt, 2003) places it in a position where it can regulate events associated with cell migration and wound healing.

Both PKA and PDE which have been targeted to the plasma membrane by gravin, shown to be involved in various cellular functions including cell migration, cell adhesion, cell cycle and cytoskeletal re organization (O'Connor and Mercurio, 2001; Howe et al., 2002; Howe and Juliano, 2000; O'Connor and Mercurio, 2001; Whittard and Akiyama, 2001). Thus, based on the results obtained from scratch wound and cell migration assays in present study, it is reasonable to hypothesize that suppression of gravin levels

attenuated PKA and PDE4 mediated cAMP regulation near the plasma membrane of the EC and affected -PKA-PDE4 dependent control of cell migration.

III. Gravin plays a role in actin cytoskeletal remodeling in EC

What could be the downstream targets of gravin and PKA activation and how could these targets be related to EC migration? Previous studies have indicated that PKA/cAMP affect regulation of actin cytoskeleton and cell migration (Howe, 2004; Howe et al., 2005; Whelan and Senger, 2003) and this actin remodeling could be one of the possible mechanisms through which gravin regulates EC migration. The present study further tested the hypothesis that reduced cell migration in antisense oligonucleotide and siRNA treated cells was associated with a change in cytoskeletal organization that inhibited the cell migration. It was found that antisense mediated gravin knockdown significantly reduced the length of cortical actin bands which were localized at the cell cortex near to membrane ruffles. It was also observed that the distribution of these bands was restricted in the area near to the free perimeter (edge not touching neighboring cell) of migrating cells. These actin bands were distinct from the actin stress fibers which were characterized as long and straight bundle of microfilaments crossing the cell body. In contrast, actin bands in the cortical regions of migrating cells were localized near the cell membrane indicating that these bands might be involved in formation of membrane ruffles and other specialized structures (i.e. lamellipodia) associated with cell migration. Gravin knockdown reduced the normalized length of actin band and indicated that it may play a role in regulation of cortical actin cytoskeleton remodeling during cell migration. This finding of the current study is consistent with previous work by Gelman and his group, which showed that overexpression of SSeCKS induced the formation of filopodia

and lamellipodia like extensions (Gelman, 2002; Gelman et al., 1998). Therefore, this study further determined a possible mechanism through which gravin may mediate cell migration.

The precise mechanism underlying AKAP mediated actin re-organization was not identified further in the present study. However, studies have shown that AKAP-lbc regulates vasodialator-stimulated phosphoprotein (VASP) mediated actin remodeling and EC migration (Paulucci-Holthauzen et al.,2009; Zhang et al., 2010). In addition, several known structural and regulatory proteins of actin remodeling such as small Rho family of GTPases (Rho, Rac, cdc42 and PAK) are also controlled by cAMP/ PKA activation (Hall, 1992; Howe, 2004; Howe et al., 2005). Recently, Tkachenko et al. (2011) has demonstrated that PKA was involved in the regulation of RhoA activation and formation of protrusions at the leading edge of migrating cells indicating that a precise mechanism of PKA activation was required for controlling Rho activity in cell migration (Tkachenko et al., 2011).

Finally, the current study proposes a possible mechanism by which gravin may regulate migration and cytoskeletal organization in endothelial cells. Gravin, known to form multivalent signaling complex, has been conceptualized to involve in compartmentalized regulation of cAMP signaling near the plasma membrane. Electrophysiological studies has revealed that propagation of cAMP signal is selectively increased in discreet area within cells ,where membrane is exposed to β_2-agonist (Jurevicius and Fischmeister, 1996; Rochais et al., 2006). Direct imaging of cAMP dynamics in the intact living cells using a fluorescence resonance energy transfer (FRET) with genetically modified sensors, further confirmed that cAMP regulation was

segregated over space and time.(Zaccolo et al., 2000; Zaccolo and Pozzan, 2002). Several groups had demonstrated that gravin was involved in resensitization and recovery of desensitized β_2 AR (Fan et al., 2001; Lin et al., 2000a; Shih et al., 1999). Based on these studies it can be speculated that gravin may be involved in the regulation of β_2 AR mediated activation of G_s subunits to activate AC leading to cAMP synthesis at various subcellular locations. Besides synthesis, gravin is also shown to be involved in cAMP hydrolysis. Using a FRET based real time measurement assay in HEK293 cells, Willoughby et al demonstrated that gravin was involved in the regulation of local cAMP dynamics (Willoughby et al., 2006). This group also showed that gravin formed a functional complex of gravin, PKA and PDE4D and targeted to plasma membrane to generate a discrete and dynamic cAMP signaling pathway. PDEs are the only known enzymes that can hydrolyze intracellular cyclic nucleotide and terminate their signaling. PDE3 and PDE4 are reported to be the main cAMP hydrolyzing PDEs which are involved in regulation of EC migration (Netherton and Maurice, 2005). Selective inhibition by PDE4 using rolipram, showed a significant decrease in cell migration in several vascular EC including HUVEC. Taken together, it may be reasonable to postulate that in EC, gravin plays a role in spatio-temporal control of cAMP by localizing cAMP degrading enzyme activity to the plasma membrane. Thus, a tight control of cAMP at the site of synthesis may play an important role in regulating cAMP or PKA dependent activity and their downstream signaling associated with EC migration.

IV. Further studies

1. Determining the molecular mechanism involved in density dependent regulation of gravin expression in cultured EC

The current study showed that gravin expression in cultured EC was regulated by

cell density. Although, molecular mechanism of the cell density dependent regulation was not studied, a few possible mechanisms of regulation were outlined here. Further study is required to find more specific mechanism involved in gravin regulation.

2. Investigating the mechanism of cAMP regulation mediated by gravin anchored signaling complex in cultured EC.

The present study demonstrated that gravin knockdown impaired wound closure and cell migration. It can be hypothesized that the mechanism involves a regulation of cAMP signaling through a gravin, PKA and PDE4 complex. To explore it, putative PDE4 binding site of gravin has to be identified and a fluorescence resonance based energy transfer (FRET) based analysis can be used to explore the effect of gravin, PKA and PDE4 scaffolding on EC migrations, wound healing and cytoskeletal organizations

3. Exploring a role for gravin, PKA and PDE4D complex in regulation of cytoskeletal organization.

As described previously, studies have shown that SSeCKS is involved in cytoskeletal regulation. Studies also showed that PKA regulates several small GTPase binding proteins such as Rho, Rac, and cdc42 which are involved in actin cytoskeleton rearrangement, formation of lamellipodia and filopodia in migrating cells. Since gravin knock down has been shown to affect cortical actin distribution, the mechanism for gravin to mediate this actin organization should be addressed.

V. Summary and conclusions

In summary, the current study identified a functional role for gravin in cultured EC. Earlier studies have demonstrated that gravin/SSeCKS has a role in cell cycle progression, cell adhesion, cell motility and tumor metastasis. The present study adds to these earlier works by providing new insights into the control of gravin expression in

cultured EC by cell density and demonstrating a link between gravin and EC wound healing and migration. Antisense oligonucleotide or siRNA mediated gravin knockdown impaired EC migration and wound healing *in vitro*, and also antisense mediated gravin knockdown affected the redistribution of the actin cytoskeleton indicating that this protein may be involved in actin cytoskeletal organization associated with EC migration. Although no precise mechanism underlying the role of gravin in EC migration was revealed, a possible mechanism of gravin mediated EC migration is speculated in this current study. It is believed that PKA and PDE4 are localized to the plasma membrane by gravin mediating a localized cAMP/PKA regulation which in turn induces proteins such as Rho, Rac, cdc42,VASP and PAK to promote actin reorganization and *in vitro* EC migration.

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