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Coronin 1B Regulates Platelet-derived Growth Factor-induced Migration and Reactive Oxygen Species Production

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B.S., Winston-Salem State University, 2003

Advisor: Kathy K. Griendling, Ph.D.

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Abstract

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Platelet derived growth factor (PDGF) plays a pivotal role in cardiovascular disease progression, partially by initiating vascular smooth muscle cell (VSMC) migration Lamellipodia formation is the first step in migration and the creation of this actin rich protrusion is under the tight control of actin polymerizing proteins such as the Arp2/3 complex and actin depolymerizing proteins such as cofilin. Studies show that the actin binding proteins known as coronins regulate actin polymerization via binding to and inhibiting the ARP2/3 complex. Coronins are known to regulate various actin dependent cellular processes including migration. However, the existence and role of coronins in vascular smooth muscle cell (VSMC) migration has yet to be determined. Therefore, the goal of this dissertation was to define the mechanism by which coronins regulate platelet-derived growth factor (PDGF)-induced VSMC migration.

Coronin 1B (Coro1B) and 1C (Coro1C) are both expressed in VSMCs at the mRNA and protein levels. Downregulation of Coro1B by siRNA increases PDGF-induced migration, while downregulation of Coro1C has no effect. Through kymograph analysis, it was confirmed that Coro1B-mediated increases in migration are directly linked to increased lamellipodial protraction rate and protrusion distance in VSMC. Additionally, PDGF induces phosphorylation of Coro1B on serine-2 via PKCε, leading to a decrease in the interaction of Coro1B with the Arp2/3 complex. VSMCs transfected with a phospho-deficient S2A-Coro1B mutant showed decreased migration in response to PDGF, suggesting that the phosphorylation of Coro1B is required for the promotion of migration by PDGF. In both the rat and mouse, Coro1B phosphorylation is increased in response to vessel injury in vivo. We also found that the Coro1B phosphorylation state is redox sensitive and dephosphorylation of Coro1B is dependent on an okadaic acid sensitive phosphatase. Furthermore, the knockdown of Coro1B increases PDGF-induced NADPH oxidase-derived ROS production, thereby providing a new avenue by which Coro1B can regulate VSMC migration. Our data support the concept that Coro1B is an important participant in PDGF-induced VSMC migration, a critical step in vascular lesion formation.

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List of Abbreviations

ALK	Anaplastic Lymphoma Kinase
Akt	α Serine/Threonine Protein Kinase
Arp	Actin Related Protein
Arp2/3	Actin Related Protein 2 and 3
ARPC	Actin Related Protein Complex Subunit
BMS	Bare Metal Stent
CABG	Coronary Artery Bypass Graft Surgery
CAD	Coronary Artery Disease
CAMK	Ca^{2+}/camodulin-dependent protein kinase 2
CapZ	Capping Protein Muscle Z-Line
CDC42	Cell Division Cycle 42
CDK4	Cyclin Dependent Kinase 4
CHD	Coronary Heart Disease
CK2	Casein Kinase 2
cMyc	Myelocytomatosis Viral Oncogene Homolog
Coro	Coronin
DAG	Diacylglycerol
DES	Drug Eluting Stent
EGFR	Epidermal Growth Factor Receptor
ERK1/2	Extracellular Signal-related Kinase
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FGR	Gardner- Rasheed Feline Sarcoma Virus Oncogene Homolog
GAP	GTPase-activating Protein

GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GEF	Guanine Nucleotide Exchange Factor
GRB2	Growth Factor Receptor-bound Protein 2
GRK-1	G protein-coupled Receptor Kinase 1
GSK-3	Glycogen Synthase Kinase 3
H_2O_2	Hydrogen Peroxide
HASM	Human Aortic Smooth Muscle
HCoASMC	Human Coronary Artery Smooth Muscle Cell
HDF	Human Dermal Fibroblast
ISR	In-stent Restenosis
JAK2	Janus Kinase 2
LCK	Lymphocyte Specific Kinase
LDL	Low Density Lipid
LIM Kinase	LIM Domain Containing Protein Kinase
LMW-PTP	Low Molecular Weight Protein Tyrosine Phosphatase
MARCKS	Myristoylated Alanine-rich Protein Kinase C Substrate
Max	Myc Associated Factor X
MyoD	Myogenic Differentiation 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NOX	NADPH Oxidase
Noxa1	NADPH Oxidase Activator 1
Noxo1	NADPH Oxidase Organizer 1
NPF	Nucleation Promoting Factor
O^-_2	Superoxide
PAK1	p-21/CDC42/Rac1-activated Kinase 1

PCI	Percutaneous Coronary Intervention
PDGF	Platelet-derived Growth Factor
PDGFR	Platelet-derived Growth Factor Receptor
PDK1	Phosphoinositide-dependent Kinase
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
β-Pix	PAK-interacting Exchange Factor Beta
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C gamma
PMA	Phorbol 12-myristate 13-acetate
Poldip2	Polymerase Delta Interacting Protein 2
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PTEN	Phosphatase and Tensin Homolog
Rab	RAS-associated Protein
Rac1	Ras-related C3 Botulinum Toxin Substrate 1
RASM	Rat Aortic Smooth Muscle
ROS	Reactive Oxygen Species
SCAR	Suppressor of Cyclic AMP Receptor
SCID	Severe combined Immunodeficiency
SHP-2	Protein Tyrosine Phosphatase, Non-receptor Type 11
SLE	Systemic Lupus Erythematosus
SMAD	Mothers Against Decapentaplegic Homolog 2
Src	Rous Sarcoma Oncogene
SSH1L	Slingshot Homologue 1 Like Phosphatase

TGFβ	Transforming Growth Factor beta
TK	Tyrosine Kinase
VSMC	Vascular Smooth Muscle Cell
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASp Verprolin Homology Domain-containing Protein

Chapter 1:

Introduction:

1.1 Coronary Heart Disease: Causes, Common Treatments, and Complications

It is estimated that every 25 seconds, an American will have a coronary event, and approximately every minute, someone will die from this event (1). In 2008 coronary heart disease caused 405,309 deaths in the United States (about 1 of every 6 deaths in the United States) (1). It has been projected that this year approximately 785,000 Americans will have a new coronary event, and about 470,000 will have a recurrent event (1). Thus, finding new methods to treat and prevent coronary heart disease (CHD) is warranted. CHD describes a condition in which the coronary arteries that supply the heart with oxygen and nutrient rich blood become obstructed or stenotic (2). The most common cause of CHD is coronary artery disease (CAD) although it is not only source of CHD (2-4). CAD is defined as atherosclerosis of the coronary arteries.

1.1.1 Atherosclerosis

Atherosclerosis is a slow and progressive disease in which the large and intermediate arteries of the body develop fatty lesions called artheromatous plaques inside the vessel wall (2). Generally these plaques contribute to luminal narrowing and hardening of the vessel (5). Initially, these lesions are often asymptomatic until lumen stenosis is so great that blood supply to downstream tissues is insufficient resulting in ischaemia (2). As plaques increase in size they can become unstable and rupture, with the end result being thrombosis or embolism formation, which can cause total vessel occlusion. Ischaemia caused by atherosclerosis of the coronary artery can lead to angina, myocardial infarction and heart failure (Figure 1.1).

The causative events that lead up to atherosclerotic plaque formation in the vessel wall are currently under intense investigation. However, early atherogenesis is hypothesized to begin with damage to the endothelial layer of the vessel caused by such risk factors such as hypertension, hyperlipidemia, tobacco use, increased homocysteine

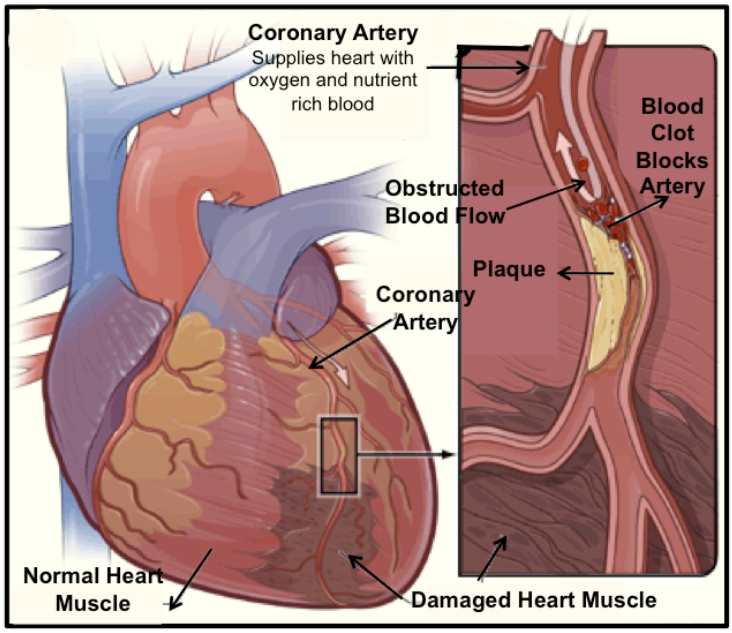


Figure 1.1 Coronary Heart Disease is Commonly Caused by Atherosclerotic Plaque Formation in the Coronary Arteries. The formation and growth of atherosclerotic plaques in the coronary arteries can obstruct blood flow through the vessels. As a result, the myocardium does not receive the blood or oxygen it needs. This causes ischaemia and can lead to angina, myocardial infarction and heart failure. Most myocardial infarctions occur when thrombosis or embolism formation suddenly obstruct blood supply, causing permanent damage to the myocardium. Image adapted with permission from National Heart, Lung, and Blood Institute; National Institutes of Health; U.S. Department of Health and Human Services.

levels, and infection (6-11). These insults to the vessel stimulate the expression of inflammatory proteins on the endothelial layer and activate a cascade of signaling events that stimulate inflammation of the vessel wall (12). During this response, T-lymphocytes, monocytes and low density lipid (LDL) enter the artery wall from the bloodstream and platelets begin to adhere to the area of insult (13). Furthermore, LDL molecules become susceptible to oxidation by free radicals produced by the immune response and monocytes that have infiltrated the area differentiate into macrophages, which ingest LDL and oxidized LDL (10). Slowly, these macrophages turn into large foam cells that contain numerous internal cytoplasmic vesicles with high lipid content. These white blood cells are not able to process the oxidized-LDL, and ultimately grow and then rupture, depositing a greater amount of oxidized cholesterol into the artery wall (14-16). This triggers the accumulation of more white blood cells, continuing the cycle. Growth factors and other cytokines such as platelet derived growth factor (PDGF), interferon γ and transforming growth factor β (TGF-β) produced from damaged endothelial cells, platelets and immune cells encourage the migration and proliferation of vascular smooth muscle cells (VSMCs) from the media to the intima of the blood vessel (17, 18). These neointimal VSMCs are then capable of ingesting lipids, and transforming into foam cells as well (19). Also, VSMCs and fibroblasts migrate into the area and secrete matrix proteins such as collagen and fibrin to provide a hard fibrous cap that covers the affected lesion causing narrowing of the artery and reduction of blood flow (18, 20). Concurrently, intracellular small calcifications form inside VSMCs of the surrounding smooth muscle layer, particularly in the smooth muscle cells neighboring the atheroma (21, 22). Eventually, as cells expire, extracellular calcium deposits are left between the vascular smooth muscle wall and outer portion of the plaque (23, 24). These events contribute to the hardening and non-contractility of the vessel observed in atherosclerosis.

Multiple treatment strategies have been implemented to combat the formation and complications of atherosclerosis. In general, most available pharmacological agents such as HMG-CoA reductase inhibitors (statins), niacin, bile acid sequestrants, cholesterol absorption inhibitors, and combination therapies are aimed at lowering circulating lipid levels and thus attenuating atherosclerosis formation (20). However, these drugs show only a modest effect on the regression of plaques that are already formed (25). Consequently, physical methods are then implemented to expand stenotic arteries. These methods include minimally invasive angioplasty procedures that may include stents to physically expand narrowed arteries (Figure 1.2) and major invasive surgery, such as bypass surgery to create additional blood supply connections that go around the more severely narrowed areas. In the case of coronary artery disease these procedures are known as percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery (CABG), respectively.

1.1.2 Percutaneous Coronary Intervention

PCI was initially introduced as an alternative less invasive treatment option to CABG surgery in revascularization of the coronary arteries (26). Since then, PCI has become widely accepted as an effective and safe treatment to reduce or eliminate the symptoms of CAD, including unstable angina, acute myocardial infarction, and multivessel CAD (26). The term balloon angioplasty, which describes the inflation of a balloon within the coronary artery to crush the plaque into the walls of the artery, is commonly used to describe PCI (2). Although balloon angioplasty is still done as a part of most PCI, it is seldom the only procedure performed. The major drawback of balloon angioplasty-only PCI was restenosis of the treated vessel, resulting in renewed symptoms and the need for repeat intervention (26). Additional procedures that are

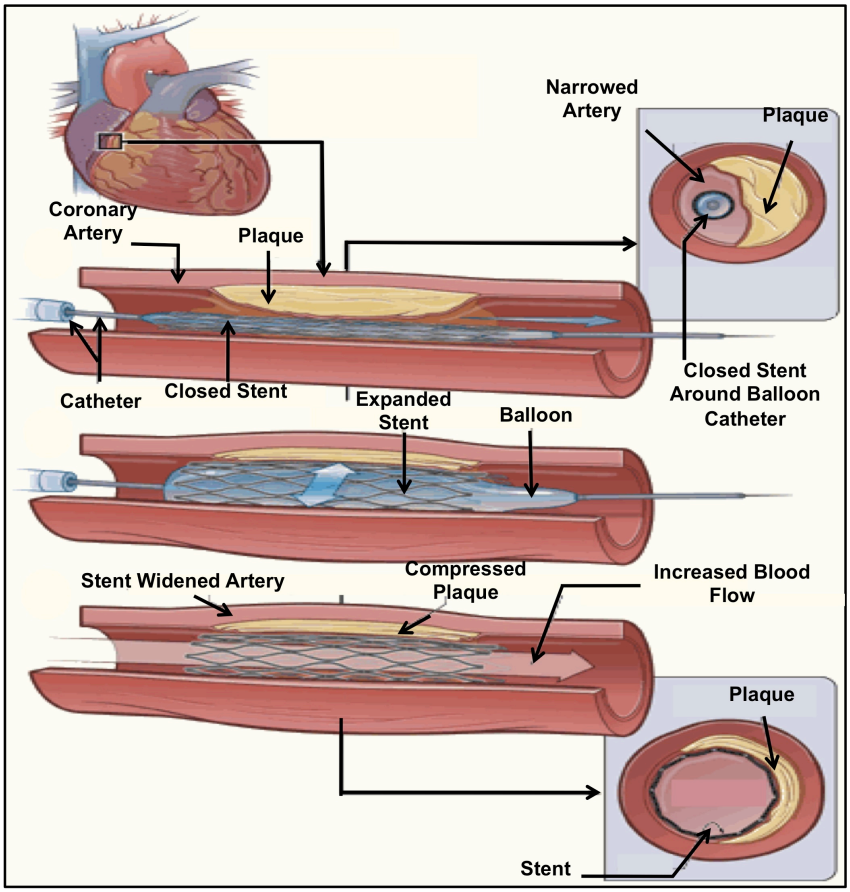


Figure 1.2. Percutaneous Coronary Intervention that Includes Stent Implantation is a Common Treatment for Coronary Artery Disease. An angioplasty catheter with a deflated balloon encased in a wire mesh tube or stent is gently threaded through the femoral artery and aorta until it finally reaches the coronary arteries. The balloon is then inflated when it reaches the plaque region. This action compresses the plaque and stretches the artery wall to expand the lumen. The balloon is then deflated and removed. However, the stent is left behind to support the new enlarged lumen, allowing for sustained increased blood flow. Image adapted with permission from National Heart, Lung, and Blood Institute; National Institutes of Health; U.S. Department of Health and Human Services.

performed during PCI include implantation of stents and atherectomy to decrease restenosis (2).

1.1.3 Restenosis After Percutaneous Coronary Intervention

Restenosis refers to the re-narrowing of an artery after angioplasty or stent application (27). This process usually occurs approximately 3 to 6 months following the procedure and is a reactive response to tissue injury created by the PCI procedure (28). Prior to the introduction of stents, approximately 50% of patients that received balloon angioplasty showed signs of restenosis (29). Only after the introduction of bare metal stents (BMS) did the incidence of restenosis begin to diminish (20–30%) (30). BMS provide a scaffold that holds the artery wall open preventing restenosis of coronary arteries (26). PCI with stenting is superior to angioplasty alone by maintaining unobstructed arteries for an increased time period (26, 31). Despite the beneficial effects of stenting, rates of restenosis remained persistently high, giving rise to a new setback known as in-stent restenosis (ISR) (31, 32). The development of ISR prompted the launch of newer drug eluting stents (DES). DES help prevent restenosis of the artery through several different physiological mechanisms, which rely upon the suppression of tissue growth at the stent site and local modulation of the body's inflammatory and immune responses (32). Rapamycin, its derivatives umirolimus, zotarolimus, and everolimus as well as paclitaxel, have demonstrated safety and efficacy in this application in controlled clinical trials by stent device manufacturers (31). Despite the fact that restenosis occurs less frequently with DES when compared to the BMS, DES restenosis remains a significant problem due to the increasing number of implanted DES as well as the targeting of more complex lesions (26). In addition, worse outcomes after repeat revascularization compared to BMS restenosis are reported in DES restenosis. Moreover, restenosis beyond 1 year and late stage thrombosis after DES implantation

has been recognized (33-36). This suggests that further investigation into pathways that promote restenosis are required to provide insight into possible new drug targets that effectively inhibit ISR.

The primary cause of restenosis is the migration of VSMCs from the medial layer across the damaged endothelium into the lumen of the vessel and their subsequent proliferation (26). Furthermore, the infiltration of lymphocytes into the area seems to trigger the inflammatory component of this event (37). Taken together, these events result in the re-narrowing of the blood vessel. Multiple growth factor and cytokine signaling pathways such as PDGF (38-40), fibroblast growth factor (FGF) (41, 42) and TGF-β (43) signaling have been proven to be responsible for neointimal formation during restenosis. These signaling molecules originate from endothelial cells, platelets, mononuclear cells, and VSMCs. While numerous factors participate in the events leading to restenosis, it is generally accepted that PDGF stimulation of the PDGFR-β found on VSMCs of the media is a major cause of the neointimal formation, although FGF also plays a significant role (38, 44). PDGF produced at the site of injury acts both as a mitogen and chemoattractant and is responsible for the migration of the VSMC and the formation of the neointima (44). Therefore, identifying the mechanisms that induce PDGF-mediated VSMC migration are of tremendous importance.

1.2 Platelet-derived Growth Factor Expression and Signaling

In the vasculature, the importance of PDGFR-β signaling during development has long been identified. During angiogenesis, PDGFB is expressed in the primitive vascular endothelium that in turn attracts and stimulates the proliferation and migration of PDGFR-β expressing VSMCs (45). These VSMCs tightly coat the nascent endothelium and stabilize the new vessels by producing pro- survival and anti-proliferative factors (45). Although PDGF-B signaling plays a positive role in vascular

development and angiogenesis, increased PDGF signaling in the adult vessel generally leads to pathophysiological disease progression. Genetic manipulation of vascular cells combined with multiple inhibitory and overexpression strategies have provided strong evidence that PDGF plays an important role in the migration of VSMCs into the neointima following acute injury and in atherosclerosis (39, 46). Thus, PDGF signaling can have both positive and harmful effects in the vasculature.

1.2.1 Platelet-derived Growth Factor

PDGF belongs to a large family of structurally and functionally related growth factors that include the vascular endothelial growth factors and are conserved throughout the animal kingdom (45, 47). PDGF and related growth factors are also members of a much larger superfamily containing cysteine knots that are thought to have evolved from a common ancestor (48). Members of the PDGF family contain a growth factor core domain that is required for receptor binding and activation (47). PDGF is a hydrophilic cationic glycoprotein (MW 28-35kDa) that can be produced by a host of cells such as platelets, monocytes/macrophages, endothelial cells and VSMCs (49). Human serum contains ~17.5 ng/ml PDGF and exogenous PDGF is cleared rapidly (50, 51). In other species such as baboons, the serum levels of PDGF are lower (~ 1.3- 5.1 ng/ml) (50). There are 4 genes that encode 4 different PDGF chains (PDGF-A, B, C, D) (47). These chains are disulphide linked to form biochemically active homo- or hetero-dimers (PDGF-AA, -AB, -BB, -CC and DD) (47). The N-terminal propeptide removal processing of PDGF-A is necessary for receptor binding; it is thought that PDGF-B also requires N-terminal propeptide removal to become active (47, 52). Conversely, these isotypes differ in receptor specificity and binding to components of the extracellular matrix (47, 53-55). The PDGF isotypes that are produced varies depending on cell type and, to some extent, on the species. PDGF-AB is the major isoform released by human

platelets, but in most other species, the PDGF-BB isoform predominates (49). PDGF-BB is produced by endothelial cells and activated macrophages (50). PDGF-AB or -BB usually have similar efficacies, but PDGF-AA has generally been reported to possess lower mitogenic activity (56-59). However, PDGF-BB was shown to be a greater chemoattractant than PDGF-AB (60). Strikingly, PDGF-AA inhibits PDGF-BB or –AB-induced VSMC migration (60). PDGF-CC is expressed in VSMCs and both PDGF-CC and –DD can both stimulate VSMC proliferation at extremely high concentrations of 100ng/ml (61). More importantly, VSMCs found in the neointima express more PDGF-B than their medial layer counterparts (62), and *in vivo* coronary arteries from patients that have undergone PCI have increased PDGF-B expression (63). Similarly, PDGF-B expression is increased in neointimal smooth muscle cells from balloon injured rabbit femoral arteries (64). Furthermore, increased neointimal formation was observed in rats infused with PDGF-BB after carotid injury (39), or when PDGF-BB was overexpressed in porcine arteries (65). In contrast, inhibition of PDGF-AA signaling had no effect on neointimal formation in the rat carotid injury model (66). In short, PDGF-BB is responsible for initiating signals that promote restenosis.

1.2.2 Platelet-derived Growth Factor Receptor Signaling

PDGF receptors (PDGFR) are members of the receptor-tyrosine kinase superfamily and show structural similarity to many of its other members, as well as a number of cell adhesion molecules (67). The PDGF receptor is composed of two monomeric receptor subunits, PDGFR-α and PDGFR-β (68, 69). Both receptors contain common domain structures including five extracellular immunoglobulin loops and a split intracellular tyrosine kinase (TK) domain. PDGFR-α is synthesized as a 140 kDa protein precursor that when processed and glycosylated to its mature form is 170 kDa (70). PDGFR-β is synthesized as a 160 kDa precursor and glycosylated to 190 kDa (71, 72).

Ligand binding to the PDGFR results in dimerization of two transmembrane subunits, autophosphorylation, receptor clustering and activation of PDGFR tyrosine kinase activity. PDGFR-β activation is known to stimulate a vast array of intracellular pathways involved in proliferation, migration and reactive oxygen species generation in VSMCs (Figure 1.3). Proteins that include phospholipase Cγ (PLCγ), c-Src, phosphatidylinositol 3 kinase (PI3K), and growth factor receptor-bound protein 2 (GRB2) bind directly to phosphorylated moieties on the dimerized PDGFR-β (73-77). These phosphorylated moieties serve as docking sites for the aforementioned proteins that lead to their activation and stimulation of downstream signaling. In particular, the activities of Src, PI3K, and PLCγ have been directly proven to regulate PDGF-induced VSMC migration. Weber et al (78) demonstrated that Src could modulate PDGF-induced VSMC migration through the activation of the phosphoinositide-dependent kinase 1 (PDK1) and p21/Cdc42/Rac1-activated kinase-1 (PAK1) pathway. In addition, the inhibition of PI3K was shown to decrease VSMC migration, and is accredited as one of the targets of red wine polyphenols (79). PI3K also stimulates the activity of the small GTPase ras-related C3 botulinum toxin substrate 1 (Rac1) to regulate migration (80). Moreover, both PI3K and PLCγ also activate a set of serine/threonine kinases known as protein kinase Cs (PKCs). Inhibition of PKC decreased PDGF-induced VSMC migration by 40% (81). Furthermore, Caglayan and colleagues (82) demonstrated that PDGFR-β activation of Src, PI3K, and PLCγ is required for chemotactic responses and inhibition of this signaling leads to decreased neointimal formation in a mouse model of restenosis (82). The activation of the PDGFR-β also stimulates the production of reactive oxygen species (ROS) that can mediate signaling to downstream effectors in VSMCs (83, 84). ROS act as major intracellular signaling molecules and can activate pathways that lead to

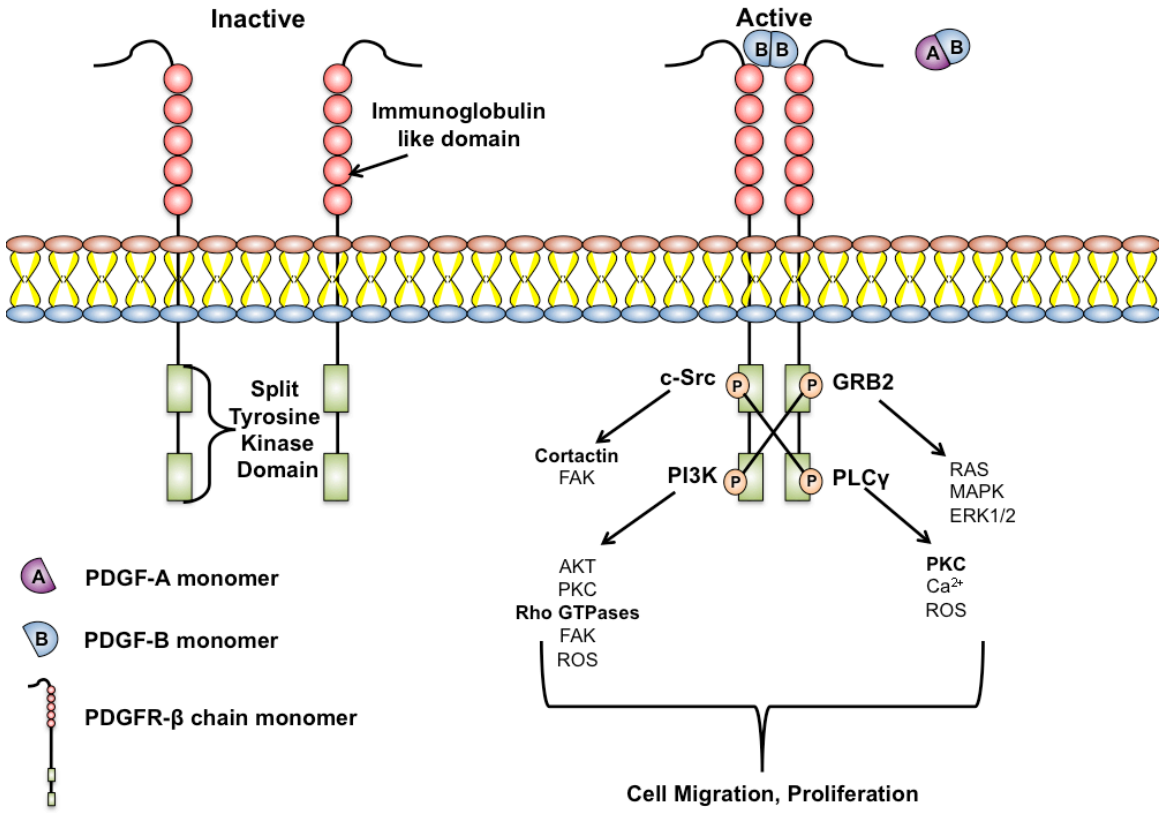


Figure1.3. Activation of the PDGFR- β on VSMCs Leads to Stimulation of Signal Transduction Pathways that are Required for Cell Migration and Proliferation. PDGF-BB binding to the PDGFR-β leads to receptor dimerization, tyrosine autophosphorylation and transactivation. These steps then lead to the docking and activation of proteins such as Src, PI3K, PLCγ, and GRB2. Direct activation of these proteins lead to the recruitment and stimulation of down stream effectors that are required for cell migration and proliferation.

migration and proliferation (85-88). PDGF has previously been shown to stimulate ROS production in VSMCs and the use of antioxidants inhibits PDGF-induced migration and Src activity in these cells (78, 89). These data indicate that ROS regulate pathways that lead to migration.

1.3 Reactive Oxygen Species and NADPH Oxidase Modulation of PDGF Signaling

ROS are generally small molecules that are highly reactive due to the presence of unpaired valence electrons and are a normal byproduct of metabolism. These small molecules derived from oxygen are able to modify cellular functions by reacting with macromolecules such as deoxyribonucleic acid (DNA), proteins, and lipids (90). In the vasculature, ROS play important roles as signaling molecules to regulate growth factor signaling, extracellular matrix dynamics, inflammation, cell proliferation, migration and differentiation, as well as vessel contraction and relaxation (90). ROS can be produced by several cellular systems such as xanthine oxidases, cyclooxygenases, mitochondrial electron transport chain uncoupling, cytochrome P450, and nitric oxide synthases, as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (91-93).

When ROS production in cells becomes altered so that pro- oxidant generating systems exhaust the capacity of antioxidant systems, oxidative stress ensues. This excess superoxide and hydrogen peroxide causes malignant signaling in the vasculature, which can lead to various cardiovascular complications that include but are not limited to restenosis and atherosclerosis (94). In the vasculature, NADPH oxidases are among the most common producers of ROS and their activity has been implicated in normal physiological processes in the vessel as well as the progression of multiple cardiovascular diseases (91-93).

1.3.1 Reactive Oxygen Species-Regulated PDGF Signaling

ROS include oxygen ions, free radicals, and both organic and inorganic peroxides. Common forms of ROS found in the vasculature include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) (95). H_2O_2 is thought to act as a signaling molecule by altering the redox state of sensitive proteins (91). The effects of H_2O_2 are largely due to covalent modification of specific cysteine residues found within redox-sensitive target proteins (96, 97). The oxidation of these reactive residues can lead to the reversible modification of proteins and enzymatic activity (96, 97). Superoxide is generally thought to mediate reversible signaling after its conversion to hydrogen peroxide. Never-the-less, it has been hypothesized that superoxide can directly modify proteins reversibly through a nucleophilc mechanism that involves the deprotonization of alcohols, phenols, and thiols and the hydrolysis of esters (98). However, this hypothesis has not been fully tested.

ROS commonly act as signaling molecules. In response to PDGF stimulation ROS are known to regulate cellular pathways that include protein phosphatases, in addition to kinases. PDGF-induced ROS reversibly, temporally and spatially inactivates low molecular weight protein tyrosine phosphatase (LMW- PTP), phosphatase and tensin homologue (PTEN), as well as tyrosine-protein phosphatase non-receptor type 11(SHP-2) (99). These phosphatases are responsible for de-phosphorylation and inactivation of kinases and signaling pathways associated with PDGF receptor activation. At the same time PDGF stimulates ROS-mediated activation and phosphorylation of kinases such as alpha serine/threonine protein kinase (AKT), extracellular signal-related kinase 1/2 (ERK1/2), and p38 mitogen activated protein kinsae (p38MAPK) all of which are necessary for migration and proliferation (100). However, it is not completely known if the decreased phosphorylation of these proteins in the presence of antioxidants and ROS inhibitors is solely due to the effects of ROS on

kinase activity or rather to lack of inhibition of phosphatase activity. Current consensus suggests both contribute to the process. Additionally, NADPH oxidase-dependent H_2O_2 production is obligatory for full activation of the PDGFR (101). Thus ROS, inactivation of tyrosine phosphatases in concert with increased PDGF receptor and non-receptor kinase activity is required for PDGF-mediated signaling.

1.3.2 NADPH Oxidase Regulation of PDGF Signaling

The activity of NADPH oxidases is important in normal cardiovascular signaling during migration and proliferation, as well as disease pathology (102). The NADPH oxidase (Nox) is a multi subunit, membrane-associated protein that catalyzes the one electron reduction of oxygen using NADPH as an electron donor (95). First and most extensively studied in neutrophils, the NADPH oxidase was found to have two membrane bound subunits, gp91phox (Nox2) and p22phox, known collectively as cytochrome b558 (103). Cytoplasmic subunits of functional importance in the phagocytic oxidase are p40phox, p47phox, p67phox and the small GTPase Rac (95). In the vasculature, Nox2-based oxidases are found in endothelial cells, fibroblasts of the adventitia and VSMCs of resistance arteries. Novel Nox subunits Nox1, Nox4, and Nox5 are also expressed throughout the vasculature, but have different modes of regulation. NADPH oxidases containing Nox1, Nox2, and Nox4 subunits require the membrane bound p22phox subunit for functionality. Nox1 is regulated by Rac and, in VSMCs, by p47phox and Noxa1, a p67phox homologue (104). Co-expression studies have clearly demonstrated that Nox4 does not require Rac, but does require the expression of Poldip2 for full functionality (105) (106). In aortic VSMCs, the main Nox subtypes expressed are Nox1 and Nox4 (and Nox5 in humans). Collectively, research in the field has demonstrated that ROS, through NADPH oxidase activity, can modulate signaling pathways that are important in migration and other PDGF- dependent processes (107).

Nox1 is found in caveolin-enriched areas of the cell membrane along with many activated growth factor receptors such as PDGF (108, 109). PDGF stimulation of the Nox1 oxidase is regulated by PI3K activation of the Rac guanine nucleotide exchange factor (GEF) β-PIX. β-PIX constitutively associates with the Nox1 complex and is compulsory for PDGF-dependent ROS production via Rac1 activation (110). Knockout mice lacking Nox1 have reduced neointimal formation in response to femoral injury. In addition, VSMCs from these animals have blunted PDGF-induced migratory responses and NADPH oxidase- dependent ROS production (111). Mechanistically, Nox1-derived ROS activate slingshot 1L phosphatase (SSH1L), which de-phosphorylates and activates cofilin, a known actin binding protein required for migration (112, 113). These data imply that Nox1 plays a considerable role in VSMC migration and restenosis.

Nox4 co-localizes with vinculin in focal adhesions and also can be found in the nucleus (108). Currently, it is thought that Nox4 regulates basal redox homeostasis in the cell through its constitutive activity. Therefore, Nox 4 activity is generally regulated by expression. Cytokines such as TGF-β regulate Nox4 by increasing its protein expression (114). To date data on the role of Nox4 in PDGF-induced signaling and migration in VSMCs is limited. However, antisense against Nox4 was shown to inhibit PDGF-induced VSMC and monocyte migration (106, 115). One hypothesis for its regulation of PDGF-induced migration stems from its localization. Nox4 focal adhesion localization suggests that it may be involved in integrin and focal adhesion signaling. The production of Nox-dependent ROS at these sites of attachment are required for integrin mediated signaling and efficient cell migration (116, 117). Hence, Nox4 can regulate PDGF-induced migration indirectly by modulating integrin signaling. Moreover, in cells from patients with leprechaunism, deficiencies in PDGF-induced tyrosine phosphorylation are reversed with ectopic Nox4 expression (118). However, to date there are no studies that demonstrate PDGF can stimulate Nox4 activity; in fact, PDGF

decreases Nox4 mRNA levels (89).

In summary, ROS generation by PDGF plays a significant role in VSMC migration. A major source of ROS in the vasculature originates from NADPH oxidases. Furthermore, both Nox1 and Nox4 have both been shown to regulate PDGF-induced VSMC migration.

1.4 Directed Cell Migration

The migration of cells is important in multiple facets of normal and aberrant cell behavior. In the vasculature, the migration of VSMCs is vital for physiological processes such as angiogenesis in addition to vascular remodeling, and is a critical component of pathophysiological lesion formation occurring during atherosclerosis and restenosis after PCI (119, 120). In these latter diseases, one of the major characteristic changes is the accumulation of VSMCs within the intimal layer of the blood vessel caused partially by PDGF-induced migration (121, 122).

Migration is a dynamic and cyclical process, which includes the extension of a protrusion at the leading edge, the formation of new adhesion sites, contraction of the cell body, and the release of focal adhesions in the rear (123). When the cell senses a chemotactic gradient, it extends protrusions called lamellipodia at its leading edge in the direction of the chemoattractant. The formation of lamellipodia requires the polymerization of actin at the leading edge of the protrusion and depolymerization of actin at the base of the protrusion. These protrusions attach to the substratum by creating focal complexes, which later will mature into focal adhesions. These adhesions are highly specialized attachments between actin filaments and the extracellular matrix that allow cells to generate force necessary for the next stage in migration, contraction. Contraction moves the cell body forward towards the protrusion and is regulated by contractile bundles composed of actin and the motor protein myosin II. Finally, the

release of rear focal adhesions mediated by calpain protease activity allows for forward progression (Figure 1.4) (124). Each segment of this process generally involves the activation of the Rho family of small GTP binding proteins (GTPases) (124).

1.4.1 Rho GTPase Regulation

G-proteins act as molecular switches that are inactive when bound to GDP and bind a variety of effector proteins when in their GTP bound active state (125). G-proteins are partially controlled by GEFs that destabilize the inactive GDP bound complex, causing release of GDP and binding of GTP (125). The conformational change upon GDP/GTP exchange activates the G-protein. GTPase activating proteins (GAPs) induce the hydrolysis of GTP bound to G-proteins thus deactivating the G-protein switch, and guanine nucleotide dissociation inhibitors can sequester G-proteins bound to either GTP or GDP (125). The Rho family of GTPases consists of Rac, Cdc42, Rho and other atypical isoforms. Lamellipodia formation, the first step in migration, is largely regulated by Rac. Rac participates in migration by regulating actin polymerization during lamellipodial protrusion and ruffle formation (126). Inactivation of Rac completely inhibits PDGF-induced VSMC migration (78). Thus, stimulation of Rac activity is a rate-limiting step in PDGF-induced VSMC migration.

1.4.2 Rac Regulation of Actin Polymerization and Lamellipodia Formation

Actin polymerization in the direction of migration generates the protrusive force necessary for lamellipodia formation and extension at the leading edge of the cell. The polymerization of actin is a dynamic treadmilling process that includes the addition of subunits at the barbed end and the loss of subunits at the pointed end (127). Rac regulates actin polymerization during lamellipodial extension in multiple ways. For

Step 1: Protrusion
The cell senses a chemotactic gradient and extends a lamellipodium towards the gradient.

Step 2: Attachment
The cell attaches the lamellipodia to the substratum by the formation of new focal contacts.

Step 3: Contraction
The cell body contracts and the cell is propelled forward. The formation of new focal contacts are continued in the front while older focal contacts in the rear are destroyed.

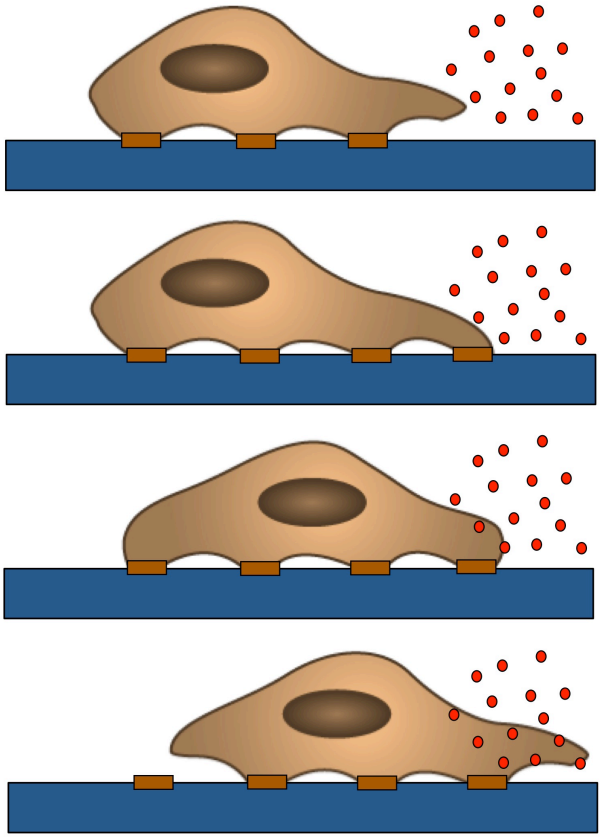


Figure 1.4 Cell Migration is a Dynamic Process. During chemotactic or directed cell migration, cells sense a chemotactic gradient, become polarized and begin to migrate towards the source of the gradient. The three distinctive phases protrusion, attachment and contraction occur in a cycle that is continued until the cell has reached its destination. This image was modified from Wikimedia commons.

example, Rac can regulate the availability of free barbed ends on actin filaments through the removal of barbed end capping proteins. Rac activation stimulates phosphatidylinositol 4,5-bisphosphate (PIP2)-mediated gelsolin and CapZ actin filament uncapping (126, 128). Additionally, Rac may also participate in lamellipodia formation through the activation of Nox1. The activation of Nox1 is indispensable for the successive activations of SSH1L and cofilin in VSMCs (113). Cofilin activation is required for the cleavage and degradation of older ADP-containing actin filaments. This action increases the pool of free actin monomers that are required to form new actin filaments at the leading edge of the cell. Finally, Rac can stimulate multiple actin nucleating proteins, such as the actin related protein 2/3 (Arp2/3) complex through suppressor of cAR /WASP family Verprolin-homologous protein (SCAR/WAVE) (129, 130) and p21protein (CDC42/Rac)- activated kinase-1 PAK1 (131). Rac activation of this pathway leads not only to de novo actin nucleation, but also actin filament branching, a requirement of lamellipodia formation.

1.4.3 Arp2/3 Complex Regulation

The Arp2/3 complex is a seven subunit complex that binds to the side of an existing parent filament and nucleates the formation of new actin filaments at a distinctive 70° angle, leading to the formation of dendritic actin filament networks that are required for efficient cell migration (132). Arp2/3 activity is required for not only motility, but also many actin dependent processes such as endocytosis and intracellular trafficking (128). The subunits of the complex include Arp2, Arp3, p40/ARPC1, p34/ARPC2, p21/ARPC3, p20/ARPC4 and p16/ARPC5. Each subunit is essential for complex stability and activity.

The active nucleating complex is thought to initiate actin polymerization by forming a pseudo actin dimer composed of its two actin related protein subunits, Arp2

and Arp3 (128). However, alone Arp2/3 has relatively weak actin nucleating abilities; for full activation the complex must bind nucleation promoting factors and actin. Multiple proteins directly regulate and increase the activity of the Arp2/3 complex. Nucleation-promoting factors (NPFs) are a large class of proteins that are known to positively regulate the Arp2/3 complex activity. WAVE, WASP and cortactin are common NPFs that when bound to the Arp2/3 complex can increase complex nucleation activity to promote lamellipodia formation (Figure 1.5) (133). When the Arp2/3 complex is not bound by a NPF, the complex is inactive and in an open conformation (134). The V domain of NPFs such as WASP or WAVE interacts with actin while the CA region associates with the Arp2 and Arp3 subunits of the ARP2/3 complex to create a nucleation core. The binding of WASP specifically causes the complex to take on a closed conformation that activates ARP2/3 nucleation activity (134). Recent data have shown that two WASP proteins are required to activate the ARP2/3 complex. One WASP protein interacts with the p40/ ARPC1 as well as Arp2, and the other WASP interacts with the Arp 3 subunit (135).

The phosphorylation of Arp2/3 complex subunits may also play a role in complex activity. Recent *in silico* data have suggested ARP2/3 complex activation also requires Arp2 phosphorylation. Narayanan et al. (136) proposed a model in which a network of auto inhibitory salt-bridge interactions maintains the Arp2 subunit in an inactive state. The phosphorylation of Arp2 destabilizes the auto-inhibitory interactions and allows Arp2 to reorient to an activation-competent configuration (136). However, these data need to be validated *in vivo* to fully examine the role of Arp2 phosphorylation on Arp2/3 complex activation. Phosphorylation regulates other subunits of the Arp2/3 complex as well. The inhibition of PAK-1-mediated phosphorylation of p40/ARPC1 subunit on threonine 21 inhibits epidermal growth factor mediated migration and targeting of p40/ARPC1 to the Arp2/3 complex (131). Hence, Arp2/3 complex formation is stimulated and regulated at

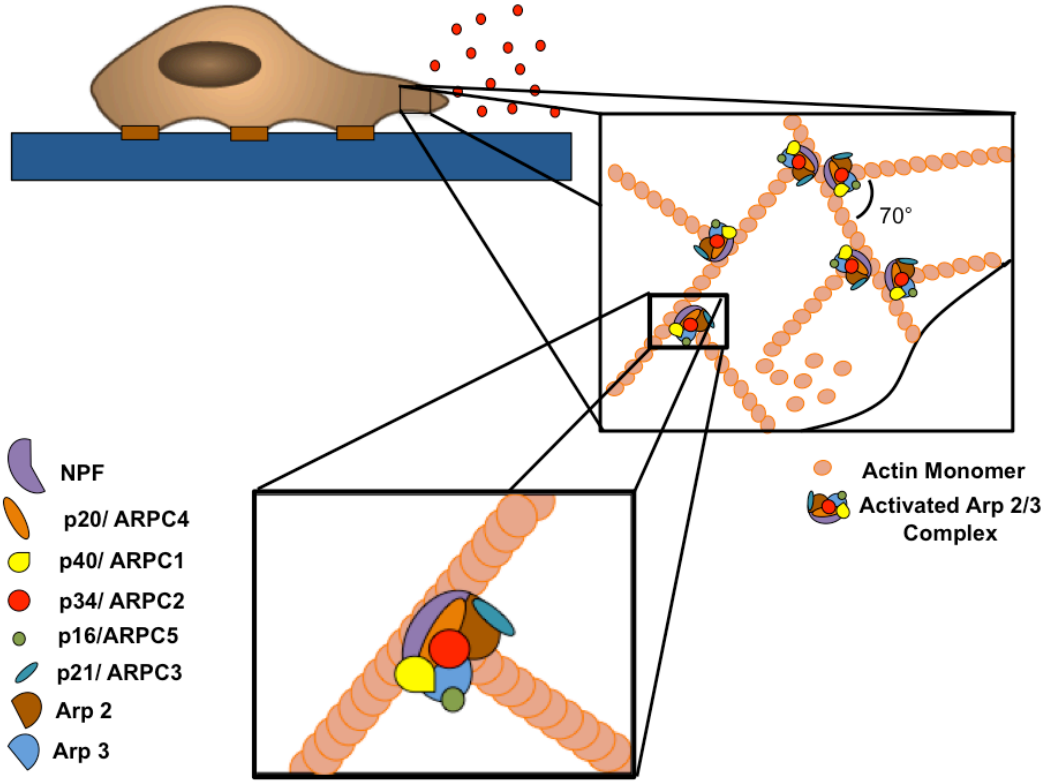


Figure 1.5. The Arp2/3 Complex Regulates Lamellipodia Formation. The actin-polymerizing complex, Arp2/3, regulates the formation of lamellipodia. This seven-subunit complex consists of two actin related proteins Arp 2 and Arp 3 that serve as the attachment point and nucleation core for actin monomers. The other five subunits p21, p40, p34, p16, and p20 stabilize the complex and regulate interactions with other proteins such as actin. The binding of NPFs to the actin filament and Arp2/3 complex subunits Arp2, Arp3, and p40/ ARPC1 stimulates actin nucleation.

multiple levels.

The Arp2/3 complex is also regulated structurally by the p34/ARPC2 and p20/ARPC4 subunits. The p34/ARPC2 and p20/ARPC4 subunits are required for interactions with actin filaments and filament branching (137). Multiple mutations in either protein lead to complex inactivity (137). The p34/ARPC2 subunit directly interacts with Arp2, Arp3, and p20/ARPC4 subunits. Generally, the p34/ARPC2 subunit is thought to maintain the structural integrity of the complex (138). The Arp2/3 complex member, p34/ARPC2, is a scaffold that relays signals and conformational changes induced by NPFs and actin filament binding to other complex subunits (134, 139). The p34/ARPC2 subunit can also interact with the actin binding protein coronin (134, 140, 141). When coronin binds to the p34/ARPC2 subunit of the Arp2/3 complex it stimulates a conformational change in p34/ARPC2 that keeps the complex in an open inactive orientation (134). The binding of coronin acts as an inhibitor for the Arp2/3 complex, and is the focus of this dissertation.

1.5 The Coronin Family of Actin Binding Proteins

Coronins were first discovered as a primary protein contaminant in isolations of actin-myosin preparations from *Dictyostelium discoideum* (142). These proteins were initially found in crown or “corona”-like cell protrusions of the cellular slime mold (142). The coronin family of proteins is evolutionarily conserved and is found in all eukaryotes except plants (143). All coronin proteins bind actin and regulate a host of actin-mediated processes such as endocytosis, phagosome formation, migration and cytokinesis in multiple cell types (144-147). Lower eukaryotes express varying quantities of coronin isotypes that range from one in *Sacchromycetes* and *Xenopus* to three in *Dictyostelium*. In mammals there are seven isoforms, separated into three subclasses (type I, II, and III) based on phylogenetic similarity (148-150). The type I coronins consist of coronin 1A

(Coro1A), 1B (Coro1B), and 1C (Coro1C) and are the most studied coronin subfamily. They all bind and inhibit Arp2/3 complex activity and regulate actin dependent processes such as migration. Coro1A is highly expressed in cells of hematopoietic lineage in addition to various tissues of the nervous system, while having significantly lower expression in other tissues of the body (151). On the other hand, Coro1B and Coro1C are more ubiquitously expressed at higher levels in most tissues (140).

The type II coronin subfamily members have been considerably less explored. Type II coronins also bind actin and include coronin 2A (Coro2A) and 2B (Coro2B). Coro2A is expressed at moderate levels is most tissues and plays a role in migration in adenocarcinoma cells by regulating cofilin activity at focal adhesions (140, 152). Coro2A also participates in the Toll-like receptor (TLR) inflammation response pathway in macrophages. In this pathway Coro2A is a component of the nuclear corepressor complex (NCoR) and mediates TLR-induced NCoR turnover by a mechanism involving interaction with oligomeric nuclear actin (153). Coro2B is enriched in cells from the nervous system (154). Coro2B immunofluorescent staining colocalizes with stress fibers as well as focal adhesions and Coro2B immunoprecipitates with vinculin (154). This suggests that both Coro2A and 2B may have similar mechanisms of action with regard to focal adhesion regulation.

The third mammalian coronin subfamily consists of only one protein known as coronin 7 (Coro7). Coro7 is quite unique in that it is about twice as large as the type I and II coronin subfamily members and has distinct functions not found in others coronins. Coro7 is conserved across species in *Caenorhabditis elegans*, *Drosophila*, *Dictyostelium* and humans (150). Interestingly, Coro7 does not bind actin in humans (155). Instead, Coro7 localizes to the Golgi complex and participates in the maintenance of Golgi morphology and membrane trafficking (155-157).

1.5.1 Structure of Type I Coronins

Type I coronins have high sequence homology. For instance Coro1B and Coro1C are 96% homologous, and Coro1C is 89% homologous to Coro1A. This conserved sequence homology imparts identifiable and predictable domain structures. Coronins that make up the type I subclass are composed of multiple domains that endow coronins with their unique functions. These regions include a N-terminal domain that contains a N-terminal motif and a β-propeller region composed of 7 WD repeats, a middle domain that contains part of the C-terminal extension followed by a unique region, and a C-terminal domain that contains a coiled-coil domain (Figure 1.6). Each domain imparts binding sites for protein interactions as well as structural stability.

1.5.1.1 N-Terminal Domain: N- Terminal Motif and β-Propeller Region

All type I coronins contain an N-terminal domain composed of a conserved N-terminal motif and WD repeats that form the β-propeller region. The N-terminal region contains 12 basic amino acids that are conserved among coronin proteins (158). This conserved area is necessary for Coro1A binding to actin (159). Phosphorylation of Coro1A and 1B on the conserved Serine-2 also occurs in this region and regulates the interaction of the coronins with the Arp2/3 complex (140).

Coronin proteins also harbor five canonical WD clustered repeats with two non conventional repeats found in the N- terminal region and the C-terminal extension (160). WD repeats were first identified in the β-subunit of the GTP-binding protein transducin (161). This structural motif is composed of approximately 40 amino acids that usually end with the amino acid sequence tryptophan (W) and aspartic acid (D), hence the name WD. Proteins that contain this domain form β propellers with 4-8 blade-shaped anti-parallel β–sheets arranged radially around a central tunnel (162). These domains form a

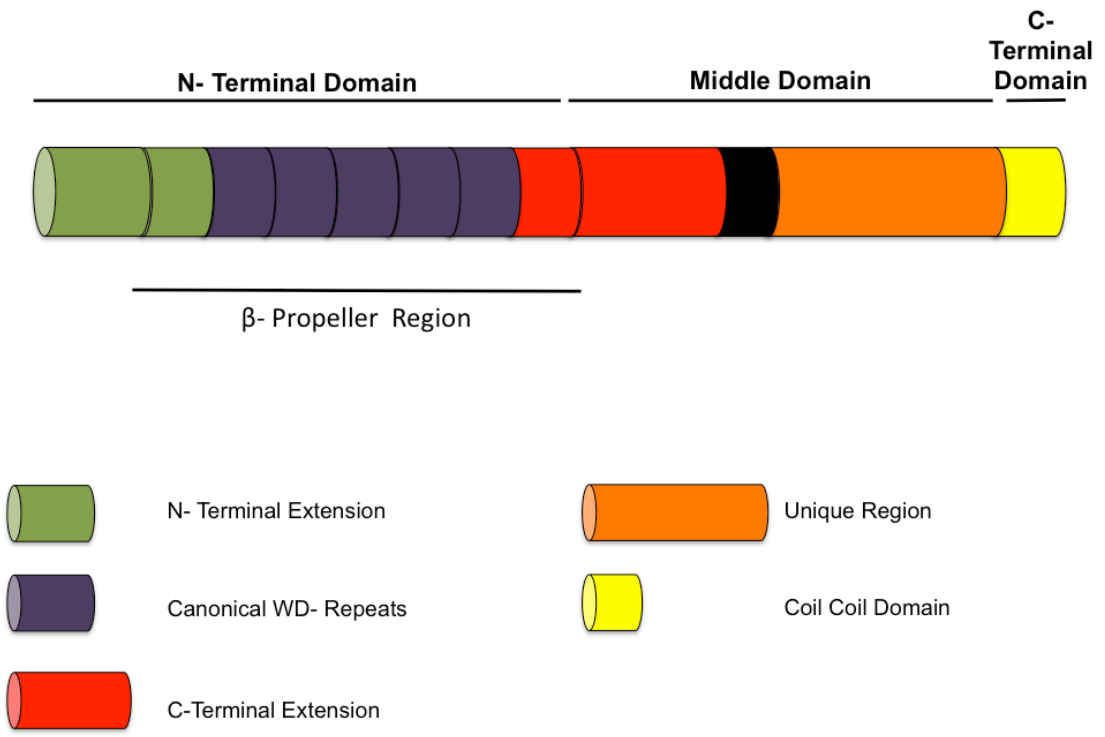


Figure 1.6 Schematic of Type I Coronin Secondary Structure. Type I Coronins are composed of three domains. The N-terminal domain consists of an N-terminal extension and the β-Propeller region. The middle domain contains the C-terminal extension, and the unique region. Lastly, the C- terminal domain is composed of a coiled-coil domain.

platform on which multiple protein complexes bind and assemble reversibly without any catalytic activity (162). Coronin 1A (lacking coiled-coil domain) crystal structure revealed seven β-propellers that are encoded by the WD repeats (160). Based on sequence similarity, other type I coronins should also have a similar confirmation. In all type I coronins, direct actin binding has been traced to various areas of the β propeller region, and in Coro1B the Arg30 residue regulates actin binding in the region (159, 163-165). Coro1A also binds to the cell membrane via its β propeller region (166). Hence, the β propeller region of coronin is required for protein interactions such actin filament binding and for its interaction with the membrane in Coro1A, making it vital for coronin functions.

1.5.1.2 Middle Domain: C-Terminal Extension and Unique Region

The middle domain of type I coronins contains both the C-terminal extension and a unique region (160). Both regions of the middle domain are important for actin binding in Coro1A and 1C (163, 164, 166, 167), but specific residues that regulate this interaction remain to be elucidated. The C-terminal extension contains two conserved strands of amino acids (160). These conserved regions are packed against the bottom side of the last β-propeller in the previous N-terminal domain. Studies of the Coro1A crystal structure demonstrate that the C-terminal extension is necessary for the stability of the β-propeller region (168).

Furthermore, each coronin contains a unique divergent sequence between the WD domain and C-terminal coiled coil region. The number of amino acids that compose this region varies greatly depending on sub family and species. Mammalian type I coronins contain a unique region that is about 50 to 70 amino acids (140, 169). The function of the unique region has been linked to actin binding; however, other functions have yet to be determined. In the *S. cerevisia* coronin homologue the unique region is

responsible for microtubule interactions (170), but this sequence is not conserved in mammalian type I coronins.

1.5.1.3 C-Terminal Domain: Coiled-Coil Domain

A coiled-coil is a structural domain found in proteins in which the α-helices are coiled together commonly in dimers or trimers that have most of their non-polar side chains on one side, so that they twist around each other with these chains facing inward (128). These domains generally contain a heptad repeat consisting of hydrophobic (h) and charged (c) amino acids in a hxxhcxc pattern (160). Coro1A contains 3 repeats of this pattern and this region is responsible for the trimeric oligomerization of the protein (160). Currently, it is accepted that the coiled-coil domain controls the oligomerization of all coronins (160). Coro1C oligomerization can be regulated by phosphorylation on Ser-463 in the coiled-coil domain. This phosphorylation inhibits trimer formation and changes its distribution with in the cell (164, 171). The coiled-coil domain is also required for Arp2/3 complex binding of Coro1A, and 1C (172, 173); however, this has not been determined for Coro1B interactions with the Arp2/3 complex. In addition, the coiled-coil domain is hypothesized to be another binding site for actin. This site and others contribute to the actin bundling capacity of the coronin protein.

1.5.2 Function and Regulation of Type I Coronins

The structures of Coro1A, Coro1B, and Coro1C are quite similar so it is not surprising that coronins regulate similar cellular functions and have similar localizations. Coro1A, 1B, and 1C localize to the plasma membrane, regulate lamellipodia formation, and bind ARP2/3 complex (140). However, recent studies have shown that some coronin functions are not so redundant.

1.5.2.1 Coronin 1A

First discovered as a co-purifying protein of PLCγ extracts in calf spleen, Coro1A is highly expressed in cells of hematopoietic and neuronal origin. Coro1A is the most studied coronin and much of what is known of type I coronin structure is based on this isotype. Coro1A was initially described as a phagosome coat protein that is retained on mycobacterial-containing phagosomes in macrophages (174). Later it was determined that microorganisms stimulate Coro1A retention to inhibit the fusion of the phagosome and lysosome and thus escape degradation (174). This eventually led to the discovery that Coro1A association with the phagosome was down regulated by a PKC-dependent phosphorylation that inhibits its interaction with actin and modulates the reorganization of the cytoskeleton (175). Additionally, Coro1A was also shown to associate with cytosolic subunit p40phox of the Nox2 containing NADPH oxidase in a PI3K dependent manner. Researchers demonstrated that Nox subunits p40phox and p67phox were required for the correct localization of Coro1A during phagocytosis in neutrophils (176). Further studies in other cell types of the immune system suggested Coro1A was required for phagosome formation and regulation of actin dependent endocytosis of LDL in macrophages (144, 177), chemokine mediated T-cell migration via a Rac and/or Arp2/3-dependent mechanism (172), T-cell receptor signaling (178), and phagosome formation and migration in neutrophils (179). Regulation of Rac activity and localization in COS and HEK 293 cells through a β-Pix/actin/PAK1 pathway has also been added to the list of Coro1A functions (145). Of importance, Coro1A expression, deficiency or mutations have been linked to diseases such as systemic lupus erythematosus (SLE), severe combined immunodeficiency (SCID), Down syndrome, and breast cancer (180-184).

Recent studies have introduced controversy into the field and suggest Coro1A may have roles in addition to regulation of the actin cytoskeleton. Coro1A has recently been demonstrated to regulate survival and calcium signaling through the activation of

calcineurin in immune cells (185, 186). When pro survival signals and calcium signaling is restored in Coro1A knockout cells previous endpoints linked to Coro1A such as decreased chemokine-dependent migration and phagocytosis in immune cells of multiple lineages were eliminated (187-190). Additionally, Coro1A expression was required for TGFβ signaling through short mothers against decapentaplegic homolog 2/ (SMAD 2/3) in lymphocytes (191). These data provide a new insight into the functional and structural mechanisms of Coro1A action.

1.5.2.2 Coronin 1B

The expression of Coro1B is ubiquitous and can be found in most tissues of the body. Coro1B was initially identified as pp66/Coronin$_{se}$ and was highly expressed in a variety of secretory-type epithelial tissues (192). Coro1B is highly phosphorylated by PKC on unknown residues in response to phorbol 12-myristate 13-acetate (PMA) and carbachol in gut parietal cells (193). Coro1B also localizes to F-actin structures in these cells (193). However the function of Coro1B in gut parietal cell processes has not been explored. Eventually, the serine-2 of Coro1B was found to be a major PKC target phosphorylation site after PMA exposure in Rat-2 fibroblasts (140).

Like yeast coronin and Coro1A, Coro1B binds to the p35/ARPC2 subunit of the Arp2/3 complex and inhibits Arp2/3 actin nucleation activity (194). The phosphorylation of Coro1B at the serine 2 position negatively regulates binding with the Arp2/3 complex in HEK 293 cells and Rat-2 fibroblasts (140). Neither endogenously phosphorylated Coro1B nor the Coro1B S2D phospho-mimetic mutant bind to the Arp2/3 complex and inhibit Arp2/3 nucleation activity efficiently (194). Additionally, Coro1B can disassemble Arp2/3-containing actin filament branches by promoting the dissociation of the Arp2/3 complex from the filament *in vitro* (195). Phosphorylation of Coro1B also diminishes its capacity to dissociate the Arp2/3 complex from filament branches (195). Hence, when

Coro1B is unphosphorylated it binds Arp2/3 complex and inhibits actin nucleation and filament branching. This implies a negative role for Coro1B in migration and lamellipodia formation. Accordingly yeast that overexpress coronin have depolarized actin patches and actin cables are replaced by aberrant actin loops as well as growth defects (141). Both actin structures and cell growth require dynamic actin assembly that is regulated by the Arp2/3 complex in yeast (196, 197).

Counterintuitively, knockdown of Coro1B inhibits migration and negatively regulates lamellipodia dynamics in Rat-2 fibroblasts (194). Additionally, overexpression of Coro1B increases migration in these cells (140). Strikingly, in Rat-2 fibroblasts, increasing PMA concentrations decreases Rat-2 cell migration and this is partially rescued by overexpression of a Coro1B S2A phospho-deficient mutant (140). In Rat-2 cells PMA stimulation decreases migration; however, it is unknown how Coro1B phosphorylation would affect cells such as in VSMCs in which PMA increases migration (81).

Mutation of the actin binding site arginine-30 on Coro1B also decreases migration in Rat-2 fibroblasts via effects on lamellipodia dynamics (165). Moreover, in these cells Coro1B not only regulates Arp2/3 activity, but also regulates the localization of SSH1L to the lamellipodia and thus cofilin de-phosphorylation and activation in the structure (194). In Rat-2 fibroblasts, Coro1B co-immunoprecipitates with tagged SSH1L and Coro1B de-phosphorylation is mediated by SSH1L (194). Conversely, data from Kurita *et al* (198) using a similar assay demonstrated that Coro1B is not de-phosphorylated by SSH1L as previously implied. Hence, the phosphatase responsible for Coro1B dephosphorylation is quite controversial.

Equally as important, Coro1B expression has been linked to wound repair following spinal injury. Following injury Coro1B and ras related protein 13 (Rab13) were induced in the injured spinal cord (199). Inhibition of either protein decreased neuronal

outgrowth (199). Furthermore, acetylation of lysine 320 (K320) of p53 specifically positively regulated the expression of Coro1B and Rab13 under conditions of injury in neuronal cells (200). Although Coro1B expression is important in wound repair and neuronal outgrowth, it remains unclear if this is mediated by its regulation of the Arp2/3 complex or some other mechanism.

Currently, all data suggest that Coro1B can bind to and inhibit the Arp 2/3 complex in multiple cell types and across species. However, the effects of this interaction or lack thereof seem to be cell type dependent. Additionally, Coro1B phosphorylation may have different consequences based on cell type and source of the stimulus.

1.5.2.3 Coronin 1C

Expression of Coro1C is also ubiquitous and found in many cell types throughout the body. In skeletal muscle three isoforms were identified. These isoforms are splice variants of a common Myo-D dependent gene and are found at neuromuscular junctions and thin filaments of myofibrils (201). In glioblastoma cells stimulated with PDGF, Coro1C expression was upregulated and it was designated as a delayed primary response gene (202). Additionally, in other experiments the Coro1C promoter was found to be the target of multiple transcription factors such as c-Myc/Max in lymphoma cells and Slug/Snail/E47 in epithelial cells (203, 204). Therefore, Coro1C expression is regulated by multiple stimuli in a variety of cell types.

Like its predecessors, Coro1C binds actin and has been shown to interact with the Arp2/3 complex in pulldown assays, as well as in HEK 293 cells, SK-CO15 and HaCat cells (164, 205, 206). In HEK293 cells, casein kinase-2 (CK2) phosphorylation of Coro1C at serine-463 inhibits interactions with the Arp2/3 complex and inhibits migration (173). In glioblastoma, 3T3- fibroblasts, and HEK 293 cell lines down regulation of

Coro1C decreases migration (205, 207). Alternatively, in SK-CO15 epithelial cells Coro1C downregulation was demonstrated to regulate focal adhesion dynamics via FAK-mediated signaling and increase wound closure (206). The discrepancies between the data suggest that the functions of Coro1C are cell type dependent.

Additionally, a role for Coro1C in secretion has been identified. In pancreatic beta cells, Coro1C regulates endocytosis of secretory membranes through its interaction with GDP-Rab27a (208, 209). Coro1C expression has also been associated with multiple types of aggressive cancers. In primary effusion lymphoma, melanoma, nonendometriod endometrial carcinoma, head and neck squamous cell carcinomas and glioblastoma, Coro1C expression is amplified (207, 210-213).

Coro1A, 1B and 1C can potentially influence migration via several different mechanisms, and their overall impact is likely to be a function of the complement of coronins expressed in a given cell type. Although, coronins have multiple unique attributes, they are all able to bind actin and interact with the Arp2/3 complex. The Arp2/3 complex binding activities of Coro1A and 1B can be regulated by PKC-mediated phosphorylation, where as known Coro1C binding is regulated by a CK2-dependent phosphorylation. Never-the-less, all coronins have multiple putative PKC phosphorylation sites. To date there is no information on the expression, regulation or function of coronins in vascular biology or disease. However, PKC a known coronin kinase, plays a pivotal role in vascular physiology and pathophysiology.

1.6 Protein Kinase C

PKCs are a large family of serine/threonine kinases that regulate diverse functions and are expressed differentially throughout the body. Activation of these proteins is associated with migration, ROS production, proliferation, phagocytosis, differentiation, survival, transformation and gene expression in many cell types (214-

217). Intriguingly, PKC isotypes may have functionally opposing roles depending on the cell type and cellular milieu in which they are expressed. Under normal physiological conditions PKCs participate in contraction, migration, proliferation, modulation of ion conductance, secretion and endocytosis in VSMCs (218, 219). Nonetheless, aberrant overexpression, changes in the isotype expression and increases in activation can have deleterious results. Specifically, in the vasculature PKC has been linked to the pathobiology of restenosis, hypertension, and vascular complications due to diabetes (219-222).

1.6.1 PKC Structure, Regulation and Activation

Generally, PKCs have a common structure that consists of a regulatory domain and a catalytic domain linked by a hinge region (219, 220, 222, 223). The N-terminal region of the protein contains the membrane targeting motifs and regulatory domains. This region is responsible for the binding of second messengers created by upstream signaling and is variable among PKC subfamilies (223). In addition, activation of PKC occurs in this region. The C-terminal region encodes the catalytic domain that binds ATP as well as substrates and is highly conserved among the different species of PKCs (223). The sequence specificity of PKC isotypes is regulated by a sequence of amino acids in this region near the catalytic domain (223).

Based on their second messenger requirements, PKCs can be divided into three subfamilies: classical or conventional, novel and atypical. Classical PKCs require Ca^{2+}, diacylglycerol (DAG) and phospholipids such as phosphatidylserine for activation and include α, βI, βII, and γ isozymes (223). The novel PKC subfamily members do not require Ca^{2+}, but need DAG and phospholipids for activation and they include the δ, ε, η, and θ isozymes (223). Lastly, the atypical subfamily members ζ and ι/ λ do not require Ca^{2+} or DAG; however, phosphatidylserine does stimulate their activity (224). Both

classical and novel PKCs can be activated by PMA; however, atypical PKCs lack the binding region for phorbol esters in their regulatory domains.

Under basal conditions PKC is found in an auto-inhibited conformation. In this conformation the activity of the kinase domain is quenched by the binding of the pseudo substrate region in the regulatory domain (220). In the case of classical and novel PKCs, DAG created by PLC activation (or chemical activator PMA) binds to the regulatory domain and leads to the translocation of the enzyme to the cell membrane (225). The interaction of the enzyme with phospholipids at the cell membrane leads to the withdrawal of the pseudo substrate region from the kinase domain and activation of the enzyme (223). For full functionality, some PKCs must also be phosphorylated in the catalytic domain. Phosphorylation in this region creates allosteric interactions that lead to conformational changes required for a mature enzyme (223).

1.6.2 Expression and Function of PKCs in VSMCs

In VSMCs, PKC activation has been linked to multiple cellular processes that include ROS production and migration. Both processes are fundamental in atherosclerotic lesion formation and restenosis after angioplasty. In studies measuring PDGF-induced ROS production, PKC inhibition was demonstrated to reduce ROS generation in VSMCs (226). In addition, the stimulation of specific PKC isotypes has also been linked to VSMC migration. Inhibition of PKC β, δ, and ε activity reduces VSMC migration under varying conditions (227-229). PKC activation is known to stimulate the phosphorylation, activation, and change in cellular localization of multiple cytoskeletal and contractile proteins such as vinculin, calponin and CPI-17, which can regulate migration (230, 231). In animal models of restenosis, PKC β, ε, ζ knockdown or inhibition decreases neointima formation (228, 232-234). Conversely, knockout of PKC δ increases neointimal formation in the femoral wire injury model through inhibition of re-

endothelialization (235). Thus, depending on cell type and the PKC isotype that is activated these proteins can have contradictory effects in the vasculature.

1.7 Dissertation Objectives

Atherosclerosis of the coronary arteries is one of the most common causes of death in the United States and is often treated with PCI. However, a common complication of this treatment is restenosis of the vessel. Experimental models have demonstrated that PDGF stimulation of VSMC proliferation and migration largely contributes to this process. Migration is a dynamic process that is initiated by the formation lamellipodia. The regulation of lamellipodia is under the tight control of actin polymerizing and depolymerizing proteins. The Arp2/3 complex creates branched actin networks that are required for lamellipodia formation. Additionally, the coronin family of actin binding proteins regulates multiple actin-dependent processes in eukaryotes, partially through their ability to bind and inhibit the Arp2/3 complex. Although there have been significant advances in identifying the functions of coronins, not much is known about physiological regulators of their function and virtually nothing is known about their part in VSMC processes. Therefore the objective of this dissertation was to explore the role of coronins in PDGF-mediated VSMC signaling *in vitro* and *in vivo*.

Chapter 2:

The Role Coro1B in PDFG-induced VSMC Migration

2.1 Introduction

The migration of cells is important in multiple facets of normal and aberrant cell behavior. In the vasculature, the migration of VSMC is vital for physiological processes such as angiogenesis and vessel remodeling, and is a critical component of pathophysiological lesion formation occurring during atherosclerosis and restenosis after PCI (119, 120). In these latter diseases, one of the major characteristic changes is the accumulation of VSMCs within the intimal layer of the blood vessel (121, 122). Narrowing of the blood vessels caused by these events can ultimately lead to thrombosis or embolus formation, both of which remain a significant clinical problem. PDGF activation of the PDGFR plays an important role in the migration of VSMCs into the neointima following acute injury and in atherosclerosis (46). Although PDGF has been shown to be the primary regulator of VSMC migration *in vivo*, the molecular mechanisms and intracellular signaling pathways that contribute to this migration are only partially understood.

Migration generally begins with the extension of actin rich protrusions called lamellipodia (123). The formation of these structures at the leading edge of the cell requires the protrusive forces that are generated by actin polymerization and increased actin branching (127). Lamellipodia formation is regulated by the Arp2/3 complex, which participates concomitantly in actin nucleation and filament branching. The Arp2/3 complex binds to the side of an existing parent filament and nucleates the formation of new actin filaments at a 70° angle, leading to the formation of branched filament networks that are required for efficient cell migration (132). For full activation, the Arp2/3 complex must bind with actin filaments and actin nucleation promoter proteins such as WAVE and WASP (129). Conversely, recent studies have demonstrated that the Arp2/3 actin nucleation activity can be negatively regulated by an interaction with actin binding proteins known as coronins (140, 141).

Coronins are a family of evolutionarily conserved WD-repeat actin-binding proteins known to control a variety of cellular processes involving actin dynamics (170). The coronin protein family includes 7 proteins in mammals, separated into three subclasses (type I, II, and III) based on phylogenetic similarity (148-150). The type I coronins consist of Coro1A, 1B, and 1C and are the most studied coronin subfamily. Coro1A, 1B and 1C localize to the cell periphery, and bind Arp2/3 and inhibit its actin nucleation abilities (140, 173, 179). Additionally, in epithelial cells, Coro1C was demonstrated to regulate focal adhesion dynamics and increase wound closure (206). Coro1A also regulates calcium signaling in leukocytes. Thus, type I coronins can potentially influence migration via several different mechanisms, and their overall impact is likely to be a function of the complement of coronins expressed in a given cell type. Additionally, there are data suggesting that coronin regulation of actin dependent processes like migration are cell type dependent (140, 141, 206).

Although there have been significant advances in identifying the functions of type I coronins, not much is known about physiological regulators or upstream signaling pathways involved in coronin activation, and virtually nothing is known about their role in VSMCs. Therefore, our present objective was to identify if type I coronins are expressed in VSMCs, and if so, to define how they participate in PDGF-dependent VSMC migration.

2.2 Methods

2.2.1 Materials

Recombinant human PDGF-BB was purchased from R&D Systems Inc. Primary antibodies were purchased from Sigma (α-smooth muscle actin, β-actin, and β-tubulin), ECM Bioscience (phospho-Coro1B Ser-2), Santa Cruz Biotechnology (Coro1B M-80, Coro1B S-20, cyclin-dependent kinase 4 (CDK4), and actin-related protein 2/3 complex

subunit 2 (ArpC2)), Millipore (ArpC2) and Cell Signaling Technologies (Myc 9B11 and 71D10, pAKT Ser-473, and pERK1/2 Thr- 202/Tyr- 204, PKCε). Mouse monoclonal antibody against Coro1C was created in the laboratory of Dr. James Bear as previously described (140). siGlo Red transfection indicator was purchased from Dharmacon RNAI Technologies. Rö-32-0423, Gö-6796 PKC, and PKCβ inhibitors were purchased from Calbiochem. Streptadividin conjugated Quantum dots with a 603 fluorescent label, Phalloidin, anti-mouse Alexa Fluor 633, and anti-rabbit Alexa Fluor 568 were purchased from Invitrogen.

2.2.2 Cell Culture

VSMCs were isolated from rat thoracic aorta by enzymatic digestion as previously described (236). Isolated VSMCs were grown in Dulbecco's Modified Eagle's Media supplemented with 10% calf serum, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 4mM L-glutamine. For experiments, cells between passages 7 and 15 were plated and allowed to grow until they reached 60% confluence. Cells were then serum starved for 24-48 hrs prior to PDGF stimulation.

Human coronary artery smooth muscle cells (HCASMC) and human aortic smooth muscle cells (HASM) were obtained from Lonza (AG) and grown in Smooth muscle Growth Media-2 (Lonza) supplemented with human epidermal growth factor 0.5 ng/mL, insulin 5 µg/mL, hFGF 2 ng/mL, 5% FBS, gentamicin 50 µg/mL, and amphotericin B 50 ng/mL. For experiments, cells between passage 5 and 9 were plated and allowed to grow until they were 70% confluent**.** Cells were made quiescent for 48 hrs by reducing the serum content of the media to 0.1% FBS prior to PDGF stimulation.

Human dermal fibroblasts (HDF) were derived from the dermis of normal neonatal foreskin and grown in Dulbecco's Modified Eagle's Media supplemented with 10% calf serum, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 4

mM L-glutamine. For experiments, cells between passages 5 and 10 were plated and allowed to grow until they reached 70% confluence. Cells were then serum starved for 48 hrs prior to PDGF stimulation.

Rat-1 fibroblasts were grown in DMEM supplemented with 10% calf serum, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 4 mM L-glutamine. Cells were allowed to grow to 60% confluence and then serum starved in serum free media for 48 hrs before stimulation with PDGF.

2.2.3 Reverse Transcriptase PCR (RT-PCR)

RNA was purified from rat lung tissue, human whole blood, HCASMCs and VSMCs using the RNeasy kit and QIAamp RNA Blood Mini kit (Qiagen), following the manufacturer's instructions. The purified RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) using random nanomers. The resulting cDNA samples were amplified by non-quantitative PCR using recombinant Platinum Taq DNA polymerase (Invitrogen). Amplification conditions were as follows: 300 nmol/L primers, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTPs, with an annealing temperature of 55°C. The following primer sequences were created for amplification of rat sequences: Coro1B (upstream primer, 5'- ACA TGT CCT TCC GAA AAG TTG TGC-3'; downstream primer, 5'-CTG ATC CAC TGG CAA TGA CTT CGT -3'), Coro1C (upstream primer, 5'-TGT CTT CAC TAC TGG TTT TAG CCG TA-3'; downstream primer, 5'-TCT AGC TTT GAA ATG CGC TCG TCT-3'), and GAPDH (upstream primer, 5'- AAT GGG GTG ATG CTG GTG CTG AGT A-3'; downstream primer, 5'-GGA AGA ATG GGA GTT GCT GTT GAA G-3').

The following primer sequences were created for amplification of human sequences: Coro1B (upstream primer, 5'- TGG AGA CTC CTG CGG ATG CCA TTA -3'; downstream primer, 5'- ACG GGA CAC GCG AAT GTC CTC ATA -3'), Coro1C

(upstream primer, 5'- GGG GTA TTT GCC CTG GGT GAT CCT -3'; downstream primer, 5'- GCC TTG GTG AGA GAT GGA GAG GCA -3') and GAPDH (upstream primer, 5'-CCC ATC ACC ATC TTC CAG GAG C-3'; downstream primer, 5'-CCA GTG AGC TTC CCG TTC AGC -3') (237).

2.2.4 Western Blotting

After treatment with PDGF, cells were washed twice with phosphate buffered saline and then lysed in Hunters buffer (25 mmol/L HEPES, 150 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, Na-orthovanadate and protease inhibitors). Lysates were then sonicated and cleared at 13,000 x g for 5 minutes. Proteins were separated using SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore), blocked with 5% non-fat dairy milk, and incubated with appropriate primary antibodies. Subsequently, blots were incubated with horseradish peroxidase-conjugated secondary antibodies and proteins were detected by enhanced chemiluminescence (ECL, GE). Band intensity was quantified by densitometry using ImageJ (NIH) or Carestream Molecular Imaging (Carestream) software.

2.2.5 Co-immunoprecipitation

Cells were washed twice with phosphate buffered saline and lysed with a KCl buffer (20 mmol/L HEPES, pH 7.0, 100 mmol/L KCl, 0.5% Nonidet P-40, 1 mmol/L EDTA, and protease inhibitors). Lysates were cleared at 13,000 x *g* for 5 min. 500 µg of protein lysate was incubated with 1 µg of primary antibody for one hour at 4 °C, followed by the addition of 30 µl of Protein A beads (Santa Cruz Biotechnology) for another hour. Beads were blocked with 1 mg/ml bovine serum albumin for one hour before use.

Immunoprecipitated proteins were collected by centrifugation, washed three times with KCl buffer, separated by SDS-PAGE, and transferred to PVDF membranes for Western blotting.

2.2.6 Plasmid Construction and Site- directed Mutagenesis

C-terminal Myc-tagged Coro1B-WT pcDNA3 was constructed by amplifying the open reading frame of human Coro1B from pCMV6-AC-Coro1B (Origene) using Phusion High-Fidelity DNA polymerase (Thermo-Fisher Scientific) as well as primers that contained the Myc coding sequence. Amplification conditions were as follows: 300 nmol/L primers, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTPs, and 3% dimethyl sulfoxide (DMSO) with an annealing temperature of 72°C. Primer sequences were as follows: upstream primer -5' TAC GGA TCC GCC ACC ATG TCC TTC CGC AAA GTG GTC CGG CAG AGC A -3' downstream primer -5' GTA TCT AGA TCA GAA TTC CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC CGC ATC CCC GTT CTC CAT GCG GCC CAG CT -3'. Myc-tagged Coro1B-S2A pcDNA3 and Myc-tagged Coro1B-S2D were generated from Myc-tagged Coro1B-WT pcDNA3 using Quick Change site-directed mutagenesis kit (Strategene) and mutation-encoding primers. Primer sequences were as follows: S2A primers (upstream primer 5'- GAT CCG CCA CCA TGG CCT TCC GCA AAG TG -3'; downstream primer 5'-CAC TTT GCG GAA GGC CAT GGT GGC GGA TC-3') and S2D primers (upstream primer 5'-CGG ATC CGC CAC CAT GGA CTT CCG CAA AGT GGT C-3'; downstream primer 5'- GAC CAC TTT GCG GAA GTC CAT GGT GGC GG ATC CG -3').

2.2.7 Small Interfering RNA and Plasmid Transfection Experiments

Cells were transfected by electroporation using the Amaxa Nucleofector system (Lonza AG) set to the U25 program with 5 µg of plasmid per $1.5x10^6$ cells or with 3 µg of

annealed siRNA duplexes for Coro1B, Coro1C or nonsilencing control sequence no. 1 from Qiagen per $1.5x10^6$ cells. siRNA target sequences were as follows: Coro1B (5'-CAG CAC CTT CTG CGC AGT CAA -3') and Coro1C (5'- ACG AGA GAA AGT GTG AAC CTA -3'). The cells were transfected, allowed to attach and recover for 24 hours and then serum starved for 24-48 hours. VSMCs that were used for single cell tracking and kymography experiments were also co-transfected with 1 µg siGlo to visually detect siRNA transfection.

2.2.8 Modified Boyden Chamber Assay

Migration was measured using a modified Boyden chamber assay as previously described (78, 238). Briefly, cells were grown to 60% confluence and then made quiescent in serum-free media for 48 hours before migration. Membrane inserts were coated with 5 µg/cm^2 of type I rat tail collagen (BD Bioscience). VSMCs were added at a density of $5x10^4$ cells/well to the upper chamber of a Transwell dish with a 6.5-mm polycarbonate membrane insert containing 8-µm pores (Costar). VSMCs were then exposed to PDGF (10 ng/mL) in the lower chamber and allowed to migrate for 4 hours. Nonmigrated cells were removed from the upper membrane using a cotton swab. The remaining cells were methanol fixed and fluorescently stained with 4', 6-diamidino-2-phenylindole (DAPI) (1 µg/mL). Membranes were removed from the insert and mounted on slides with Fluoromount-G (Southern Biotech). Migrated cells were visualized using a Zeiss Axioskop microscope and five images from five random fields per membrane were quantified by counting nuclei from three independent experiments. Images were quantified using ImageJ software.

2.2.9 Single Cell Tracking

VSMCs were plated on 5 μg/cm^2 collagen coated MatTek dishes (MatTek Corp.), allowed to attach to the dishes for 3 hrs and then serum starved for 24 hrs. Then VSMCs were stimulated with 10 ng/ml PDGF and monitored for 12 hrs using the Olympus Viva View live cell imaging microscope system. Ten viewing fields were chosen from each dish and images were taken of each field every 15 minutes for 12 hrs. Images were taken at a magnification of 20x and were converted to stacks using Image J software. Single cell velocity and distance traveled were obtained from the aforementioned stacks using Image J tracking software. To avoid bias in the analysis, only cells that did not divide, remained within the field of view for the entire duration of the experiment, did not touch other cells more than transiently, and were fluorescently labeled by siGLO were tracked. Quantification of individual cell speed and total distance traveled were obtained. VSMCs were transfected and migration was observed in two independent experiments.

2.2.10 Lamellipodia Kymography

Cells were plated on 5 μg/cm^2 collagen coated MatTek dishes (MatTek Corp.) and allowed to attach to the dishes for 3 hrs then serum starved for 24 hrs. Cells were then stimulated with 10 ng/ml PDGF for 20 minutes. Cells with lamellipodia (broad thin protrusions) and labeled with siGLO were identified and images were taken every 4 s for 4 min at 80x on a Nikon BioStation IM. Images were converted to stacks and kymographs were created using Image J software. Lamellipodial protrusion rate, protrusion distance and protrusion duration were quantified as previously described (194, 239).

2.2.11 Immunocytochemistry

VSMCs were plated onto 22-mm diameter round No. 1 German glass coverslips

coated with collagen (BD Bioscience) and serum starved for 24 to 48 hrs. Before fixation cells were rinsed with ice-cold PBS, then fixed in 10% formaldehyde for 10 min at room temperature and permeabilized in 0.2% Triton X-100 in PBS for 5 minutes. Subsequent incubation in 50 mmol/L NH_4Cl for 10 minutes was used to quench free aldehydes. After 1 hour of blocking in 3% bovine serum albumin (BSA) in PBS, the cells were incubated with antibodies overnight, and incubated for 1 hour with secondary antibody conjugated to Rhodamine Red X (Jackson ImmnoResearch). Actin filaments were stained with phalloidin Alexa-488 (Molecular Probes) and nuclei were stained with DAPI. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Inc.). Images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope System using a 63x oil objective lens (numerical aperture: 1.40) and Zeiss ZEN acquisition software. Controls with rabbit IgG antibody showed no fluorescence. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant. All images are maximum intensity projections of Z-series from the base through the top of the cell.

2.2.12 Rat Carotid Balloon Injury and Mouse Carotid Artery Wire Injury

Sprague-Dawley rats (375 to 400 g) subjected to left common carotid artery injury by means of a 2F arterial embolectomy balloon catheter introduced into the external branch were purchased from Zivic-Miller Laboratories. Carotid arteries were harvested 7 and 10 days after surgery. Arteries were embedded in OCT (Tissue-Tek) and cut into 7μm sections.

Wild type C57BL/6 mice were subjected to wire injury of the carotid artery as previously reported (240). At 7 and 14 days after injury, the mice were sacrificed. Six animals were used per time point. Carotids were pooled together into groups of two before protein extraction and analysis by SDS-PAGE gel.

2.2.13 Immunohistochemistry

Sections were incubated with primary antibodies overnight at 4°C. The sections were washed and incubated with secondary streptavidin-labeled antibody for 30 minutes at room temperature. Sections were washed again and incubated with anti-streptavidin 633 fluorescently labeled Qdots from Invitrogen. Cells were then counterstained with DAPI for nuclear localization. Sections treated with secondary antibodies alone did not show specific staining. Carotid arteries from 3 animals per treatment group were analyzed and 2 to 3 sections were stained per animal. Images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope System using a 20x air objective lens and Zeiss ZEN acquisition software. When comparing sections from different experimental groups, all image threshold settings of the confocal microscope remained constant.

2.2.14 Statistics

Results are expressed as means ± SEM. Differences among groups were analyzed using student's t-test as well as one-way and two-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. A value of $P<0.05$ was considered to be statistically significant.

2.3 Results

2.3.1 Expression of Type I Coronins in VSMC Migration

There have been no studies examining coronin expression or function in VSMCs. To elucidate if type I coronins are expressed in VSMCs, we used reverse transcription PCR under two conditions. Using RNA from rat lung tissue homogenates as a positive control, we discovered that only Coro1B and 1C proteins are expressed in VSMC samples with 25 PCR amplification cycles (Figure 2.1A). When the number of PCR

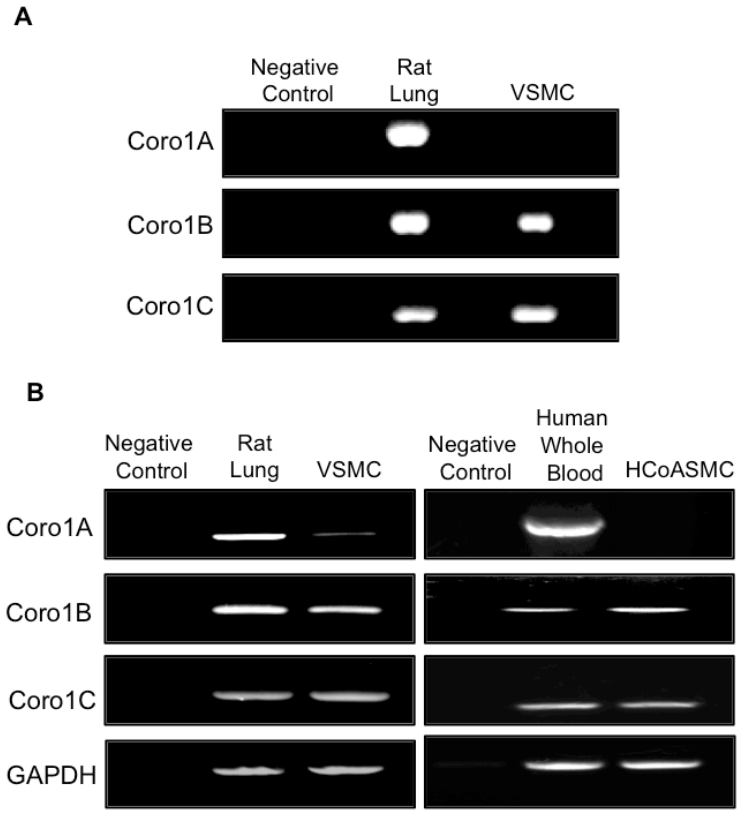


Figure 2.1 Coro1B and 1C are Expressed in VSMCs. (A) VSMCs were grown in serum for 48 hours and then harvested for RNA. Coro Coro1A, 1B, and 1C mRNA was detected using sequence specific PCR primers. Rat lung cDNA was used as a positive control. cDNA was amplified with 25 PCR cycles. **(B)** VSMCs and HCoASMCs were grown in serum for 48 hours and then harvested for RNA. Coro1A, 1B, 1C, and GAPDH mRNA was detected using sequence specific PCR primers. Rat lung cDNA was used as a positive control for VSMCs and human whole blood was as a positive control for HCoASMCs. cDNA was amplified with 34 PCR cycles.

cycles was increased to 34 amplification cycles to increase sensitivity, Coro1A, which was previously undetectable in our cells, was expressed at much lower levels compared to the positive control (Figure 2.1B). To determine if VSMCs from other vascular beds express a similar complement of coronins, we tested human coronary artery smooth muscle cells (HCoASMC) and found that these cells also express only Coro1B and 1C mRNA in PCR reactions that had 34 PCR amplification cycles (Figure 2.1B). Coro1A was undetectable in HCoSMCs. (Figure 2.1B). This suggested Coro1A is not expressed at meaningful levels in VSMC and confirms the idea that Coro1A is expressed to much lower levels in cells outside the immune system. Both, Coro1B and 1C proteins were detectable by western blot, and we were able to specifically knock down each protein (Figure 2.2). On average Coro1B was downregulated by 87 ± 3% and Coro1C by 68 ± 5% (Figure 2.2). These data demonstrate for the first time that VSMCs express coronin proteins.

2.3.2 Coronin 1B Down Regulation Potentiates PDGF-induced VSMC Migration

Coronins serve a significant function in actin dependent processes such as migration and can variably inhibit or promote migration. Since Coro1B and 1C were more highly expressed in VSMCs we decided to explore their role in PDGF-induced VSMC migration. To determine if either Coro1B or 1C plays a role in VSMC motility stimulated by PDGF, we used siRNA against each protein. Single cell tracking was used to measure the distance the cells traveled and to calculate cell velocity in response to PDGF treatment. As expected, in all samples stimulated with PDGF, there was a significant increase in the total distance traveled that mirrored changes in velocity (Figure 2.3, Figure 2.4A and B). Interestingly, Coro1B knockdown increased PDGF-induced VSMC motility when compared to siNegCtrl (210.3 ± 8.2 μm v. 166.6 ± 8.2 μm, $p < 0.001$, siCoro1B vs siNegCtrl) (Figure 2.4A). However, the down-regulation of

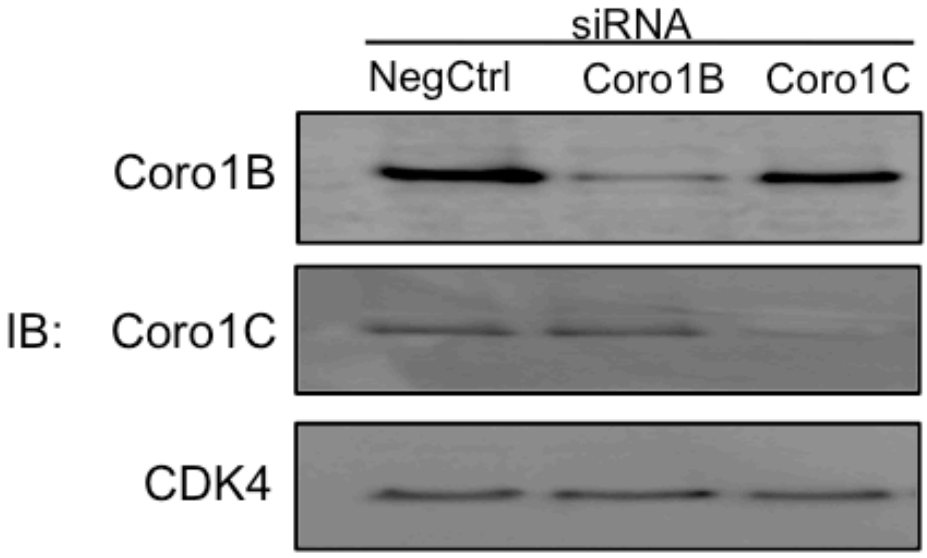
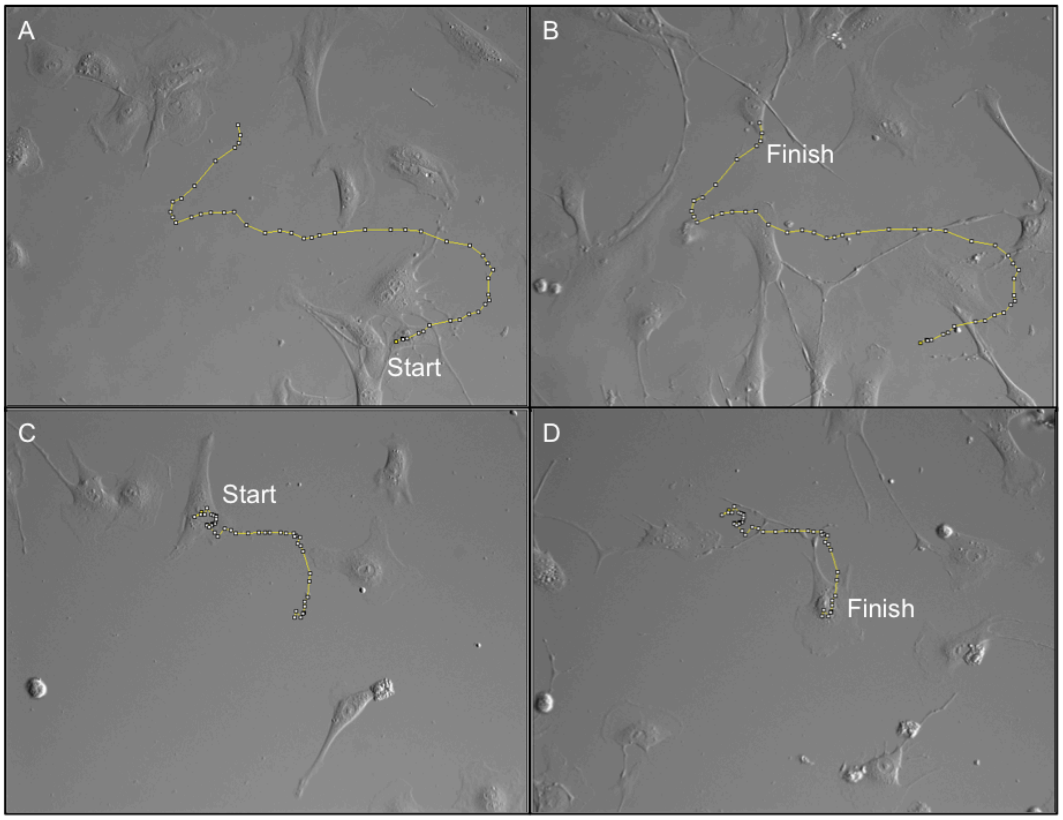
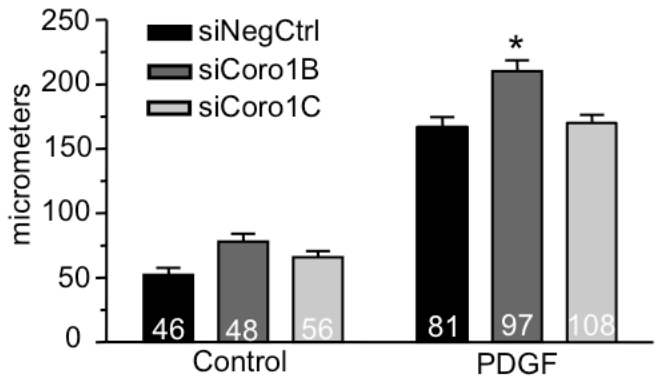


Figure 2.2 Coro1B and 1C can be Specifically Knocked Down in VSMCs. VSMCs were transfected with siNegCtrl, siCoro1B or siCoro1C with the Amaxa electroporation system and then serum starved for 48hrs. Cell lysates were harvested and immunoblotted for Coro1B and Coro1C and CDK4 as a loading control. On average Coro1B was down regulated by 87 ± 3% (Representative blot, N= 10) and Coro1C by 68 ± 5% (Representative blot, N=5).



2.3 Live Cell Image: siCoro1b Increases VSMC Migration in Response to PDGF. Cells were plated on collagen coated MatTek dishes, allowed to attach to the dishes and then serum starved for 24 hrs. Cells were stimulated with PDGF and monitored for 12 hrs using the Olympus Viva View live cell imaging microscope system. Images were taken of each field every 15 minutes for 12 hrs. Images were taken at a magnification of 20x and were converted to stacks and tracked using Image J software. Examples of tracked cells **(A)** siCoro1B starting position, **(B)** siCoro1B ending position, **(C)** siNegCtrl starting position, and **(D)** siNegCtrl ending position. Yellow line represents the track of each cell.

A



B

	Control			PDGF		
siRNA	**Velocity (µm/hr)**	**Std. Error**	**N**	**Velocity (µm/hr)**	**Std. Error**	**N**
siNeg Ctrl	4.3450	0.4621	46	13.8800	0.6791	81
siCoro1B	6.5620	0.5136	48	17.5200 *	0.6868	97
siCoro1C	5.501	0.3966	56	14.200	0.5224	108

Figure 2.4 Knockdown of Coro1B Increases PDGF-induced VSMC Migration. Live cell imaging was used to measure the **(A)** distanced traveled and **(B)** velocity of VSMCs transfected with siNeg Ctrl, siCoro1B, or Coro 1C. Cells were serum starved for 24hrs and then stimulated with 10 ng/ml of PDGF for 12hrs. The graph represents the mean ± SEM velocity of each cell. The N (number of cells) is indicated within each column (*p< 0.001, PDGF treated siNeg Ctrl compared to siCoro1B).

Coro1C had no effect on PDGF-induced VSMC motility when compared to siNeg Ctrl (170.0 ± 6.3 μm v. 166.6 ± 8.2 μm, p>0.05, siCoro1C vs siNegCtrl) (Figure 2.4A and B). These data suggest that Coro1B is a negative regulator of VSMC motility and that Coro1C does not modulate PDGF-induced motility in these cells.

In Rat-2 fibroblasts, Coro1B knockdown decreased random basal migration and suggests a positive role for Coro1B in migration (194). Because this effect is in direct contrast to our results in VSMCs, we posited that this discrepancy might be due to cell type, species or transformation. To test this hypothesis, the effects of Coro1B downregulation on PDGF-induced migration in transformed Rat-1 fibroblasts, primary HDF, and primary VSMCs of human and rat origin (HASM and RASM, respectively) were assessed. Initially, we confirmed that Coro1B was knocked down in each cell type (Figure 2.5 A, B, C, D). In the modified Boyden chamber assay, down regulation of Coro1B in Rat-1 fibroblasts led to a trend towards decreased basal migration (114 ± 18 vs 84 ± 12, p>0.05, siNegCtrl vs siCoro1B N=3-4) (Figure 2.6A). This is in accordance with previously published data. However, this trend for basal decline in migration was not seen in RASM (38 ± 14 vs 35 ± 3 cells, p>0.05, siNegCtrl vs siCoro1B N=3-4), HASM (50 ± 13 vs 48 ± 7 cells, p>0.05, siNegCtrl vs siCoro1B N=3-4) or HDF (93± 14 vs 111 ± 16 cells, p>0.05, siNegCtrl vs siCoro1B N=3-4) (Figure 2.6B, C, D). Interestingly, stimulation of Rat-1 cells with PDGF did not increase migration (114 ± 18 vs 110 ± 13 cells, p>0.05, siNegCtrl: control vs PDGF N=3-4) (Figure 2.6A). Conversely, PDGF significantly stimulated RASM (38 ± 14 vs 150 ± 1 cells, * p<0.05, siNegCtrl: control vs PDGF N=3-4), HASM (50 ± 13 vs 94 ± 12 cells, * p<0.05, siNegCtrl: control vs PDGF N=3-4), and HDF (93 ± 14 vs 169 ± 18 cells, * p<0.05, siNegCtrl: control vs PDGF N=3-4) cell migration (Figure 2.6B, C, D). Coro1B down regulation tended to increase PDGF-induced migration non-significantly in RASM (150 ± 1 vs 173 ± 14 cells, p>0.05, siNegCtrl vs

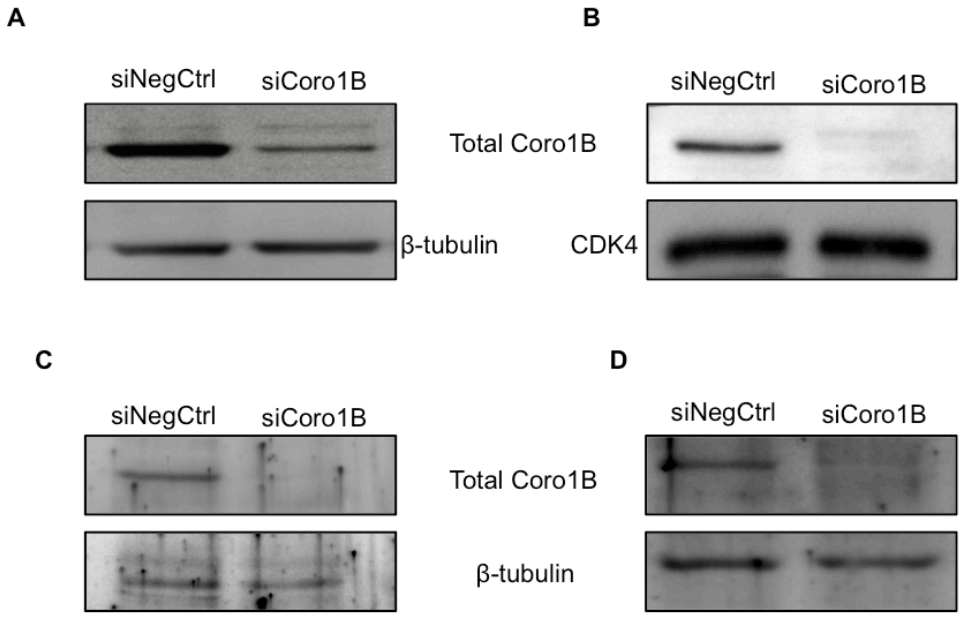


Figure 2.5 siCoro1B Down Regulation in Rat-1, RASM, HASM and HDF. (A) Rat-1 fibroblasts**, (B)** RASM**, (C)** HASM and **(D)** HDF were transfected with siRNA against Coro1B or NegCtrl. Cells were serum starved for 48hrs, harvested for protein and immunoblotted with Coro1B, β- tubulin, or CDK4. (Representative blot, N=3-4)

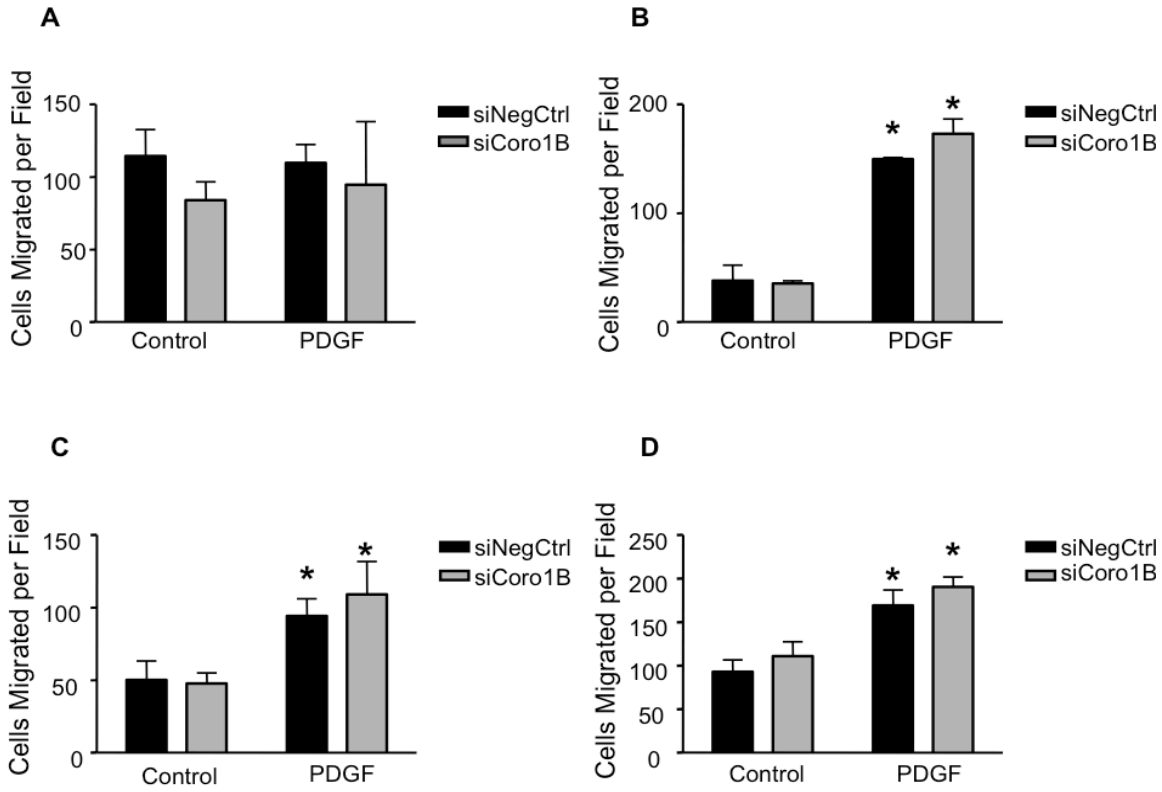


Figure 2.6 The Effect Coro1B Downregulation on PDGF-induced Migration is Cell Type Specific. (A) Rat-1 fibroblasts**, (B)** RASM**, (C)** HASM and **(D)** HDF were transfected with siRNA against Coro1B or NegCtrl. Cells were serum starved for 48hrs, harvested and migration was assessed via modified Boyden chamber assay (* $p<0.05$, control vs PDGF N=3-4).

siCoro1B, N=3-4), HASM (94 ± 12 vs 109 ± 22 cells, p>0.05, siNegCtrl vs siCoro1B, N=3-4) and HDF (169 ± 18 vs 190 ± 11 cells, p>0.05, siNegCtrl vs siCoro1B, N=3-4) cells (Figure 2.6B, C, D). It is also of importance to note that basal migration in the siNegCtrl treated samples is significantly higher in Rat-1 cells when compared to RASM (114 ± 18 vs 38 ± 14 cells, *p<0.05, Rat-1 vs RASM, N=3-4) and HASM (114 ± 18 vs 50 ± 13 cells, #p<0.05, Rat-1 vs HASM, N=3-4); however, there was not a significant difference in the basal migration of Rat-1 cells when compared to HDF (114 ± 18 vs 93 ± 134 p>0.05, Rat-1 vs HDF, N=3-4) (Figure 2.7). Although siNegCtrl HDF and Rat-1 cells had similar basal cell migration, Rat-1 cells showed impaired chemotactic migration compared to HDFs (110 ± 13 vs 190 ± 11, +p<0.05, PDGF: Rat-1 vs HDF, N=3-4) in response to PDGF (Figure 2.7). To determine if Rat-1 cells are able to respond to PDGF and if their response is different from VSMC, we stimulated Rat-1 and VSMCs with PDGF and evaluated the phosphorylation of ERK1/2, a known downstream target of PDGF stimulation (Figure 2.8). We found that PDGF stimulated both Rat-1 cells and VSMCs at five minutes; hence, Rat-1 cells do respond to PDGF stimulation. However, basal phosphorylation of ERK 1/2 was higher in Rat-1 cells suggesting they have a higher basal rate of activity. These data suggest that Rat-1 cells respond differently chemotactically to siCoro1B inhibition and to PDGF, suggesting that transformation alters the role of Coro1B in migration. However, in primary cells, regardless of species or cell type, Coro1B expression apparently negatively regulates migration.

2.3.3 Coronin 1B Overexpression Inhibits PDGF-induced Migration

Since the downregulation of Coro1B increased VSMC migration we performed a gain-of-function experiment to investigate the effects of Coro1B overexpression on VSMCs. Cells were transfected with a plasmid containing a wildtype (WT) myc tagged Coro1B or empty vector (pcDNA 3) and then used in a modified Boyden chamber assay.

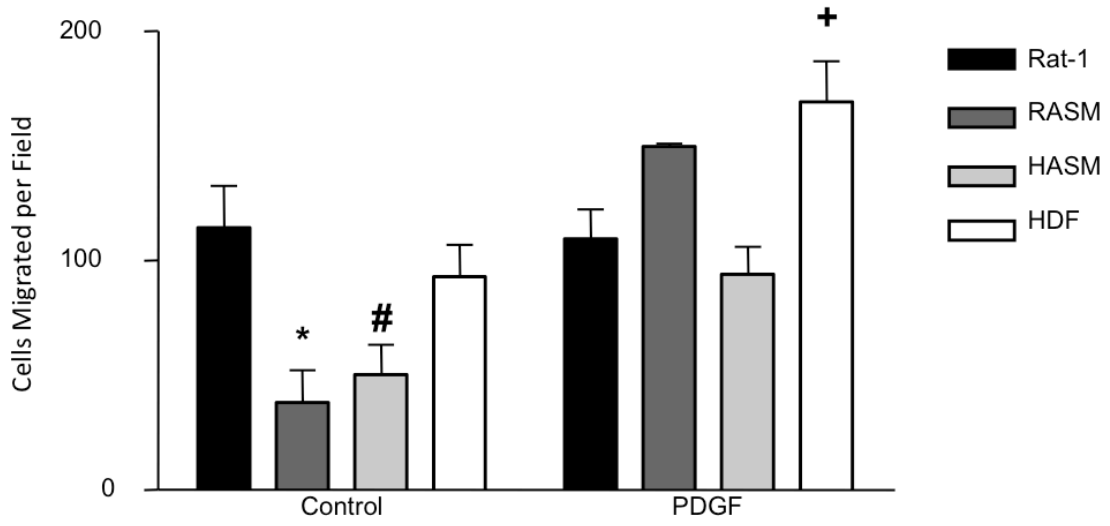


Figure 2.7 PDGF Stimulates Chemotaxis in RASM, HASM, and HDFs, but Not in Rat-1 Fibroblasts. Recombined graphical representation of siNegCtrl transfected cells. Rat-1 fibroblasts, RASM, HASM, and HDF cells were transfected with siNegCtrl siRNA , starved for 48hrs, harvested and migration was assessed via modified Boyden chamber assay (* $p < 0.05$, Rat-1 control vs RASM control, # $p < 0.05$, Rat-1 control vs HASM control, +$p<0.05$, Rat-1 PDGF vs HDF PDGF, N=3-4).

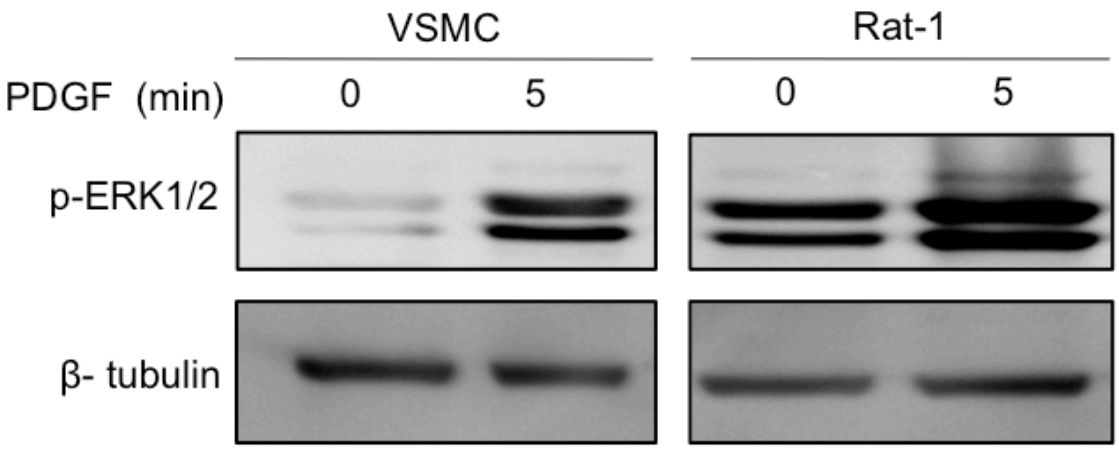


Figure 2.8 PDGF Phosphorylates ERK 1/2 in Response to Stimulation in VSMCs and Rat-1 Fibroblasts. RASM and Rat-1 cells were serum starved for 48hrs. Cells were then stimulated with 10 ng/ml PDGF for 5 minutes and harvest for protein. Lysates were immunoblotted for p-ERK1/2 and tubulin. (Representative blot, N=2)

In VSMCs, Coro1B overexpression inhibited migration in response to PDGF by 44 ± 11 % (+$p<0.05$, 56 ± 8 vs 30 ± 6 cells, pcDNA3 vs WT) (Figure 2.9A). To ensure PDGF signaling was still intact, VSMC transfected with myc tagged Coro1B or empty vector were stimulated with PDGF and harvested for protein. Western blot analysis of these lysates demonstrated that Coro1B was overexpressed in these cells and that Coro1B overexpressing cells were able to phosphorylate Akt in response to PDGF (Figure 2.9B). This suggests that overexpression of Coro1B does not interfere with PDGF receptor signaling to downstream proteins. Furthermore, these data corroborate a negative role for Coro1B in PDGF-induced VSMC migration.

2.3.4 Localization of Coronin 1B in VSMCs

To gain further insight into Coro1B regulation of PDGF-induced migration, we examined its localization in VSMCs. In cell fractionation studies enrichment fractions of proteins from the cytosolic, cytoskeletal, and membrane fractions were collected. PKCα, vimentin and epidermal growth factor receptor (EGFR) expression were used as markers of the cytosol, cytoskeleton, and membrane respectively (Figure 2.10A). The EGFR was found only in the membrane fraction. PKCα was isolated in both the cytosolic and membrane fraction; however it was enriched in the cytosolic fraction. It is important to note that PKCα translocates to the membrane when activated. Vimentin was enriched in the cytoskeletal fraction and found in much smaller quantities in the cytosolic and membrane fractions. The presence of Coro1B was examined in these fractions in response to PDGF. Coro1B was found in all fractions and PDGF did not change its compartmentalization (Figure 2.10B). Because these fractions were not pure and therefore the quantitative compartmentalization of Coro1B was not possible, we next examined the localization of Coro1B using confocal microscopy.

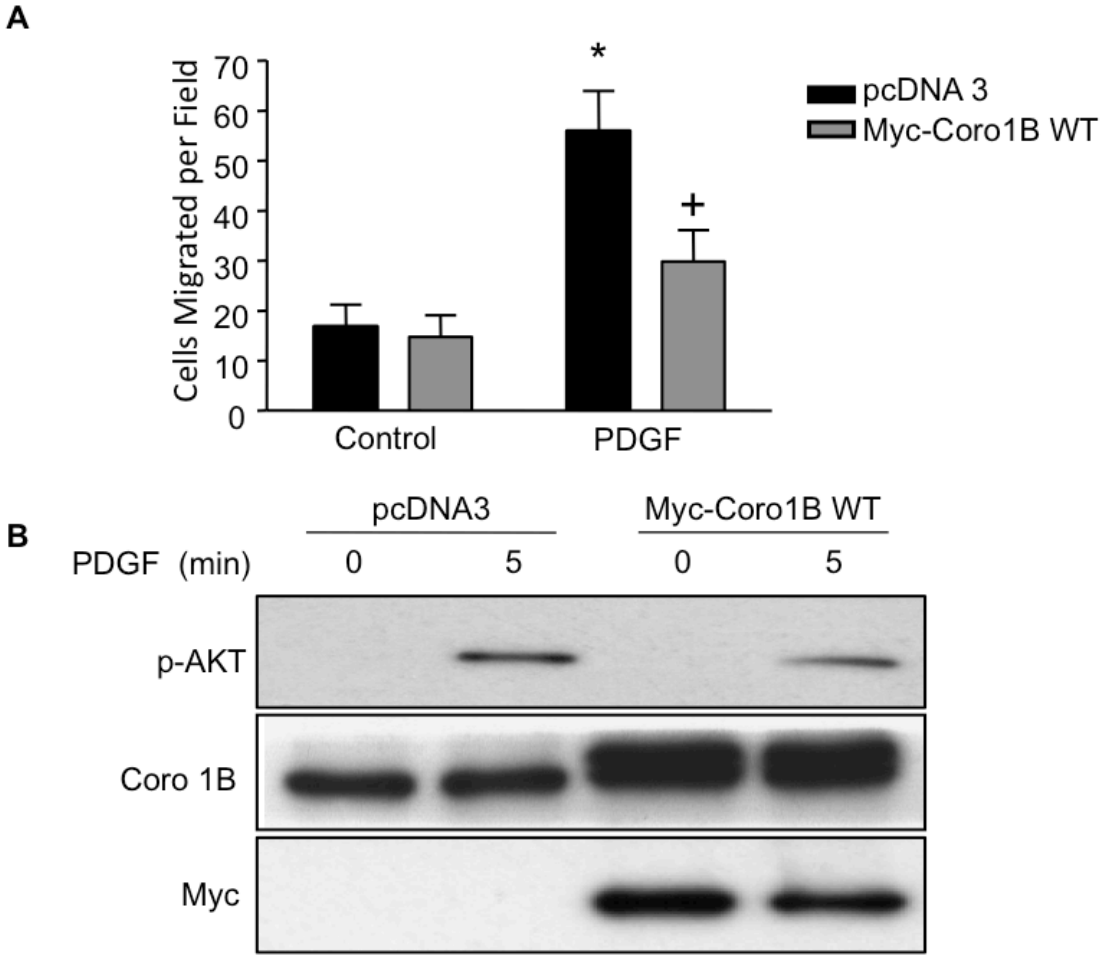


Figure 2.9 Coro1B Overexpression Inhibits PDGF-induced Migration, but Does Not Inhibit PDGF Signaling to AKT. VSMCs were transfected with myc-tagged Coro1B WT, serum starved for 48hrs, **(A)** harvested and migration was assessed via modified Boyden chamber assay (*p<0.05, control vs PDGF, +p<0.05, PDGF: pcDNA3 vs WT, N=4) and **(B)** then stimulated with 10 ng/ml PDGF for 5 minutes and harvested for protein. Lysates were immunoblotted for p-Akt, Coro1B, and Myc (Representative blot, N=2).

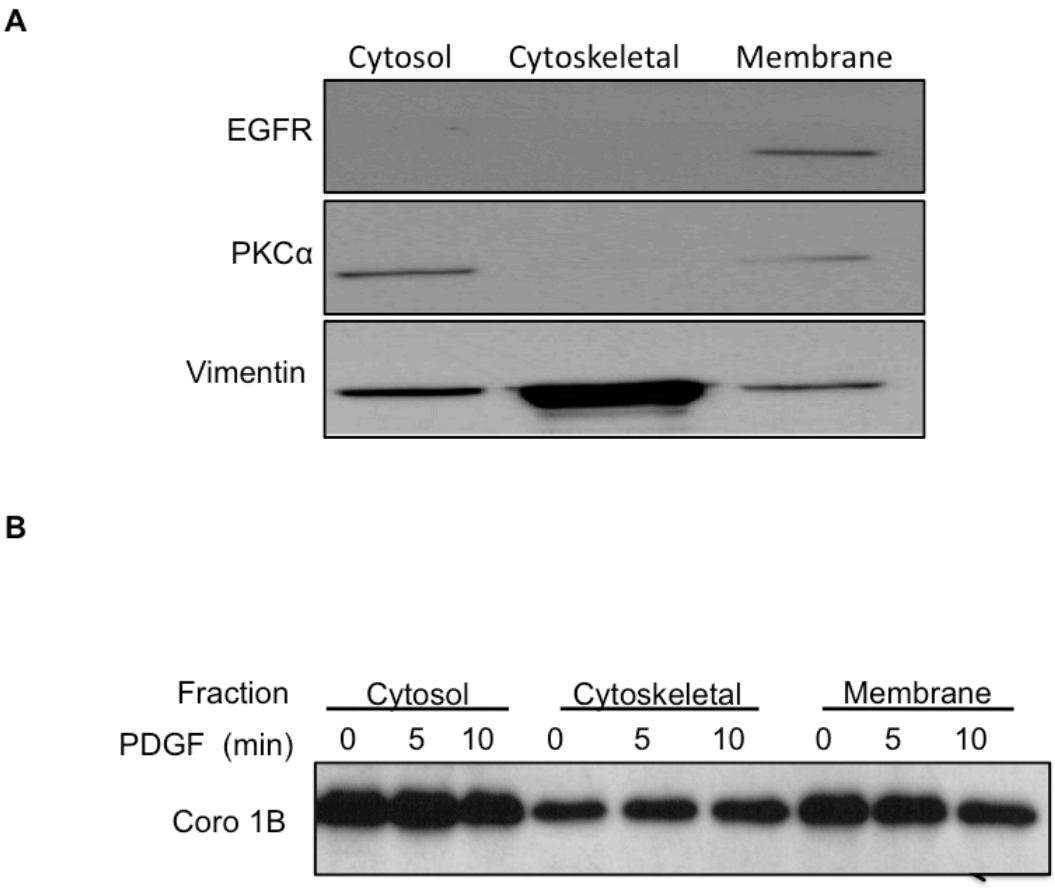


Figure 2.10 Coro1B Localizes to the Cytosol, Cytoskeletal and Membrane of VSMCs. Cells were serum starved for 48hrs and then stimulated with 10 ng/ml PDGF. Cell lysates were harvested and subcellular fractions were collected. **(A)** Lysates were harvested from each fraction and tested for enrichment by immunoblotting with antibodies against EGFR (membrane), PKCα (cytosol), and vimentin (cytoskeleton). **(B)** Lysates were immunoblotted for Coro1B.

We observed strong immunofluorescence staining at the cell periphery, with detectable signal in the cytosol and in the peri-nuclear region (Figure 2.11). Coro1B also co-localized with actin stress fibers when over expressed (Figure 2.12). These microscopy results suggest that Coro1B in VSMCs localizes primarily to the membrane, but is also found associated with the cytoskeleton and in the cytosol. Although coronins were first described as actin binding proteins, Coro1B shows minimal staining for cytoskeletal structures such as stress fibers in VSMCs. One possible reason for this is a technical one and deals with antibody recognition. The Coro1B (M-80) antibody recognizes the C-terminal coiled coil region of Coro1B. This region also participates in oligomerization of the protein and it is thought that oligomerized coronin mediates the function of actin bundling. Therefore the epitope that this antibody detects may be masked. Because the myc tag on the WT Coro1B is also at the C-terminus, this epitope could be masked in the overexpression studies as well. Another hypothesis is that Coro1B is in fact predominately located at the membrane in VSMCs. This could partially account for the differences in responses seen when expression is modulated in VSMCs and Rat-2 fibroblasts.

2.3.5 Coronin 1B Down Regulation Modifies PDGF-induced Changes in Lamellipodia Dynamics

Based on the strong peripheral localization of Coro1B in VSMCs and the fact that type I coronins are known to bind the Arp2/3 complex, a potent regulator of lamellipodia formation, we examined the effects of siRNA against Coro1B in PDGF-induced VSMC lamellipodia dynamics using kymography (Figure 2.13A). siCoro1B transfected samples showed increased PDGF-induced lamellipodia protrusion rate (4.9 ± 0.2 μm/min v. 3.7 ± 0.2 μm/min, $p<0.0001$ siCoro1B vs siNegCtrl) (Figure 2.13B) and protrusion distance

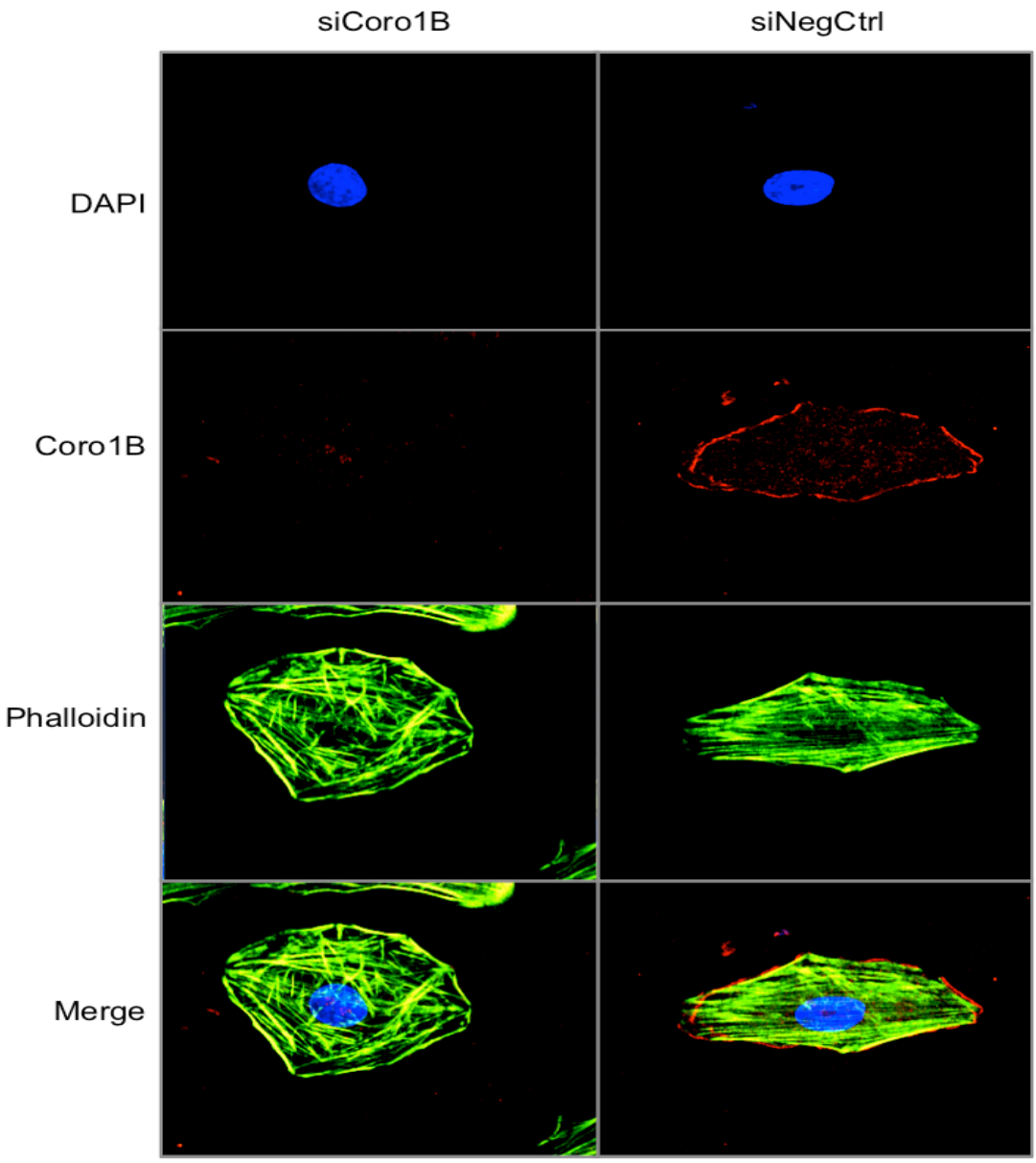


Figure 2.11 Coro1B Localizes to the Periphery of VSMCs. Confocal images were acquired after transfecting VSMCs with siNegCtrl or siCoro1B using the Amaxa electroporation system. Cells were serum starved for 48 hrs and then fixed and permeabilized. Immunofluorescence of Coro1B (red), DAPI (blue) and phalloidin (green) was then detected using the Zeiss LSM 510 META Laser Scanning Confocal Microscope.

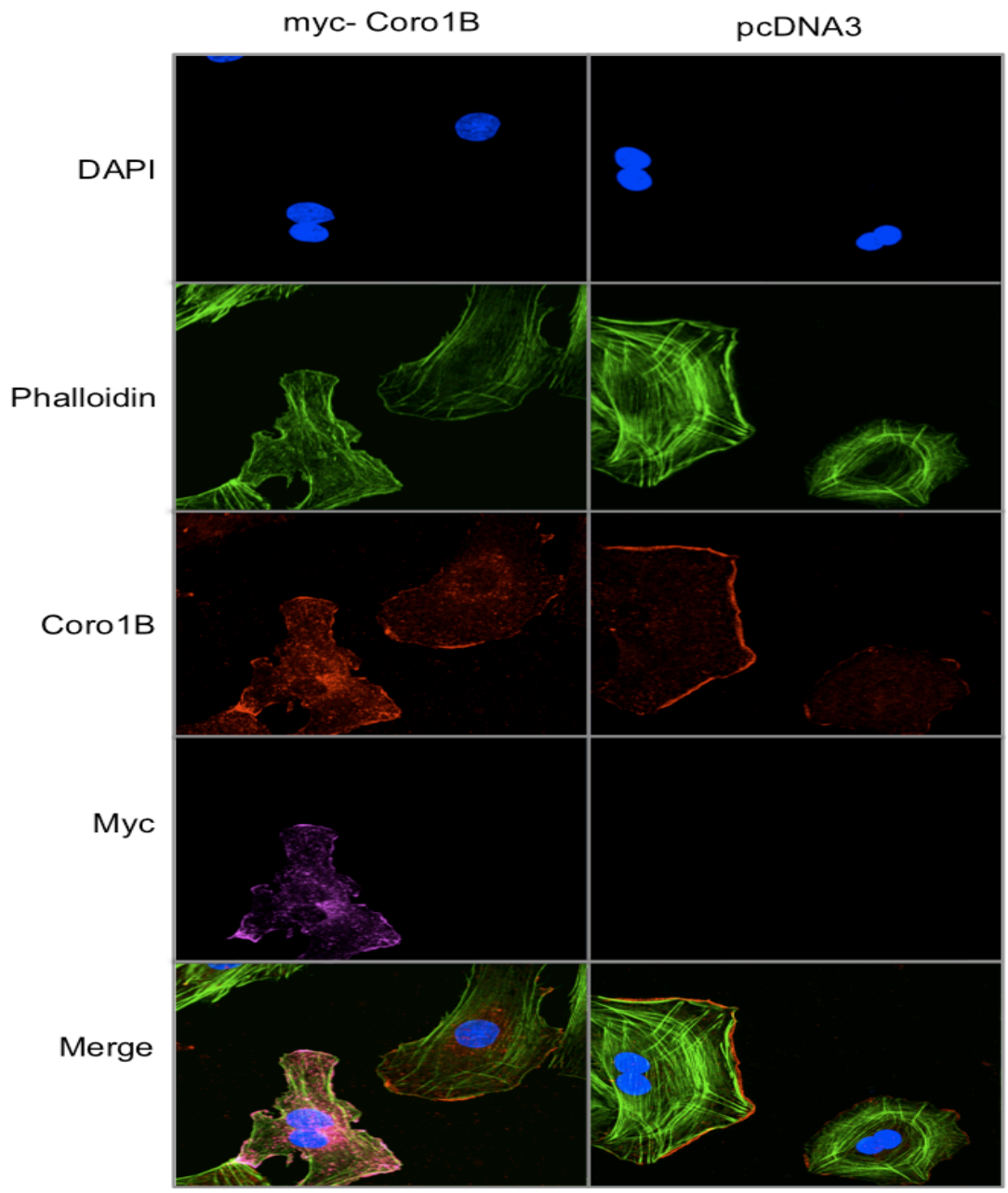


Figure 2.12 Localization of Overexpressed Coro1B in VSMCs. Confocal images were acquired after transfecting VSMCs with siNegCtrl or siCoro1B using the Amaxa electroporation system. Cells were serum starved for 48 hrs and then fixed and permeabilized. Immunofluorescence of Coro1B (red), DAPI (blue) and phalloidin (green) was then detected using the Zeiss LSM 510 META Laser Scanning Confocal Microscope (63x).

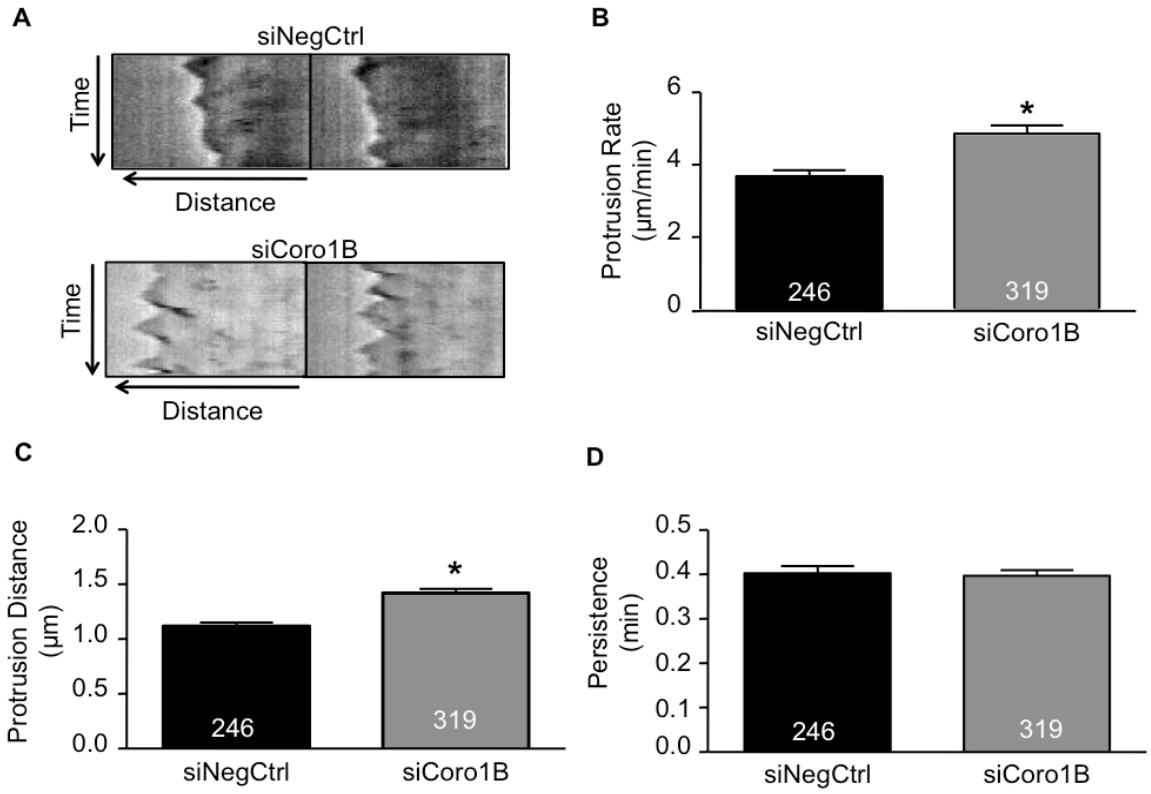


Figure 2.13 Downregulation of Coro1B Increases Lamellipodia Protrusion Rate and Distance in VSMCs. (A) Kymographs of cells transfected with siNegCtrl or siCoro1B and stimulated with 10 ng/ml PDGF were generated by taking images of cells containing lamellipodia every 4 seconds for 4 minutes. Images were stacked into movies and then converted to minimal intensity projections. Pixel intensities along a 1-pixel width line drawn through the lamellipodia were used to create kymographs. X-axis represents distance and y-axis represents time. Graphs represent protrusion rates **(B)**, persistence **(C)**, and distance **(D)** of lamellipodia in VSMCs transfected with siNegCtrl or siCoro1B. The graphs represent the mean ± SEM values. The N (number of events measured) is indicated within each column. (*p< 0.001, siCoro1B compared to siNegCtrl).

(1.4 ± 0.04 μm v. 1.1 ± 0.03 μm, p< 0.0001 siCoro1B vs siNegCtrl) (Figure 2.13C) when compared with siNeg Ctrl transfected samples. However, inhibition of Coro1B expression did not significantly affect PDGF-induced changes in lamellipodia persistence (Figure 2.13D). These changes in lamellipodia dynamics correspond to the changes that were observed in VSMC distance traveled and velocity (Figure 2.4A and B), supporting the notion that in VSMCs Coro1B negatively regulates migration. As a result, we next began to examine how PDGF could modulate Coro1B in VSMCs.

2.3.6 PDGF Stimulation of VSMCs Induces Coronin 1B Serine 2 Phosphorylation

To begin to understand how PDGF might regulate Coro1B activity, we focused on phosphorylation. Data presented by Cai *et al* (140). demonstrated that phosphorylation of Coro1B on Ser-2 can be stimulated by phorbol esters in Rat-2 fibroblasts, although whether this occurs in response to a physiological agonist is not known. Additionally, Rat-2 fibroblast migration is inhibited by PMA; thus, the phosphorylation response of Coro1B in these cells is negative and this response may not occur in VSMC treated with a promigratory agent such as PDGF. However, in VSMCs PMA stimulates migration and therefore Coro1B phosphorylation, if it occurred in VSMCs, could be promigratory. For this reason we hypothesized that PDGF would stimulate Coro1B phosphorylation on Ser-2 (p-Ser2 Coro1B) in a PKC-dependent manner. In initial studies we detected the serine phosphorylation of Coro1B in VSMCs stimulated with PDGF as early as 1 min (data not shown). The phosphorylation response peaked at 5 minutes (1009 ± 95% above basal) and was maintained for at least 30 minutes (Figure 2.14A). To verify the specificity of the antibody, siCoro1B transfection was used to deplete Coro1B in these cells. When Coro1B levels were reduced by ~50%, the p-Ser2 Coro1B signal after stimulation with PDGF was abolished,

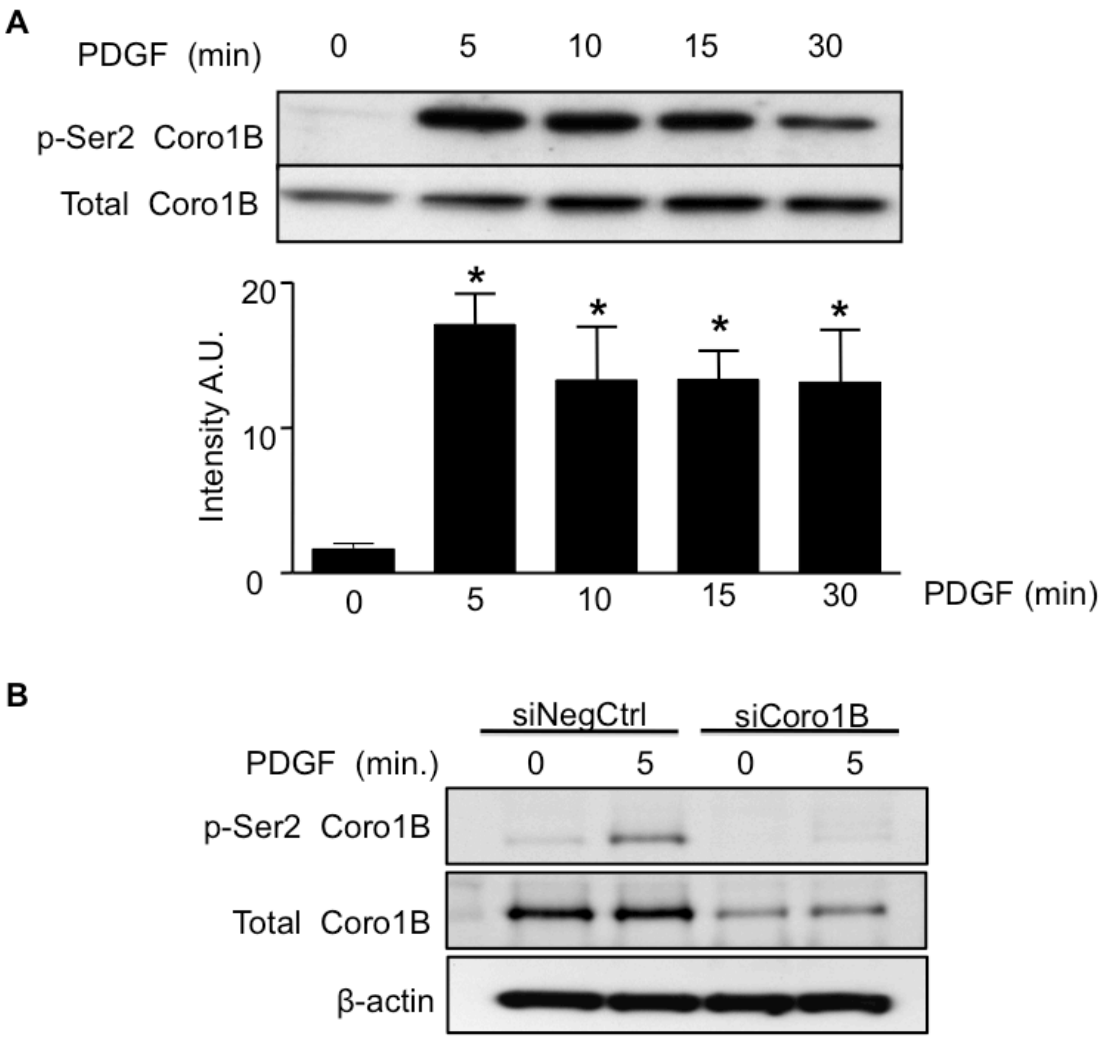


Figure 2.14 PDGF Stimulates Coro1B Ser-2 Phosphorylation in VSMCs and siRNA Against Coro1B Attenuates the Signal. (A) VSMCs were serum starved for 48 hours, and then stimulated with 10 ng/ ml PDGF for the indicated times. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibodies. The graph represents the mean ± SEM of densitometric analysis from 3-5 independent experiments. (*p< 0.01, compared to control). **(B)** VSMCs were transfected with siCoro1b or siNegCtrl via Amaxa electroporation and then serum starved for 48hrs. Cells were then stimulated with 10 ng/ml PDGF for 5 minutes. PDGF-induced Coro1B phosphorylation was markedly reduced in siCoro1B treated cells. (N=3)

demonstrating that the antibody targets Coro1B specifically and that the phosphorylated pool of Coro1B is likely only a small fraction of the total (Figure 2.14B). Coro1B phosphorylation in response to PDGF stimulation in HCoASMC was also determined. Coro1B p-Ser2 occurred in a similar manner as in VSMC, suggesting that cells from multiple vascular beds respond in a similar fashion (Figure 2.15).

The localization of p-Ser2 Coro1B was then determined. When cells were stimulated with PDGF for 15 minutes there was an increase in p-Ser2 Coro1B staining when compared to unstimulated cells (Figure 2.16). When siCoro1B transfected cells were stimulated with PDGF, p-Ser2 Coro1B detection was decreased by immunofluorescence demonstrating specificity of the signal (Figure 2.17). Ser-2 phosphorylated Coro1B stained in a stress fiber pattern (Figure 2.17) as well as the cell periphery and lamellipodia (Figure 2.16 and 2.17). Incidentally, p-Ser2 Coro1B also exhibited a somewhat diffuse distribution in the cytosol. Remarkably, p-Ser2 Coro1B nuclear staining was noted in both basal and PDGF treated samples, suggesting that Coro1B may also have a nuclear function as well.

2.3.7 PDGF-stimulates Coro1B Serine Phosphorylation via PKCε

Since PDGF was able to stimulate Coro1B phosphorylation, we then examined the upstream kinase pathway involved. Previously, in gut parietal cells and Rat-1 cells Coro1B phosphorylation was induced by PMA (140, 193). PMA is a known classical and novel PKC agonist. Hence, we began by testing the hypothesis that PKC is a possible upstream kinase for Coro1B phosphorylation. To determine if the phosphorylation of Coro1B is mediated by PKC, we used the pan-PKC inhibitor Rö-32-0432 (0.5 μM), as well as more selective inhibitors, PKC-β (10 μM) inhibitor and Gö-6976 (0.5 μM). Rö-

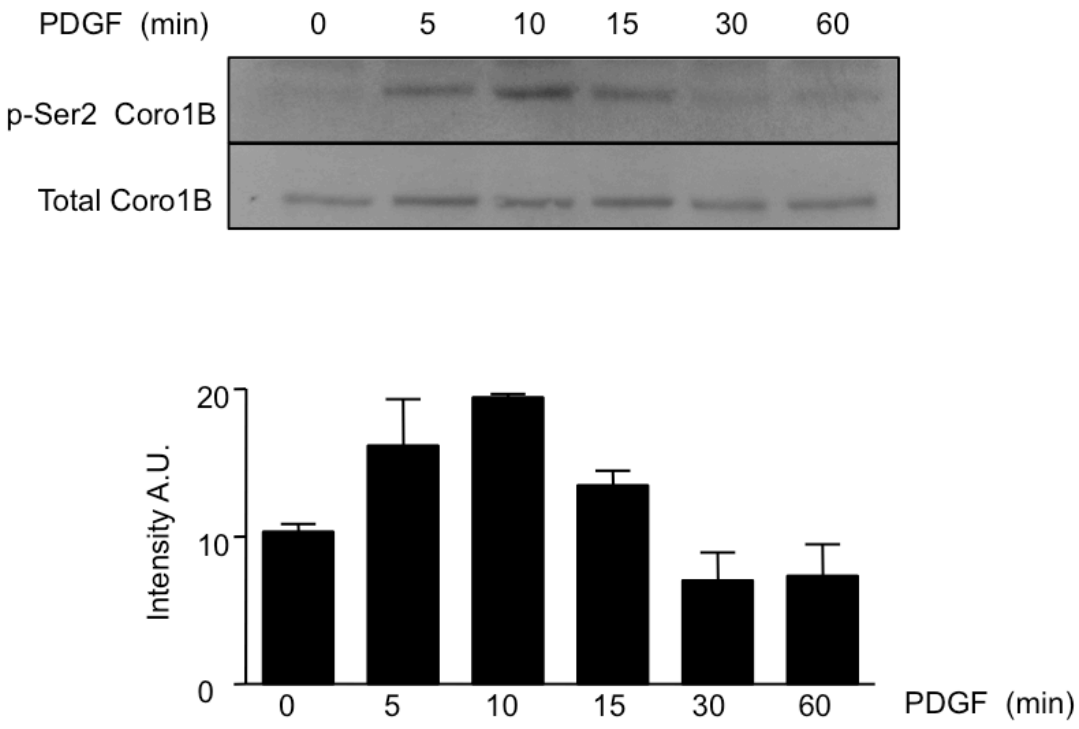


Figure 2.15 PDGF Stimulates p-Ser-2 Cor1B in HCoASMC. HCoASMCs were serum starved for 48 hours, and then stimulated with 10 ng/ ml PDGF for the indicated times. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibodies. (N=2.)

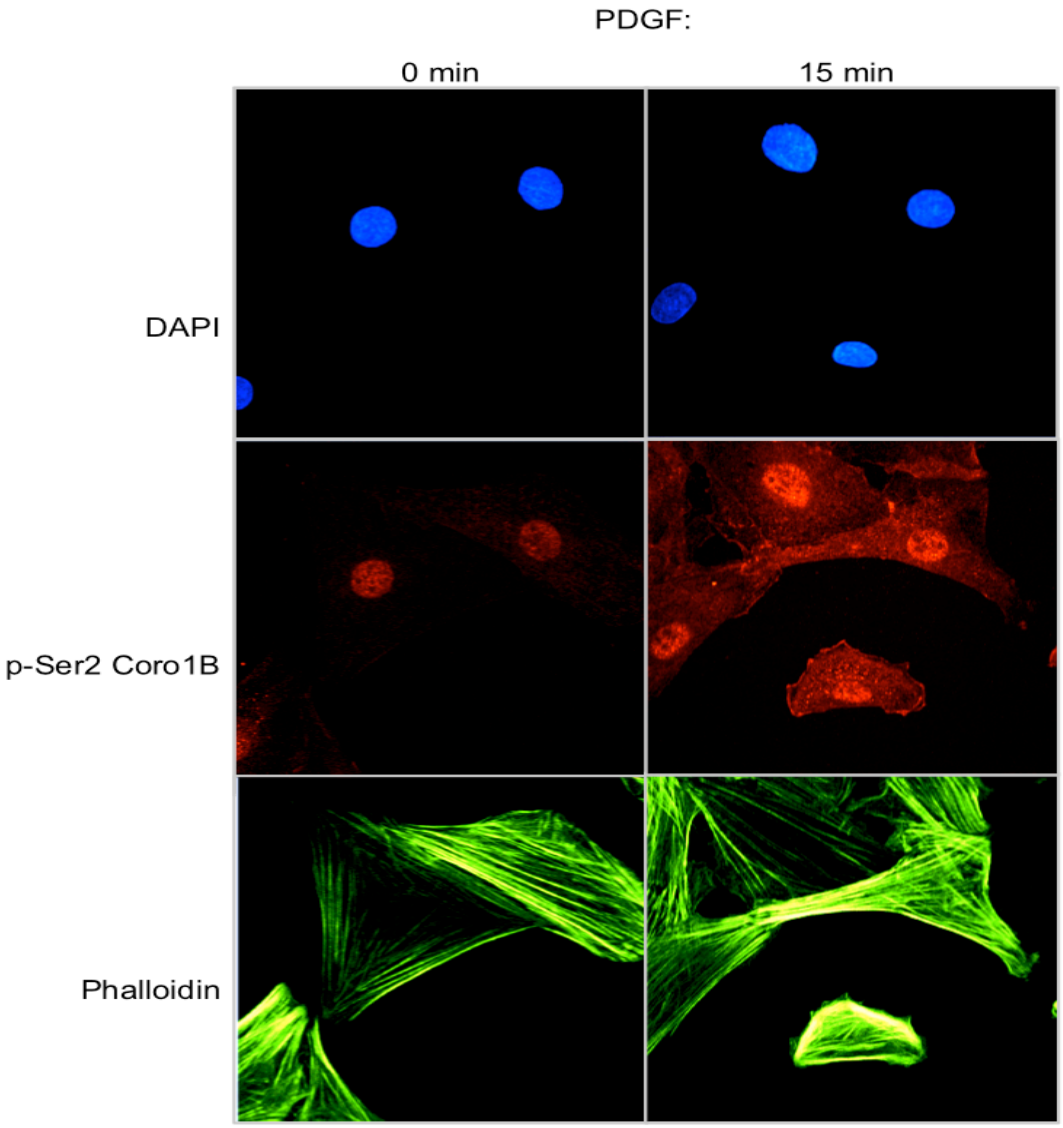


Figure 2.16 p-Ser2 Coro1B Localizes to the Cell Periphery and Cytoplasm in VSMCs. VSMCs were serum starved for 48 hrs, stimulated with 10ng/ml PDGF for 15 minutes and then fixed and permeabilized. Immunofluorescent images using DAPI (blue), phalloidin (green) and p-Ser2 Coro1B antibody (red) were then acquired using the Zeiss LSM 510 META Laser Scanning Confocal Microscope (63x).

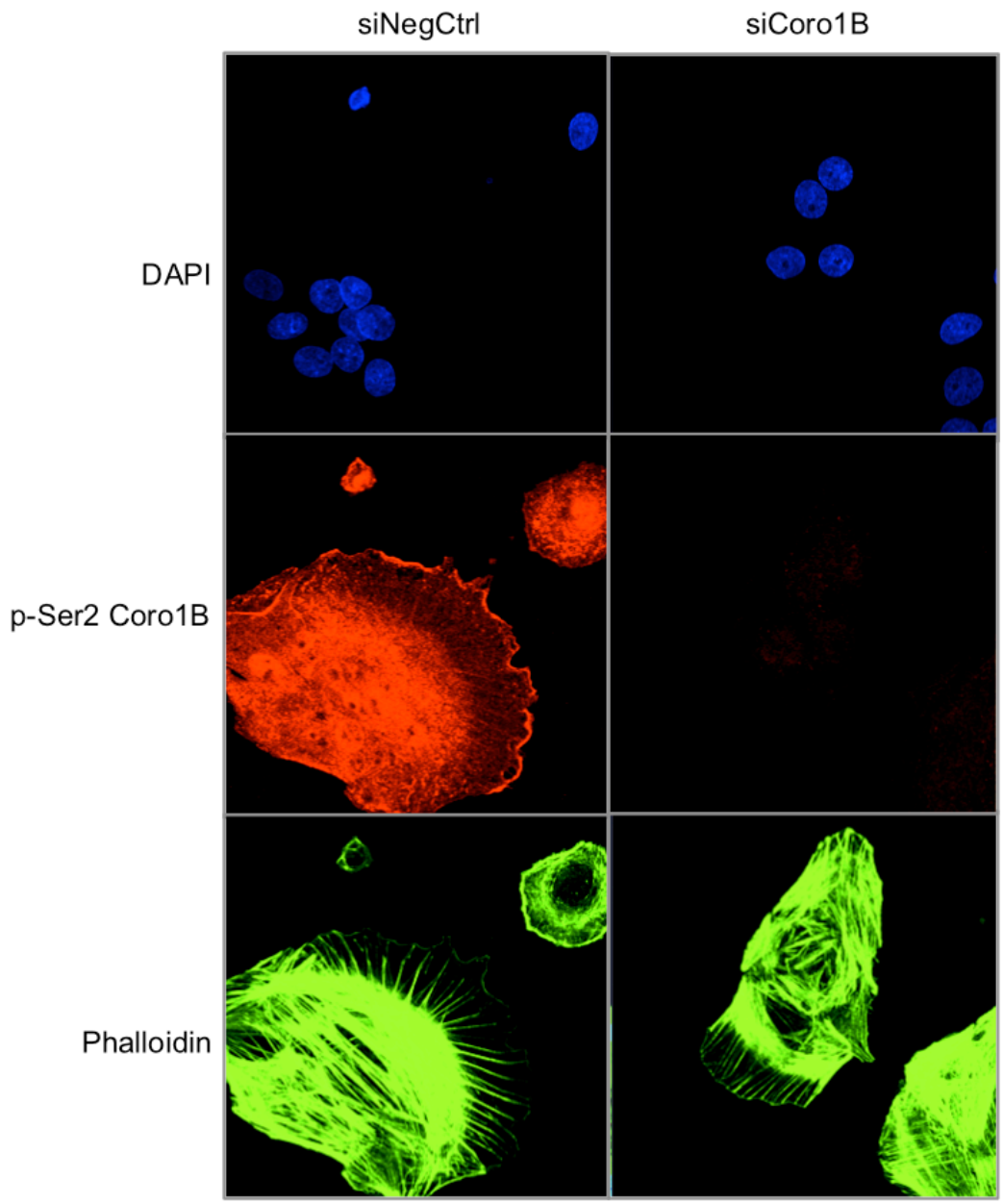


Figure 2.17 siRNA Against Coro1B Attenuates p-Ser2 Coro1B Detection by Immunofluorescence. Confocal images were acquired after transfecting VSMCs with siNeg Ctrl or siCoro1B using the Amaxa electroporation system. Cells were serum starved for 48 hrs, stimulated with 10 ng/ml PDGF and then fixed and permeabilized. Immunofluorescence of p-Ser2 Coro1B (red), DAPI (blue) and phalloidin (green) was then detected using the Zeiss LSM 510 META Laser Scanning Confocal Microscope (63X) (N=2).

32-0432 reduced PDGF- induced Coro1B phosphorylation by 88 ± 10% compared to control treated samples (Figure 2.18 A). For the PKCβ inhibitor, a dose response curve was created (data not shown); however, PDGF-induced Coro1B phosphorylation was not inhibited until a concentration of 10 μM was used (Figure 2.18B). Classical PKCs are inhibited at concentrations below 1 μM. The Furthermore, Gö-6976 had no effect on PDGF-induced p-Ser2 Coro1B (Figure 2.19 A). We then used this information to narrow down the identity of possible PKC isoforms involved in PDGF-induced Coro1B phosphorylation (Figure 2.19B). Based on reported IC50s and the inhibition profiles we observed, it was clear that the classical PKCs, PKCα and β, were not required for PDGF-induced Coro1B Ser2 phosphorylation. Subsequently, we examined PKCs that had known cytoskeletal substrates and found PKCε to be a viable candidate (241, 242). When siRNA against PKCε was transfected into VSMCs, Coro1B phosphorylation was markedly reduced (40 ± 3.7%) compared to PDGF stimulated siNeg Ctrl (Figure 2.20A). As another control we also used siRNA against PKCα to confirm our inhibitor studies. As expected, Coro1B phosphorylation was not affected by downregulation of this isoform (Figure 2.20B). In all, these data suggest that PKCε phosphorylates Coro1B in response to PDGF stimulation in VSMCs.

2.3.8 Coronin 1C is Not Phosphorylated by PKC in Response to PDGF in VSMCs

Although Coro1C expression did not modulate PDGF-induced migration, it is still possible for PDGF to modify the protein. Both Coro1A and 1B are phosphorylated by PKC at the conserved Ser-2 position. However, Coro1C does not contain this conserved motif. Nevertheless, it is possible that Coro1C could be phosphorylated at another serine. To determine if Coro1C could be phosphorylated in a p-SerPKC-dependent fashion similar to other type I coronins, the Human Protein Reference Database

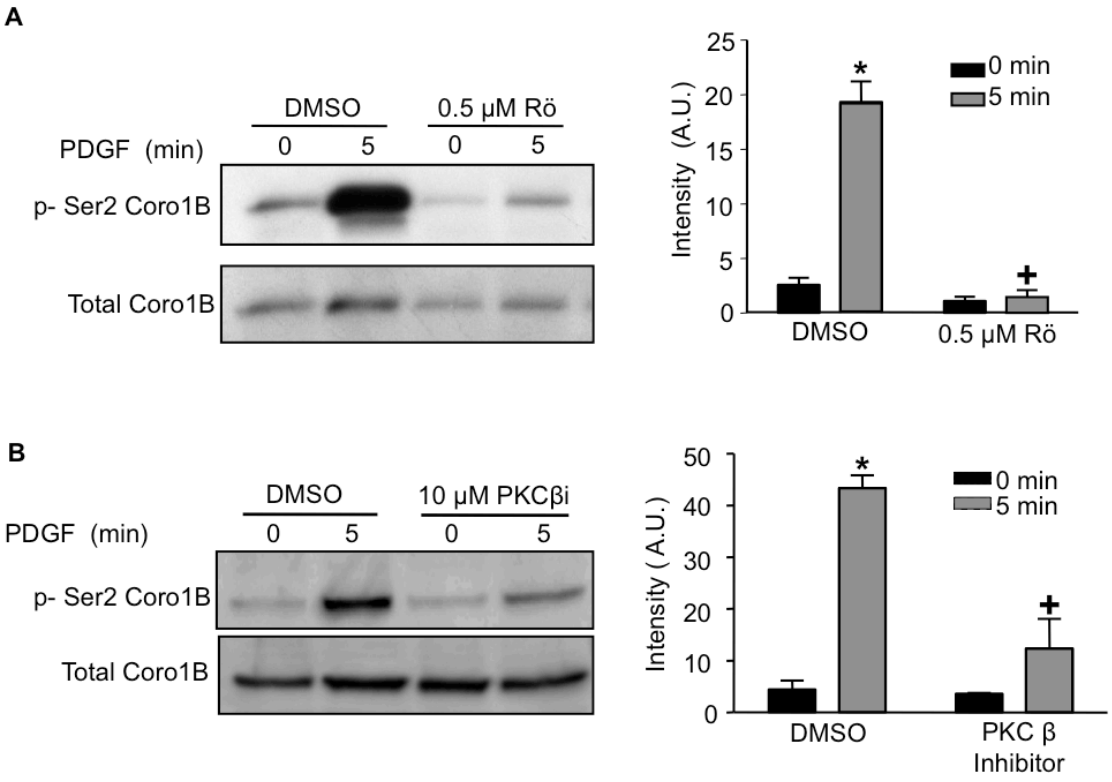
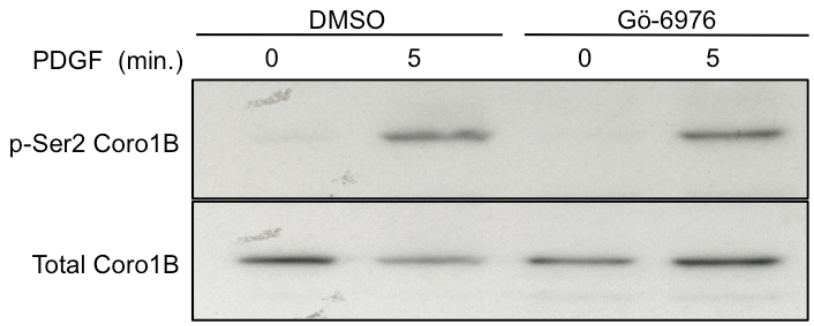


Figure 2.18 Pretreatment With PKC Inhibitors Ro-32-0432 and PKC β Inhibitor Attenuates Coro1B Phosphorylation. (A) VSMCs were serum starved for 48 hours, and then pre-incubated with 0.5 μM Ro-32-0432 (PKC inhibitor) or vehicle for 30 minutes. The cells were then stimulated with 10 ng/ml PDGF for 5 minutes. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibody. The graph represents the mean ± SEM of densitometric analysis from 4 independent experiments. (*p< 0.01 compared to control, **†** p<0.0001 compared to control PDGF treated). **(B)** VSMCs were serum starved for 48 hours, and then pre-incubated with 10 μM PKC β Inhibitor or vehicle for 30 minutes. The cells were then stimulated with 10 ng/ml PDGF for 5 minutes. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibody. The graph represents the mean ± SEM of densiometric analysis from 4 independent experiments. (*p< 0.01 compared to control, **†** p<0.05 compared to control PDGF treated).

A



B

	IC_{50}		
PKC Isoform	**Rö-32-0432 (μM)**	**PKCβ inhib. (μM)**	**Gö 6976 (μM)**
α	0.009	0.331	.0023
β	0.028	0.021	0.006
γ	0.037	>1.0	-
ε	0.108	2.8	-

Figure 2.19 Classical PKC Inhibition Does Not Inhibit Coro1B Ser2 Phosphorylation. (A) VSMCs were serum starved for 48 hours, and then pre-incubated with 0.5 μmol/L Gö-6796 (PKC inhibitor) or vehicle for 30 min. The cells were then stimulated with 10 ng/ml PDGF for 5 minutes. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibodies (N=2). **(B)** List of PKC inhibitor IC50s for specific isoforms.

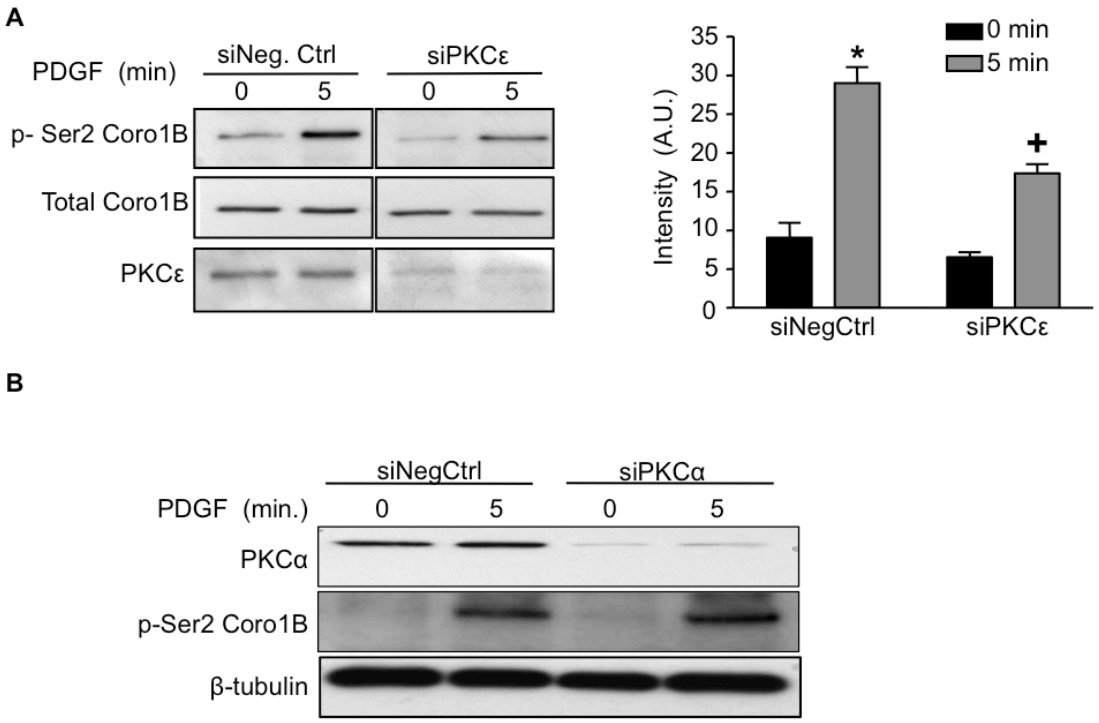


Figure 2.20 siRNA Against PKCε, but not PKCα, Attenuates Coro1B Phosphorylation. VSMCs were transfected with **(A)** siNegCtrl or siPKCε using the Amaxa electroporation system. Cells were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF for 5 minutes. Cell protein lysates were harvested and immunoblotted with p-Ser2 Coro1B, Coro1B and PKCε antibodies. Blots presented are from the same gel, but unrelated lanes were removed for clarity. The graph represents the mean ± SEM of densitometric analysis from 4 independent experiments. (*p< 0.0001, siNeg Ctrl vs. PDGF stimulated siNegCtrl, and **†** p< 0.01, PDGF stimulated siNegCtrl vs, PDGF stimulated, siPKCε transfected). **(B)** siNegCtrl or siPKCα using the Amaxa electroporation system. Cells were serum starved for 48 hours and then stimulated with 10 ng/ml PDGF for 5 minutes. Cell protein lysates were harvested and immunoblotted with p-Ser2 Coro1B, PKCα and β-tubulin antibodies. (N=2)

Phosphosite Prediction program and the NetPhosK 1.0 server were used (243-246). There were 13 predicted p-SerPKC sites that were conserved between rat and human genomes (Figure 2.21). To determine if PDGF stimulates Coro1C at any of these sites, cells were stimulated with PDGF, and Coro1C was immunoprecipitated. Pulled-down protein was then immunoblotted with a p-SerPKC motif antibody. This antibody recognizes phosphorylation of serine residues surrounded by Arg or Lys at the -2 and +2 positions and a hydrophobic residue at the +1 position. However, PDGF stimulation did not stimulate serine phosphorylation of Coro1C (Figure2.22 A). In the same samples Coro1B Ser2 phosphorylation was measured as a control for PDGF stimulation (Figure 2.22B). In short, PDGF does not modify Coro1C via PKC serine phosphorylation, which may in part explain why Coro1C is not involved in migration.

2.3.9 Phosphorylation Deficient Coro1B Mutant Decreases VSMC Migration

Next, we assessed the importance of Coro1B phosphorylation state on PDGF-induced VSMC migration. We transfected the phospho-deficient S2A mutant into VSMCs and measured its effects on PDGF-induced cell migration. Using the modified Boyden chamber assay, we observed a reduction in cell migration when cells were transfected with the Coro1B S2A mutant (91 ± 10%) compared to empty control vector transfected cells (Figure 2.23A). To confirm expression and correct targeting and localization of mutant Coro1B S2A, we used western analysis and immunocytochemistry. We observed that Coro1B S2A was expressed at or above endogenous Coro1B levels (Figure 2.23B). Coro1B S2A had a similar localization pattern as Coro1B WT (Figure 2.24), although Coro1B S2A mutant had less defined peripheral staining. Together, these data demonstrate that the phosphorylation state and expression of Coro1B is an important factor in the induction of PDGF mediated VSMC migration. Accordingly, we began to

1 mrrvvrqskf rhvfgqavkn dqcyddirvs rvtwdssfca vnprfvaiii easgggaflv

61 lplhktgrid ksyptvcght gpvldidwcp hndqviasgs edctvmvwqi pengltlslt

121 epvvilegh s krvgivawhp tarnvllsag cdnaiiiwnv gtgealinld dmhsdmiynv

181 swsrngslic taskdkkvrv idprrqeiva ekekahegar pmraifladg nvfttgfsrm

241 serqlalwnp knmqepialh emdtsngvll pfydpdtsii ylcgkgdssi ryfeitdesp

301 yvhylntfss kepqrgmgym pkrgldvnkc eiarffklhe rkcepiimtv prksdlfqdd

361 lypdtagpea aleaeewfeg knadpilisl khgyipgknr dlkvvkknil dskpaankks

421 dlicapkktt dtasvqneak ldeilkeiks ikdticnqde riskleqqla kmaa

Figure 2.21 Coro1C Rat Protein Sequence and Predicted pSerPKC Sites. The human and rat Coro1C protein sequence were entered into the Human Protein Reference Database Phosphosite Prediction program and NetPhosK 1.0 server. There were 13 predicted p-SerPKC sites that were conserved between rat and human genomes.

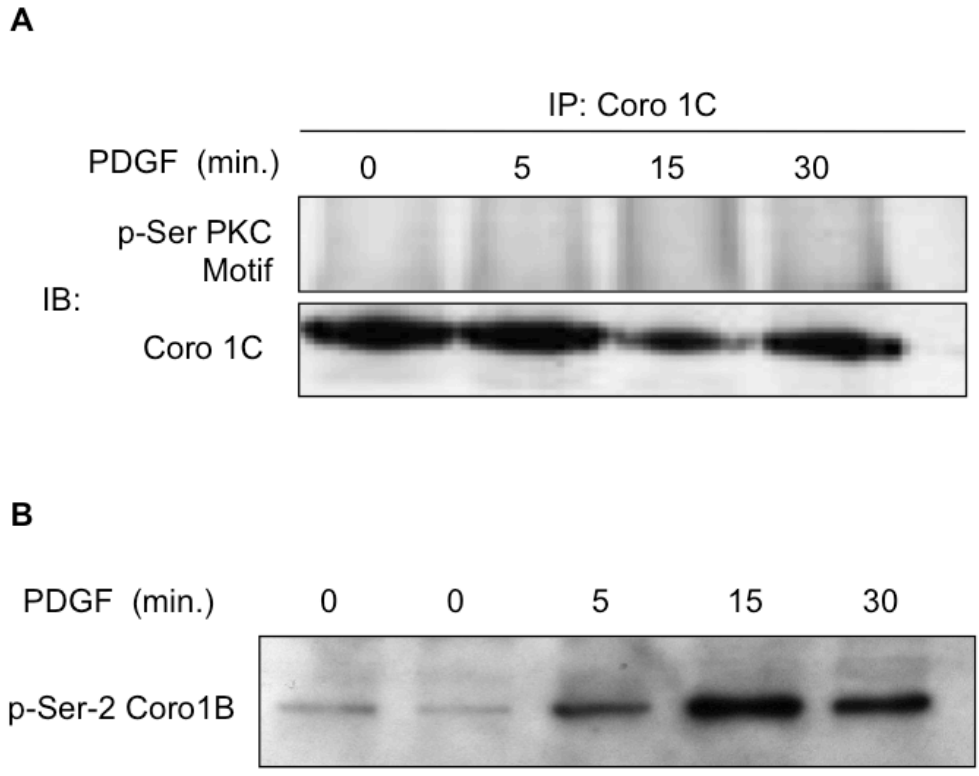


Figure 2.22 Coronin 1C is Not Phosphorylated by PKC on Serine Residues in Response to PDGF in VSMCs (A) VSMC were serum starved for 48 hrs and then stimulated with 10ng/ml PDGF. Cell lysates were harvested and immunoprecipitated with Coro 1C antibody. Membranes were immunoblotted with Coro1C and pSerPKC motif antibody. **(B)** Lysates from **A**, were immunoblotted with p-Ser2 Coro1B (representative blot, N=3).

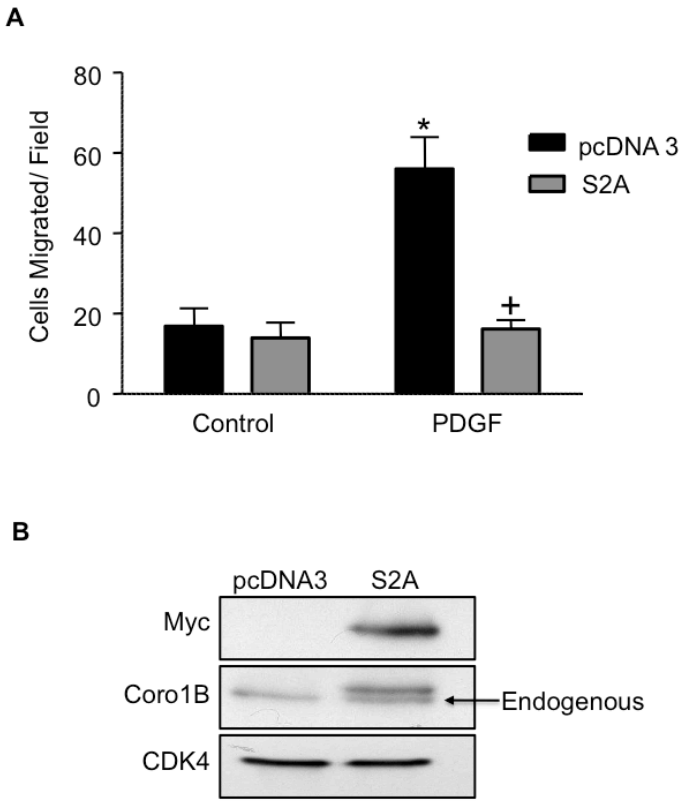


Figure 2.23 Phospho-deficient Coro1B S2A Mutant Inhibits PDGF-induced VSMC Migration. VSMCs were transfected with the Myc tagged S2A Coro1B phosphodeficient mutant using the Amaxa electroporation system and then serum starved for 24hrs. **(A)** Cells were harvested and migration was assessed via the modified Boyden chamber assay**.** This graph represents the mean ± SEM of the number of cells migrated per field (*p< 0.001, pcDNA3 vs pcDNA3 with PDGF stimulation, and **†** p < 0.001, PDGF stimulated pcDNA3 vs. PDGF stimulated Coro1B S2A mutant transfected cells, N=4). **(B)** Cells were harvested for protein. Lysates were immunoblotted for Coro1B, Myc, and CDK4 (Representative blot, N=4).

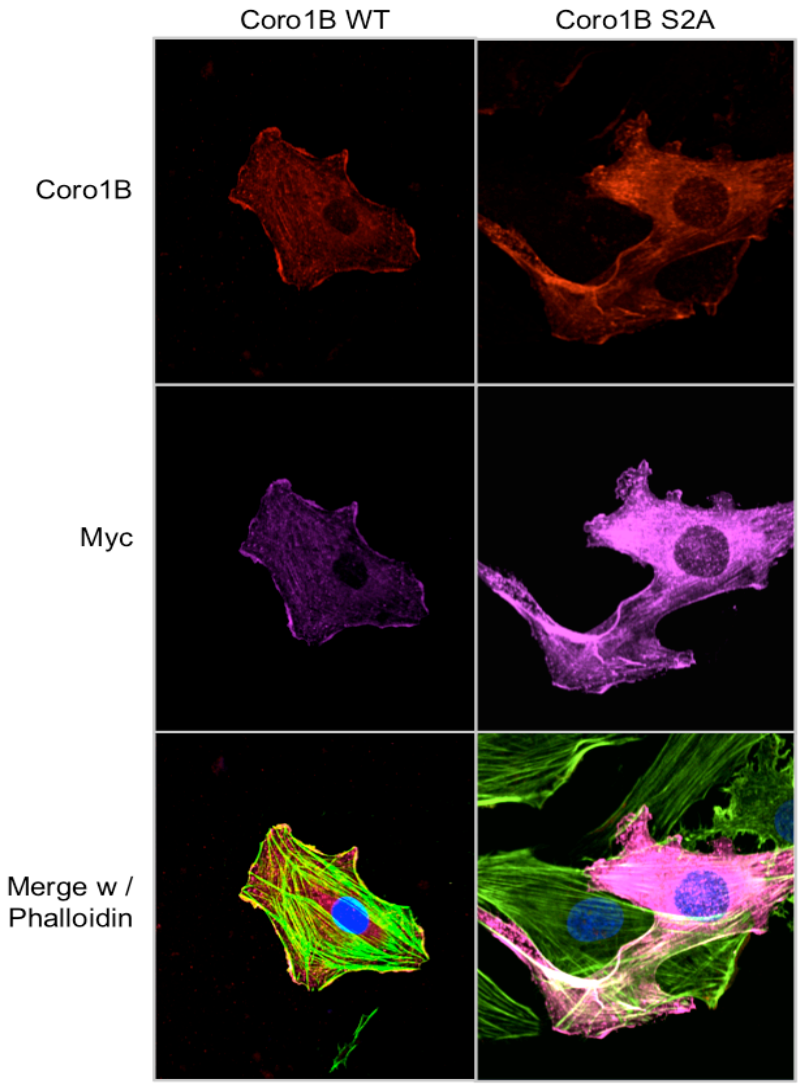


Figure 2.24 WT and S2A Coro1B Have Similar Localizations in VSMCs. VSMCs were transfected with Myc tagged WT or S2A Coro1B, serum starved for 24 hrs, then fixed and permeabilized. Immunofluorescent images using DAPI (blue), phalloidin (green), Coro1B antibody (red) and Myc antibody (magenta) were then acquired using the Zeiss LSM 510 META Laser Scanning Confocal Microscope (63x).

examine mechanistically how phosphorylation at this site regulates PDGF-induced VSMC migration.

2.3.10 Basal and PDGF-induced Changes in Coronin Protein Interactions

Coronins are a family of WD-repeat proteins, with a seven bladed β-propeller tertiary structure. Commonly this structure is necessary for interaction with protein complexes. Previous data demonstrate that coronins exert their effects through interaction with various proteins. Thus, we decided to examine the interaction of both Coro1B and 1C with various cytoskeletal proteins that were previously identified as coronin interacting partners, focusing on those known to regulate actin polymerization. In VSMCs Coro1C did not specifically interact with the Arp2/3 complex or cofilin (data not shown). Interestingly, we did find that Coro1B and Coro1C co-immunoprecipitated together in a PDGF-independent manner in VSMCs (Figure 2.25). Subsequently we tested whether Coro1B and Arp2/3 interact in VSMCs, and if this interaction could be disrupted by PDGF stimulation. Using co-immunoprecipitation, we observed a 45 ± 5 % and 51 ± 11% decrease in ArpC2 (a subunit of the Arp2/3 complex) and Coro1B interaction after 5 and 10 minutes of PDGF stimulation, respectively (Figure 2.26). The interaction of Coro1B with the Arp2/3 complex inhibits Arp2/3 actin nucleation activity (194), which can possibly negatively affect lamellipodia formation and migration. In contrast, we observed no specific interaction with the previously described binding partner SSH1L (data not shown). From these data we concluded that Coro1B and 1C exist in a complex independent of PDGF stimulation, which has not been previously reported. Furthermore, we demonstrated that PDGF could promote the dissociation Arp2/3 from an inhibitory

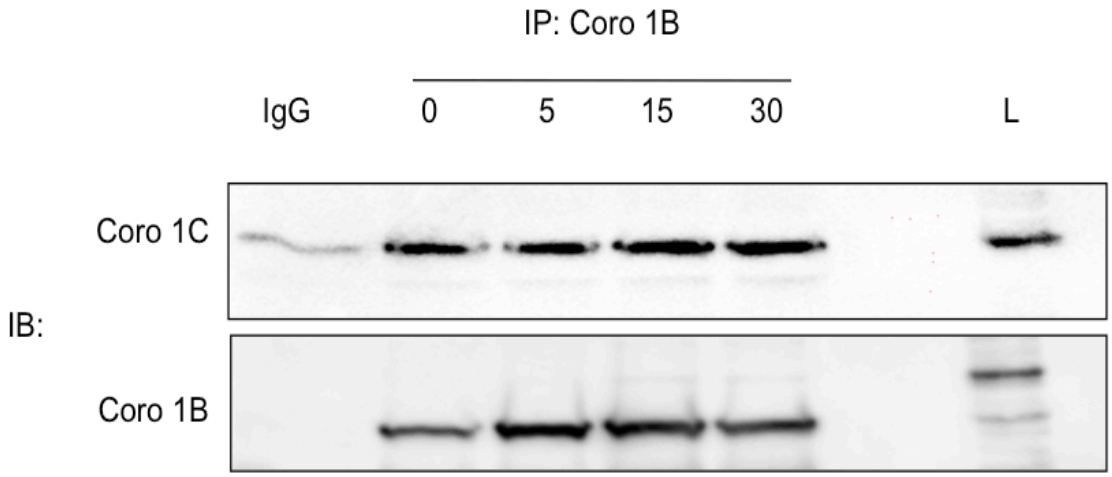


Figure 2.25 Coro1B and Coro1C Interactions are Not Regulated by PDGF in VSMCs. VSMCs were serum starved for 48hrs and then treated with 10 ng/ml PDGF for the indicated times. Cell lysates were harvested and immunoprecipitated with Coro1B antibody. Membranes were immunoblotted with Coro1B and Coro1C antibodies (Representative blot, N=3). L=lysate; IgG=IgG control IP.

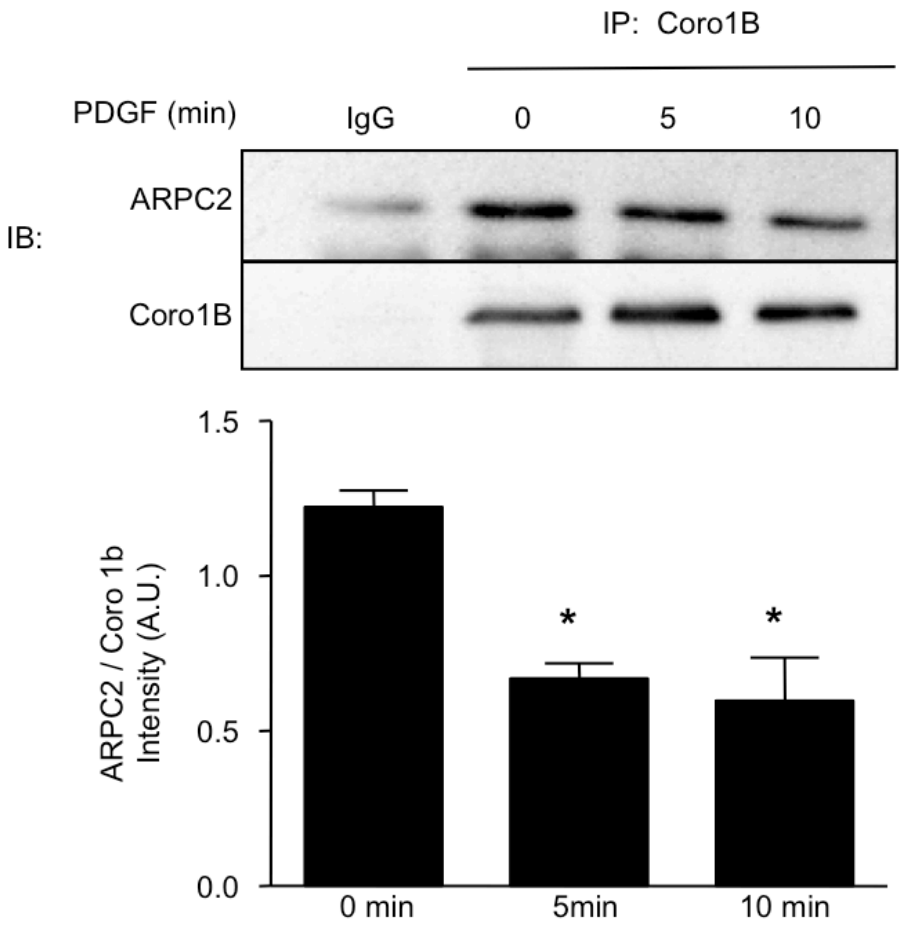


Figure 2.26 PDGF Stimulates Dissociation of Coro1B from the Arp2/3 Complex in VSMCs. VSMCs were serum starved for 48 hrs and then treated with 10 ng/ml PDGF for the indicated times. Cell lysates were harvested and immunoprecipitated with Coro1B antibody. Membranes were immunoblotted with Coro1B and ARPC2. The graph represents the mean ± SEM of densitometric analysis from 3-4 independent experiments. Data are plotted as total ARPC2/ Coro1B. (*p< 0.05 compared to control).

complex with Coro1B in VSMC.

2.3.11 Phosphorylation of Coronin1B Disrupts its Interaction with the Arp2/3 Complex

The inhibition of Arp2/3 and Coro1B interaction occurred during the time of peak PDGF induced Coro1B phosphorylation (Figure 2.14). To determine whether Coro1B phosphorylation is important for its interaction with the Arp2/3 complex in VSMC, we transfected cells with Myc tagged Coro1B WT, phospho-deficient Coro1B S2A mutant and phospho-mimetic Coro1B S2D. The phospho-mimetic S2D mutant of Coro1B showed 48 ± 7% less co-immunoprecipitation with the Arp2/3 complex subunit ArpC2, than did the phospho-deficient mutant S2A (Figure 2.27). Overexpression of Coro1B WT trended toward a decrease in interaction with the Arp2/3 complex when compared to the Coro1B S2A mutant, although it did not meet statistical significance. We found this was most likely due to the increased basal phosphorylation of the overexpressed protein (Figure 2.28). These data suggest that the function of Coro1B phosphorylation is to negatively regulate its interaction with the Arp2/3 complex, the most important modulator of actin protrusion in lamellipodia. These data also explain why the Coro1B S2A mutant inhibits PDGF-induced VSMC migration.

2.3.12 Coronin 1B is Phosphorylated in the Neointima After Mouse Carotid Artery Wire Injury and Rat Carotid Balloon Injury

Lastly, we examined the expression pattern of Coro1B phosphorylation *in vivo* in response to arterial injury to verify that the changes we observed *in vitro* reflect those that occur when cells are induced to migrate and proliferate in vivo. As shown in Figure 2.29, we examined the level of Coro1B phosphorylation in response to carotid wire injury in C57BL/6 mice. Mice were subjected to left carotid wire injury and then sacrificed 7 or

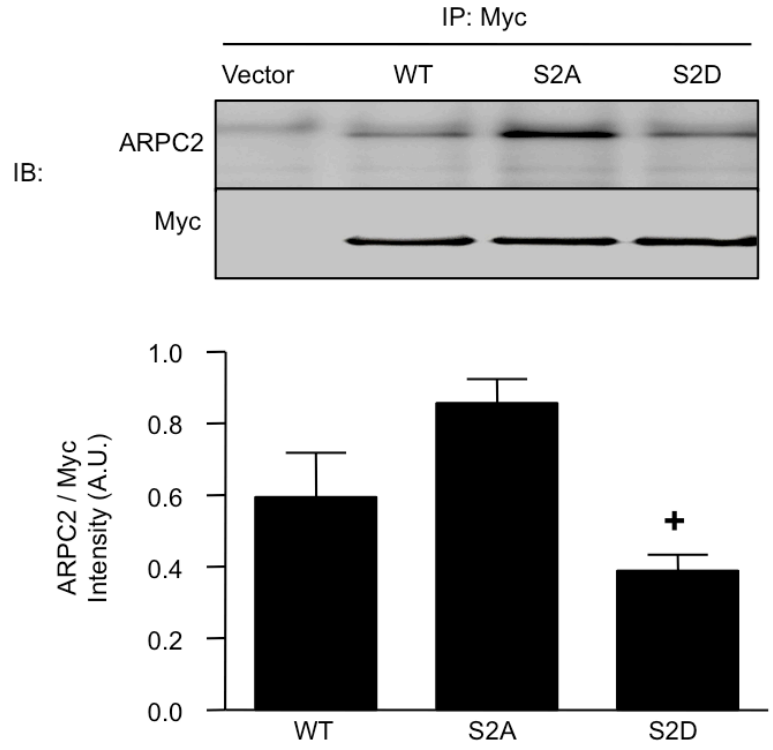


Figure 2.27 ARP2/3 subunit ARPC2 and Coro1B Interactions are Negatively Regulated by PDGF in VSMCs. VSMCs were transfected with the Myc tagged Coro1B WT, the S2A Coro1B phospho-deficient mutant, or the S2D Coro1B phospho-mimetic mutant using the Amaxa electroporation system and then serum starved for 24hrs. Cell lysates were harvested and immunoprecipitated with Myc antibody. Membranes were immunoblotted with anti-Myc and ARPC2. The graph represents the mean ± SEM of densitometric analysis from 4 independent experiments. Data are plotted as total ArpC2/Myc. (+ $p < 0.05$ compared to S2A).

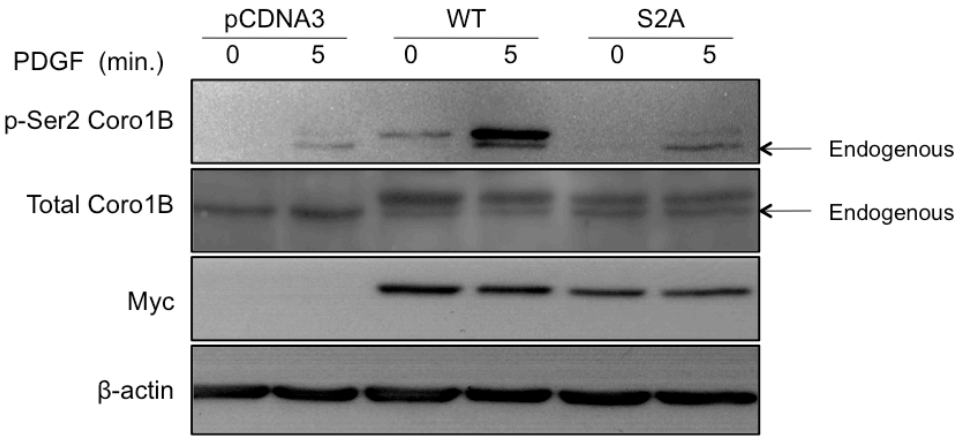


Figure 2.28 Coro1B WT Overexpression Increases Basal Phosphorylation and Coro1B S2A Mutant Overexpression Does Not Inhibit Endogenous PDGF-induced Coro1B Phosphorylation. VSMCs were transfected with empty vector (pcDNA3), Coro1B WT, or Coro1B S2A and then serum starved for 24hrs. Cells were then stimulated with 10 ng/ml PDGF for 5 minutes. Although Coro1B WT overexpression increased basal phosphorylation, cells transfected with either the mutant or wildtype Coro1B were still able to respond to PDGF.

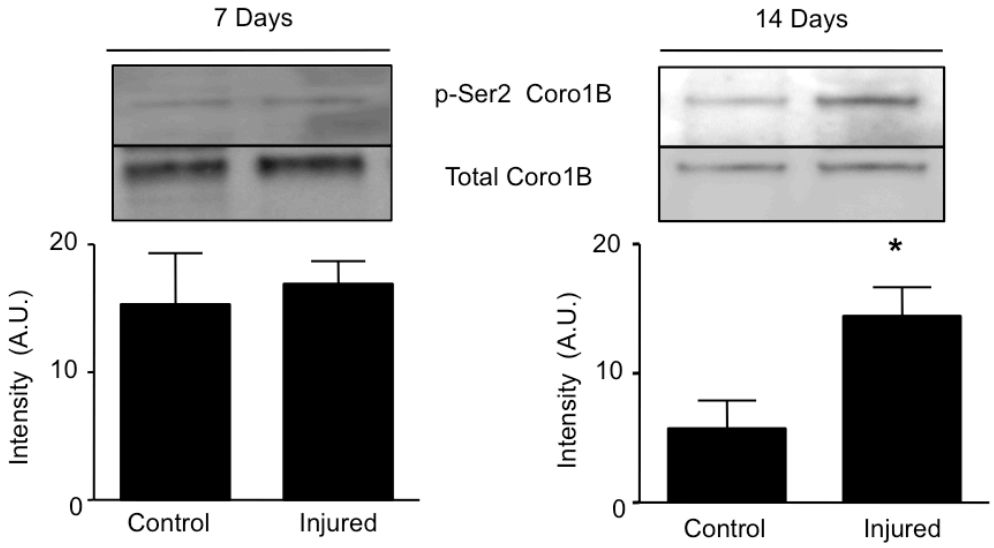


Figure 2.29 Coro1B is Phosphorylated in Response to Wire Injury in the Mouse Carotid Artery Injury. Mice were subjected to left carotid wire injury and then sacrificed 7 or 14 days post surgery. The left carotids were collected and the right carotid was used as a control. Tissue was harvested for protein and immunoblotted with p-Ser2 Coro1B and Coro1B antibodies (N=3 *p< 0.05 compared to control).

14 days post surgery. Right carotids were used as a control. At 7 days after surgery there was no significant change in p-Ser2 Coro1B levels in injured vessels compared to uninjured vessels (Figure 2.29A). However, at 14 days post surgery, there was a significant 63±11% increase in p-Ser2 Coro1B in injured vessels (Figure 2.29B). This suggests that pathways that lead to Coro1B phosphorylation are active in vivo. Next we examined the localization of phospho-Coro1B in the blood vessel. Sprague-Dawley rats were subjected to left common carotid artery injury or sham surgery and arteries were collected 10 days post injury. In the mouse, migration and proliferation begin 7 to 14 days post injury and are generally complete at 21 days (240). In the rat, the growth and migration of VSMCs into the neointima is generally complete at 14 days (39, 247). We chose 10 days in the rat to capture a similar condition as that in mice. p-Ser2 Coro1B was observed at low levels in the media of uninjured vessels (Figure 2.30A). In vessels from injured animals, there was significant staining in the neointima (Figure 2.30B). This staining pattern correlated closely to that of α-smooth muscle actin (Figure 2.30C and D). These data suggest that Coro1B phosphorylation *in vivo* occurs in cells that are phenotypically modulated to migrate and proliferate.

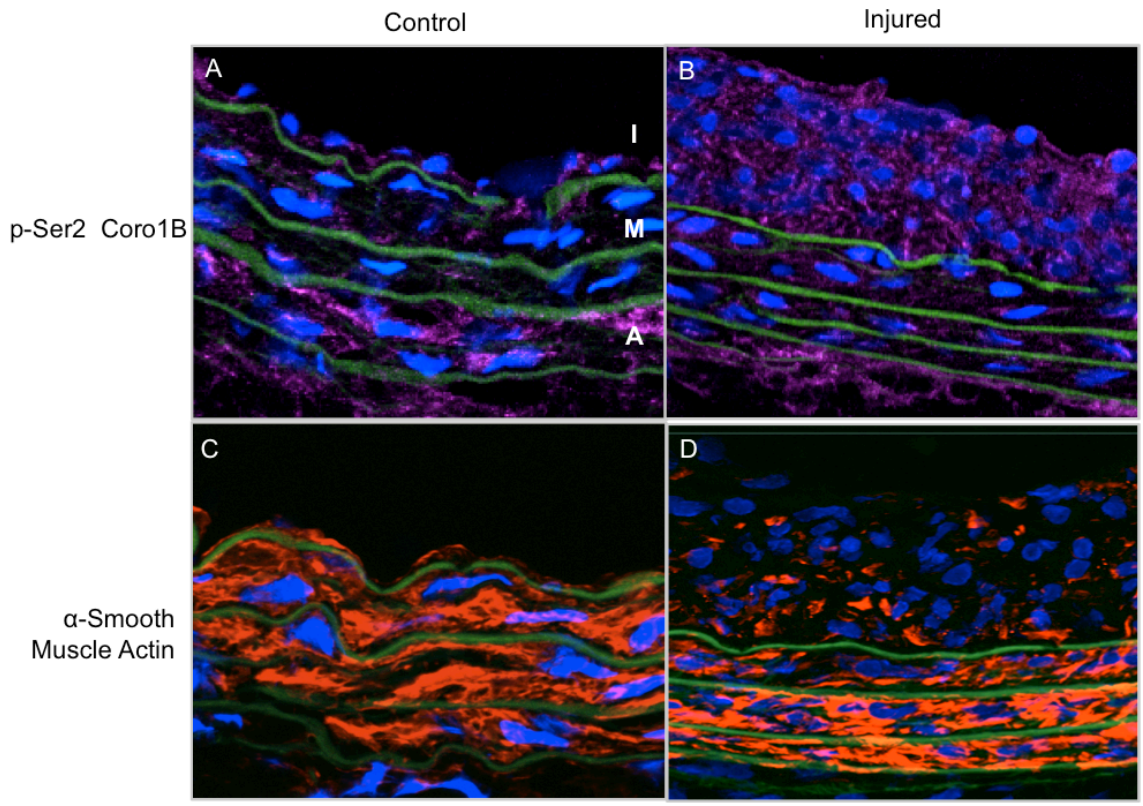


Figure 2.30 Coro1B is Phosphorylated in the Neointima After Rat Carotid Balloon Injury. Sprague-Dawley rats were subjected to balloon injury of the left common carotid artery. Ten days after injury, arteries were harvested, embedded in OTC and then cut into 7μm sections. Sections were stained for p-Ser2 Coro1B (magenta, **(A)** Control and **(B)** Injured) or α smooth muscle actin (red, **(C)** Control and **(D)** Injured) and nuclei (DAPI, blue**,** all panels). Green represents the autofluorescence of the internal elastic lamina. The blood vessel is labeled as follows; I denotes intimal region, M denotes medial region, and A denotes the adventitia.

2.4. Discussion

VSMC migration is important in both physiological and pathophysiological cellular processes. In atherosclerosis and restenosis after PCI, VSMCs migrate from the media to the intima of the blood vessel and this can eventually lead to occlusion. In vivo this process has been attributed to PDGF receptor-β activation by PDGF-BB. Here we describe a mechanism by which PDGF, acting through PKCε, regulates the phosphorylation of the actin binding protein Coro1B and its interaction with the ARP2/3 complex to modulate lamellipodia formation and migration. We demonstrate for the first time that both Coro1B and 1C are expressed in VSMCs of various lineages and that Coro1B is expressed *in vivo* in the vascular wall. In addition, we show that PDGF, a strong physiological migratory agonist, phosphorylates Coro1B on Ser-2, leading to a decrease in the interaction of Coro1B with the Arp2/3 complex. Dissociation of phospho-Coro1B from Arp2/3 releases an inhibitory effect on actin polymerization, resulting in an overall increase in lamellipodial protrusion and eventually migration.

Although the mechanisms regulating migration in different cell types are often similar involving lamellipodia formation and protrusion, focal adhesion turnover and contraction of the cell body, regulation of the actin cytoskeleton is complex. Actin polymerization in the lamellipodium is controlled by cofilin/slingshot-mediated actin depolymerization and Arp2/3/WAVE/WASP-stimulated actin extension and branching (127). A role for type I coronins in fine-tuning this process was only recently recognized. Originally identified in *Dictyostelium discoideum,* coronins make up a family of actin binding proteins that were found to be necessary for cytokinesis and cell migration in this organism (248, 249). In Rat-2 fibroblasts, Coro1B has been demonstrated to regulate actin polymerization via binding to and inhibiting Arp2/3 activity, and actin depolymerization by directing SSH1L to lamellipodia where SSH1L dephosphorylates and activates cofilin (194). SSH1L has been shown to dephosphorylate Coro1B as well

(194). However, in contrast to the present observations showing that Coro1B negatively regulates PDGF-induced lamellipodial formation and migration, previous investigators using Rat-2 fibroblasts and *Dictyostelium discoideum* found that Coro1B expression actually promotes migration and that cells deficient in Coro1B showed impaired migratory responses (194, 248). In Rat-2 cells, Coro1B inhibited the generation of free barbed end of actin filaments and coordinated actin filament assembly at the front of the lamellipodium. Using an in vitro actin polymerization activity, the authors showed that Coro1B WT and S2A, but not S2D, could inhibit ARP2/3 nucleation activity. They also found that Coro1B formed a complex with Arp2/3 and slingshot. This work was performed in unstimulated cells or in vitro. While our results support the idea that Coro1B phosphorylation regulates its interaction with Arp2/3 (Figure 2.26), we were unable to detect an interaction with slingshot phosphatase (unpublished observations). This difference might help to explain why we observed a negative regulation of migration by Coro1B, while it appears to be promigratory in Rat-2 cells. Experiments in our own laboratory have confirmed the positive role of Coro1B in transformed fibroblasts, but in general support a negative role for Coro1B in primary cells (both VSMCs and human dermal fibroblasts (Figure 2.6 and 2.7)). It should be noted that Rat-2 cells are an immortalized cell line with limited tumorgenicity (250), while the primary cells used here have no invasive potential. This suggests that cell transformation may influence the effects that Coro1B has on migration, and underlines the importance of investigating the molecular mechanisms in migration in multiple cell types, as they are obviously not identical. Moreover, in our studies we stimulated migration with PDGF, while motility in the Rat-2 cells was observed under basal conditions or in response to PMA stimulation. Furthermore, PMA treatment decreased Rat-2 fibroblast motility, where as VSMC migration has been reported to be stimulated by PMA (82). Thus, PKCs differentially regulate migration in these two cell types. Moreover, PDGF activates multiple signaling

pathways in addition to PKC, including calcium mobilization and activation of tyrosine kinase cascades, and it is the integration of those pathways that stimulates migration. Additional work will be necessary to identify all the factors that impinge on Coro1B to regulate the actin cytoskeleton.

Our examination of PDGF–induced changes in lamellipodia dynamics demonstrated that inhibition of Coro1B expression increases lamellipodial protrusion rate and protrusion distance, while not altering lamellipodial persistence (Figure 2.13). The amplitude of these changes was consistent with the amplitude of the change in velocity and migration distance, suggesting the changes in migration were due to a direct effect of Coro1B inhibition on lamellipodia formation. These data suggest that Coro1B acts by regulating the protrusion rate and distance that the lamellipodia protrude in VSMCs. The Arp2/3 complex is a known regulator of lamellipodia formation and is thought to regulate this structure by initiating actin polymerization and branching. Coro1B has been shown to a negative regulator of Arp2/3 complex activity (140, 141), and our data support that notion.

Previously, the phosphorylation of Coro1B at Ser-2 was only observed after PMA treatment of cells. Here, we describe a physiological agonist that induces this phosphorylation (Figure 2 .14). We have also shown that Coro1B phosphorylation and expression is regulated *in vivo* (Figure 2.29 and 2.30). Importantly, we identified PKCε as a PKC that phosphorylates Coro1B in response to PDGF. It is noteworthy that the data presented here identify a new PKCε substrate. Deuse et al (228) demonstrated in a rat model of balloon injury with stenting that restenosis was significantly reduced after treatment with a PKCε inhibitor (εV1-2) compared with saline. While PKCε has been shown to play a pivotal role in migration of VSMCs (251), how it does so remains to be fully explored. Of relevance, PKCε has been shown to phosphorylate other cytoskeleton associated proteins such as connexin 43 in cardiomyocytes (241), cytokeratins 8 and 18

(242), as well as diacylglycerol kinase (252). Our study shows that PKCε may also act through Coro1B to regulate cytoskeletal remodeling.

In *in vivo* studies, we demonstrated that Coro1B phosphorylation is stimulated in response to vessel injury in both the rat and mouse. These data show that Coro1B phosphorylation occurs under pathophysiological conditions associated with cell migration. In both the rat carotid balloon and mouse carotid wire injury models, Coro1B was phosphorylated at time points during which cells are actively migrating into the intima. This increased phosphorylation of Coro1B is a positive stimulus for migration by decreasing its inhibitory interaction with the Arp2/3 complex. These data support the idea that Coro1B phosphorylation may be necessary for VSMC migration and neointimal formation. However, further investigation will be necessary to establish a causal role for Coro1B in neointimal formation. In future studies the effects of Coro1B knockout and overexpression should be evaluated to more thoroughly investigate the role of Coro1B in neointimal formation and vessel remodeling.

Although Coro1C was expressed in VSMCs, it did not modulate PDGF-induced VSMC migration (Figure 2.4). However, we did observe an interaction between Coro1B and Coro1C (Figure 2.25). This interaction remained stable when cells were treated with PDGF. These proteins are thought to form homotrimers through their coiled-coil domain. In overexpression studies of tagged proteins, Coro1B was discovered to only interact with itself (140). In contrast, we found that Coro1B and 1C co-immunoprecipitate in VSMCs. One possible explanation for these disparate results is that the tag on the proteins hinders their interaction. Alternatively, the interaction between Coro1B and Coro1C could be indirect through a common binding partner. Potentially, these partners could include actin or the Arp2/3 complex; however, an interaction between Coro1C and the Arp2/3 complex alone was not observed in these cells.

In *in silico* studies we found that Coro 1C phosphorylation was possible on thirteen sites by PKC (Figure 2.21). Originally, we hypothesized that Coro1C could be phosphorylated by PDGF in a PKC dependent manner. This was based on the fact that both Coro1A and Coro1B phosphorylation can be stimulated by PMA and our *in silico* data suggested multiple possible phosphorylation sites. In contrast, we observed that Coro1C is not phosphorylated in a pSerPKC- dependent manner in response to PDGF stimulation in VSMCs (Figure 2.22A). Recent data by Xavier *et al* (173) demonstrated that Coro1C can be phosphorylated by caesin kinase 2. Casein kinase 2 is a serine/ threonine kinase whose activity is linked to the cell cycle, DNA repair, cell survival and regulation of circadian rhythms. Thus, it is very possible that Coro1C is important for processes other than migration in VSMCs.

In summary, our data demonstrate a role for Coro1B in PDGF-induced VSMC migration. This is the first report of coronin expression in VSMCs, and the apparent functional importance of Coro1B in the response to PDGF makes it clear that further investigation of this family of proteins is warranted. By understanding the intracellular signaling mechanisms by which PDGF induces migration, therapeutic options to combat lesion formation may be expanded.

Chapter 3:

Coronin 1B and Redox Signaling

3.1 Introduction

VSMC migration is important in both normal vascular development and vascular pathophysiology. The migration of these cells is largely governed by the chemokine PDGF-BB, and its activation of the PDGFR-β. However, the pathways that regulate PDGF-induced migration are still being elucidated. PDGF receptor activation leads to the autophosphoylation of its intracellular tyrosine kinase domains. Phosphorylation of these domains creates docking sites for proteins such as Src, PI3K and PLCγ in VSMC. Binding and activation of these proteins promotes the creation and/ or release of second messenger molecules such as ROS, PIP_2, Ca^{2+} and DAG. PDGF-induced signaling and migration is greatly influenced by the receptor's ability to stimulate ROS as signaling molecules. In fact, PDGF-induced VSMC migration does not occur without it, and moderate increases in ROS production coincide with increased migration, suggesting that these pathways are inextricably linked.

A major source of ROS production in the vessel wall is derived from the NADPH oxidases. In VSMCs Nox1 and Nox4 are the predominate isotypes. Nox1 resides in membrane regions of the cell enriched with caveolae, while Nox4 can be found in focal adhesions, nucleus and endoplasmic reticulum. Specifically, in VSMCs Nox1 is the major generator of superoxide in response to PDGF stimulation (111). $Nox1^{-/y}$ VSMCs have lower rates of migration and proliferation in response to PDGF (111). Furthermore, the decrease in VSMC migration found with this genotype has been attributed to a decrease in SSH1L activity due to Nox1 deletion. ROS derived from Nox1are required to activate this phosphatase in VSMCs stimulated with PDGF. On the other hand, the Nox4 oxidase has been shown to play a role in basal ROS production and its expression is downregulated in response to growth factors (253). However, inhibition of Nox4 expression reduces PDGF-induced VSMC migration. It is hypothesized that Nox4 regulation of PDGF-induced migration is not directly linked to PDGF receptor signaling

but instead is an indirect consequence of integrin based signaling. Based on its focal adhesion localization, it is possible for Nox4 to participate in integrin based redox signaling. All in all, both Nox1 and Nox4 participate in VSMC migration and signaling initiated by PDGF stimulation.

In chapter 2 we described a pathway in which Coro1B expression and phosphorylation regulates PDGF-induced VSMC migration. However, the ROS sensitivity of this pathway remains is unknown. Therefore, our present goal was to determine if PDGF-induced Coro1B phosphorylation is redox sensitive and if Coro1B could regulate the PDGF-induced ROS production.

3.2 Methods

3.2.1 Materials

Recombinant human PDGF-BB was purchased from R&D Systems Inc. Primary antibodies were purchased from Sigma (β-tubulin), ECM Bioscience (phospho-Coro1B Ser-2), Santa Cruz Biotechnology (Coro1B M-80), Cell Signaling Technologies (p-Cofilin Ser-3) and Abcam (SSH1L). Hydrogen peroxide was purchased from Sigma.

3.2.2 Cell Culture

VSMCs were isolated from rat thoracic aorta by enzymatic digestion as previously described (236). Isolated VSMCs were grown in Dulbecco's Modified Eagle's Media supplemented with: 10% calf serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 4 mM L-glutamine. For experiments, cells between passages 7 and 15 were plated and allowed to grow until they reached 60% confluence. Cells were then serum starved for 24-48 hrs prior to PDGF stimulation.

Human aortic smooth muscle cells (HASM) were obtained from Lonza (AG) and grown in Smooth muscle Growth Media-2 (Lonza) supplemented with human epidermal

growth factor 0.5 ng/mL, insulin 5 µg/mL, hFGF 2 ng/mL, 5% FBS, gentamicin 50 µg/mL, and amphotericin B 50 ng/mL. For experiments, cells between passage 5 and 9 were plated and allowed to grow until they were 70% confluent**.** Cells were made quiescent for 48 hrs by reducing the serum content of the media to 0.1% FBS prior to PDGF stimulation.

$Nox1^{-/y}$ mice were created in laboratory of Dr K.H. Krause. (254) $Nox1^{-/y}$ VSMCs were isolated by enzymatic digestion of $Nox1^{-/y}$ mouse aortas, as previously described (111). VSMCs were plated in a culture flask, and grown in DMEM supplemented with 10% Fetal Bovine Serum, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 4 mM L-glutamine. For experiments, cells between passages 5 and 10 were plated and allowed to grow until they reached 60% confluence. Cells were then serum starved for 24-48 hrs prior to PDGF stimulation.

3.2.3 Western Blotting

After treatment with PDGF, cells were washed twice with phosphate buffered saline and then lysed in Hunters buffer (25 mmol/L HEPES, 150 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, Na-orthovanadate and protease inhibitors). Lysates were then sonicated and cleared at 13,000 x g for 5 minutes. Proteins were separated using SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore), blocked with 5% non-fat dairy milk, and incubated with appropriate primary antibodies. Subsequently, blots were incubated with horseradish peroxidase-conjugated secondary antibodies and proteins were detected by enhanced chemiluminescence (ECL, GE). Band intensity was quantified by densitometry using ImageJ (NIH) or Carestream Molecular Imaging (Carestream) software.

3.2.4 Small Interfering RNA Transfection Experiments

Cells were transfected by electroporation using the Amaxa Nucleofector system (Lonza AG) set to the U25 program with 3 µg of annealed siRNA duplexes for Coro1B, SSH1L or nonsilencing control sequence no. 1 from Qiagen per $1.5x10^6$ cells. siRNA target sequences were as follows: Coro1B (5'- CAG CAC CTT CTG CGC AGT CAA -3'), and SSH1L (5'-UCG UCA CCC AAG AAA GAU AUU-3'). The cells were transfected, allowed to attach and recover for 24 hours and then serum starved for 24-48 hours.

3.2.5 Detection of Superoxide and Hydrogen Peroxide By Electron Spin Resonance

NADPH dependent superoxide and hydrogen peroxide was measured as previously stated (255). Briefly, VSMCs were harvested and washed with PBS. Cells were resuspended in PBS containing protease inhibitors. Cells were sonicated and the membrane pellet was resuspended in electron spin resonance buffer. Electron spin resonance spectroscopy using 1-hydroxy-3-carboxy-pyrrolidine (CPH spin trap) and 200 µmol/L NADPH was used for quantitative measurements of superoxide and hydrogen peroxide production as described previously. Superoxide dismutase (50 U/mL) was added directly to the sample to determine the superoxide dismutase-inhibitable signal when superoxide was measured. Production of H_2O_2 was measured by co-oxidation of CPH in HRP-acetamidophenol (AAP) reaction. Each molecule of H_2O_2 forms two molecules of CP by two consecutive peroxidase reactions of compound I and compound II. Detection of H_2O_2 was confirmed by inhibition with 50 µg/ml catalase.

3.2.6 Statistics

Results are expressed as means ± SEM. Differences among groups were analyzed using student's t-test as well as one-way and two-way analysis of variance

(ANOVA), followed by the Bonferroni post hoc test. A value of $P<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Hydrogen Peroxide Marginally Increases Coronin 1B Phosphorylation

Many pathways activated by PDGF are ROS sensitive and are obligatory for migration. In the literature, ROS have been demonstrated to increase and activate PKC (256, 257). For that reason, we stimulated VSMCs with hydrogen peroxide to determine if Coro1B serine-2 phosphorylation is ROS sensitive. In contrast to PDGF stimulation, cells treated with hydrogen peroxide demonstrated a mild non-statistically significant increase in p-Ser2Coro1B (Figure 3. 1). In preliminary experiments, the PKC inhibitor Rö-32-0432 repressed this phosphorylation (data not shown). Data gathered here imply that Coro1B phosphorylation by PKC may in fact be regulated by acute hydrogen peroxide exposure, but additional experiments will be necessary to confirm this finding.

3.3.2 PDGF-induced Coronin 1B Serine-2 Phosphorylation is Augmented by the Deletion of Nox1

NOX-derived ROS are an important source of ROS in PDGF-stimulated VSMCs. To determine if NOX1-derived ROS regulate Coro1B phosphorylation, we stimulated NOX1 knockout ($NOX1^{-/y}$) VSMCs with PDGF and analyzed protein lysate for p-Ser2Coro1b expression. Unexpectedly, and in contrast to the potential increase in Coro1B phosphorylation in response to exogenous ROS, loss of ROS in $NOX1^{-/y}$ cells

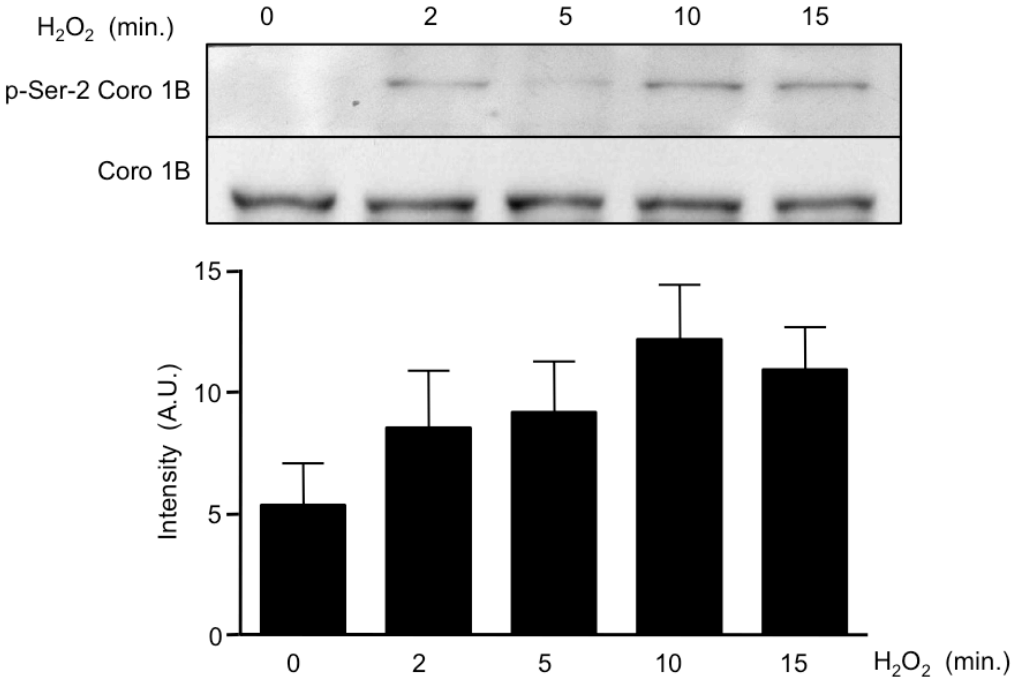


Figure 3.1 Hydrogen Peroxide Marginally Stimulates Coro1B Ser-2 Phosphorylation in VSMCs. VSMCs were serum starved for 48 hours, and then stimulated with 100 μM H_20_2 for the indicated times. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibodies. The graph represents the mean ± SEM of densitometric analysis from 3 independent experiments.

also led to increased Coro1B phosphorylation compared to wild type (WT) cells (Figure 3.2). This apparent paradox may be due to differential effects of localized vs. global ROS production. It is noteworthy that data from our lab indicate that PDGF stimulation of a potential coronin phosphatase, SSH1L, requires NOX1-derived ROS for activation, and is therefore inhibited in NOX1$^{-/y}$ cells (88). Thus, we hypothesized that the increase in Coro1B phosphorylation observed in NOX1$^{-/y}$ cells is due to decreased PDGF-induced SSH1L activity.

3.3.3 Mechanism of Coronin 1B Serine-2 Dephosphorylation

Previously, SSH1L was demonstrated to dephosphorylate Coro1B *in vitro* and in Rat-1 fibroblasts (194). In contrast studies by Kurita *et al* (198) demonstrated that Coro1B cannot be de-phosphorylated by SSH1L. Hence, there is some degree of controversy on the issue. To determine if SSH1L could dephosphorylate Coro1B in VSMCs, HASMs were transfected with siRNA against SSH1L and then stimulated with PDGF. Contrary to our hypothesis, knockdown of SSH1L did not increase Coro1B phosphorylation basally or in response to PDGF (Figure 3.3). As a control for effective functional inhibition of SSH1L, p-Cofilin was measured, as SSH1L is known to de-phosphorylate cofilin in response to PDGF in VSMCs (113). As expected, PDGF-induced, SSH1L-mediated cofilin dephosphorylation was blunted in SSH1L transfected cells (Figure 3.3). In any event, these data show that SSH1L does not de-phosphorylate Coro1B. Therefore, we examined the effects of okadaic acid on Coro1B phosphorylation. Okadaic acid is a potent inhibitor of the serine/threonine phosphatases PP1 and PP2A. When cells were pre incubated with 0.5 μM okadaic acid, PDGF-induced Coro1B phosphorylation was potentiated (Figure 3.4). Thus serine/ threonine phosphatases regulate Coro1B phosphorylation and are a potential target for Nox1-derived ROS.

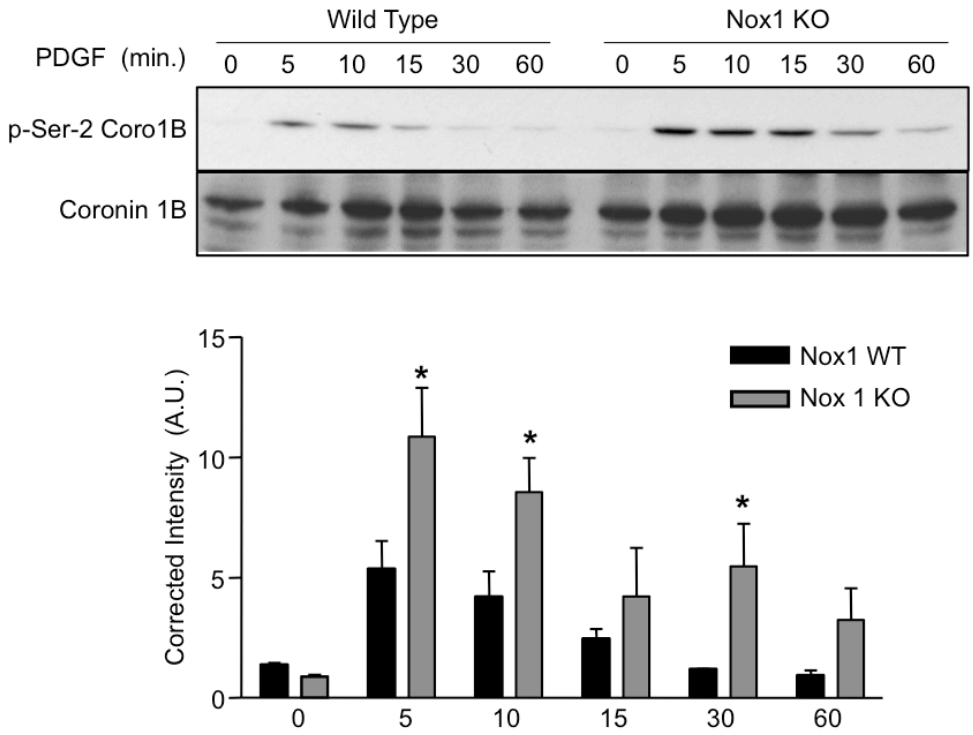


Figure 3.2 NOX1 Downregulation Potentiates PDGF-induced Coro1B Phosphorylation. WT and NOX1$^{-/y}$ cells were serum starved for 48 hours, and then stimulated with 10ng/ml PDGF for the indicated times. Cell lysates were harvested and immunoblotted with Coro1B and p-Ser2 Coro1B antibodies. The graph represents the mean ± SEM of densitometric analysis from 3 independent experiments. (*$p<0.05$, N=3)

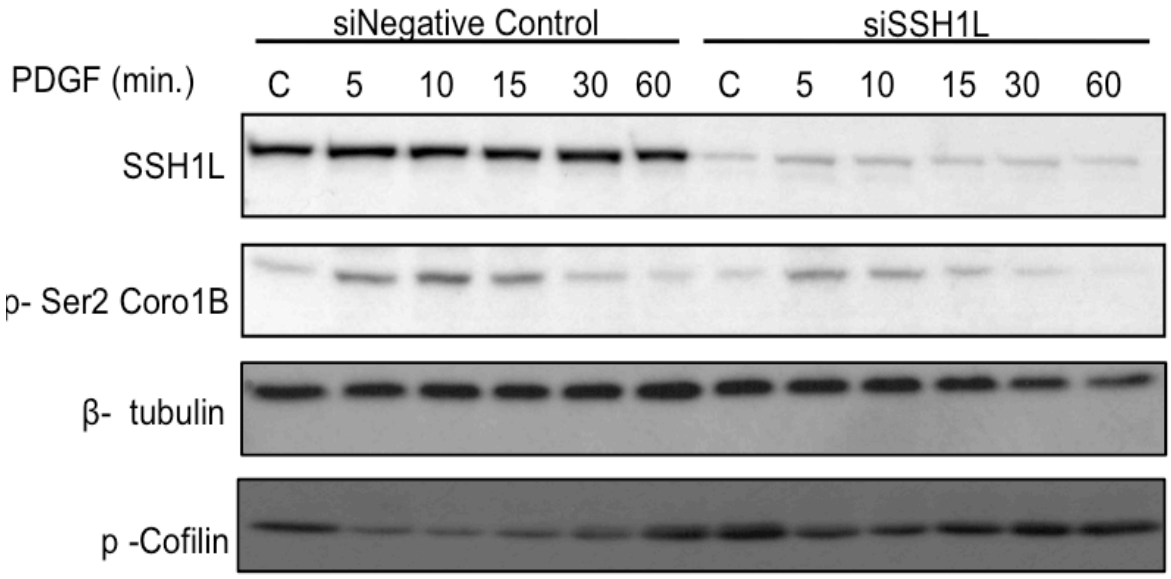


Figure 3.3 SSH1L Does Not Dephosphorylate Coro1B Basally or in Response to PDGF in HASMs. HASM were transfected with siSSHL by Amaxa electroporation, serum starved for 48hrs and harvested for protein. Membranes were probed with antibodies against SSH1L, p-Ser2 Coro1B, p-Cofilin, and β- tubulin. (Representative of N=3)

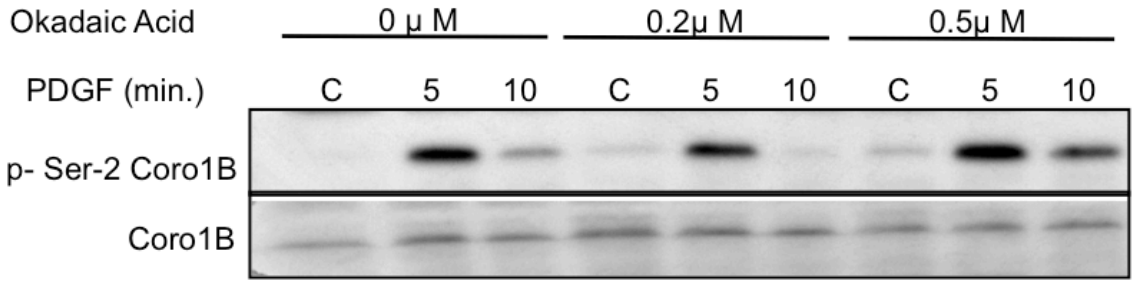


Figure 3.4 Okadaic Acid Increases Basal and PDGF-induced Coro1B Phosphorylation. VSMCs were pre-incubated with varying concentrations of Okadaic acid. Cells were then stimulated with 10ng/ml PDGF and cell lysates were harvested. Membranes were blotted for p-Ser-2 Coro1B and Coro1B (representative blot, N=2).

3.3.4 siCoro1B Increases PDGF-induced NOX-derived ROS

Multiple factors culminate to produce both atherosclerosis as well as restenosis. One of those factors is ROS production, which ultimately leads to oxidative stress and inflammation. PDGF-mediated signaling has been demonstrated to stimulate ROS production in VSMC. PDGF-induced ROS are required for both VSMC migration and proliferation mediated by this growth factor. PDGF-stimulated NOX1-derived ROS has been proven to regulate proliferation and migration in VSMCs. Coincidentally, there are data that support an interaction for Coro1A and the cytosolic subunits of the phagocytic oxidase in neutrophils. However, its expression is relegated to cells of hematopoietic origin. Since type I coronins share high degree of sequence and structural similarity, it is possible that another isotype can interact with homologous structures found in other oxidases. Thus, based on Coro1B membrane localization which puts it in close proximity to the Nox1 oxidase and data collected from the literature, we next asked whether Coro1B might also regulate ROS production, in addition to being regulated itself by ROS. We examined the effects of Coro1B downregulation on PDGF-induced NADPH oxidase derived ROS production.

PDGF-stimulated superoxide production in both siNegCtrl (5.25 ± 0.9 vs 11.75 ± 0.7 A.U, *$p<0.05$, siNegCtrl: control vs PDGF, N=4) and siCoro1B samples (5.75 ± 1 vs 15.5 ± 0.7 A.U, *$p<0.05$ siCoro1B: control vs PDGF, N=4) (Figure 3.5A). siCoro1B transfected cells demonstrated enhanced Nox-derived superoxide production (11.75 ± 0.7 vs 15.5 ± 0.7 A.U, +$p<0.05$, PDGF: siNegCtrl vs siCoro1B N=4) (Figure 3.5A). Interestingly, PDGF-stimulated NADPH-derived hydrogen peroxide production was increased in siCoro1B transfected cells (16 ± 1.0 vs 27.0 ± 3.3 A.U, +$p<0.05$, PDGF: siNegCtrl vs siCoro1B); however, there was no increase in PDGF-stimulated NADPH-dependent hydrogen peroxide in siNegCtrl trasfected samples (13.0 ± 1.8 vs 16.0 ± 1.0 A.U, $p>0.05$, N=4 siNegctrl: control vs PDGF) (Figure 3.5B). These data suggest that

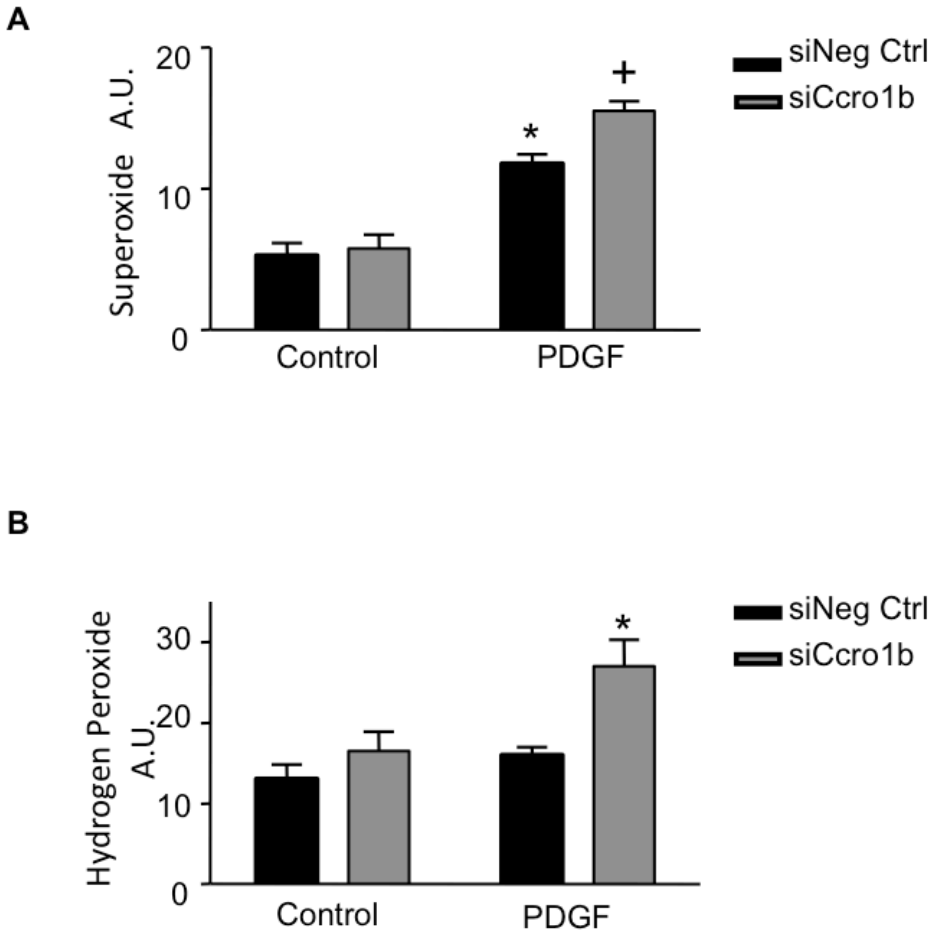


Figure 3.5 siCoro1B Increases PDGF-induced NOX-derived ROS production. VSMCs were transfected with siRNA against Coro1B or siNegCtrl with the Amaxa electroporation system. Cells were then serum starved for 48 hrs and stimulated with 10 ng/ml PDGF for 3 hrs. (**A)** NOX-dependent superoxide production (*p< 0.05, siNegCtrl: control vs PDGF, N=4), (+p<0.05, PDGF: siNegCtrl vs siCoro1B N=4) and **(B)** NOX-dependent hydrogen peroxide as measured by ESR (*p<0.05, PDGF: siNegCtrl vs siCoro1B, N=4).

Coro1B also functions to decrease PDGF-induced ROS production, and possibly demonstrates another mechanism by which Coro1B can regulate migration. These data are consistent with its negative regulation of migration.

3.4 Discussion

PDGF receptor stimulation of ROS is required for migration induced by the growth factor in VSMCs. PKC is a known regulator of VSMC migration and its activation can be stimulated via ROS in many types. Previously, in chapter two, we demonstrated that the phosphorylation of Coro1B was mediated by PKC in VSMCs. This phosphorylation was required to induce PDGF-mediated VSMC migration. Since PDGF-induced VSMC migration is ROS sensitive we tested the ROS sensitivity of Coro1B phosphorylation and the effects of Coro1B expression on PDGF-induced ROS production. With the use of 100 μM hydrogen peroxide we observed a mild increase in Coro1B phosphorylation (Figure 3.1) and this phosphorylation was PKC dependent (data not shown). However, addition of exogenous hydrogen peroxide does not exactly mimic intracellular ROS signaling. ROS produced during signaling is generally localized in discrete areas and is low in concentration. Although the concentration used was low, hydrogen peroxide was allowed to permeate the cell non-specifically, which can activate and inhibit simultaneously. Hence, we examined a more specific source. In $Nox1^{-y}$ cells we observed an increase in Coro1B phosphorylation (Figure 3.2). Previous data from our lab demonstrated that Nox1 was required for SSH1L activity. These two observations together suggested that Coro1B could be de-phosphorylated by SSH1L and that the up-regulation in Coro1B phosphorylation was due to reduced SSH1L activity in these cells. However, when VSMC were transfected with siSSH1L Coro1B phosphorylation was not affected (Figure 3.3). Consequently, we pretreated cells with

okadaic acid to try and determine what family of phosphatases were involved in Coro1B de-phosphorylation. Okadaic acid did increase basal and PDGF-induced Coro1B phosphorylation (Figure 3.4). This suggests a serine/threonine phosphatase governs Coro1B de-phosphorylation. However, whether the okadaic acid-sensitive phosphatase acts directly on Coro1B or on PKC needs to be determined. PKC phosphorylation in the catalytic domain stimulates and increases PKC activity. PKCs are known serine/ threonine phosphatase substrates (258). Serine/threonine phosphatases include such family members as protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2). It is therefore possible that Coro1B dephosphorylation is mediated by an okadaic acid-sensitive phosphatase. Future experiments must be designed to test this possibility.

Lastly, we examined the effects of Coro1B down regulation on PDGF-induced NOX-derived superoxide and hydrogen peroxide production. Intriguingly, both were significantly increased in the siCoro1B transfected samples (Figure 3.5). Data collected by our lab and others have attributed PDGF-induced NOX-derived superoxide production to activation to NOX1. Taken together these data suggests that Coro1B negatively regulates superoxide and hydrogen peroxide production derived from the NADPH oxidase in response to PDGF stimulation in VSMCs. Interestingly, an increase in hydrogen peroxide was not detected in siNegCtrl PDGF treated samples; however, in siCoro1B, PDGF-stimulated samples hydrogen peroxide production was significantly increased. This suggests Coro1B acts normally to reduce ROS production in these cells. Coro1B may exert these effects by multiple avenues. One way in which Coro1B could possibly regulate PDGF-induced superoxide production is by sequestering known cytosolic subunits p47phox and/or NoxA1 (p67phox homologue) in the cytosol. Phosphorylation of either protein could possibly lead to a disruption in the interaction between the proteins. Both p47phox and Coro1B are known kinase substrates for PKC.

However, for this hypothesis to be true, Coro1B would have to bind to or co-immunoprecipitate with p47phox or NoxA1.

In conclusion our data demonstrate a role for Coro1B in PDGF-induced VSMC ROS production. Additionally, our data suggest that Coro1B phosphorylation is ROS sensitive. Therefore, Coro1B is both upstream and downstream of PDGF-induced NADPH oxidase activity, and therefore may fine tune redox-sensitive migration pathways.

Chapter 4:

Discussion

Lamellipodia formation is critical in many cell types for efficient cell migration. Recent data have suggested that a group of actin binding proteins known as coronins can potentially regulate lamellipodia formation, which is the first step in migration. Here we describe a mechanism by which PDGF, acting through PKCε, regulates the phosphorylation of Coro1B and its interaction with the Arp2/3 complex to modulate lamellipodia formation and migration. We show for the first time that both Coro1B and 1C are expressed in VSMCs of various lineages and that Coro1B is also expressed *in vivo* in the vessel wall. Knockdown of Coro1B, but not Coro1C. increases PDGF-induced VSMC migration. Additionally, we demonstrate that PDGF phosphorylates Coro1B on Ser-2, leading to a decrease in the interaction of Coro1B with the Arp2/3 complex. Dissociation of phospho-Coro1B from Arp2/3 releases it from an inhibitory complex, resulting in an overall increase in lamellipodial protrusion and eventually migration. Furthermore, Coro1B phosphorylation is negatively regulated by a serine/ threonine phosphatase. Coro1B and Coro1C were also found to co-immunoprecipitate in a PDGF-independent manner. More importantly, Coro1B down regulation leads to an increase in PDGF-induced NADPH-derived superoxide and hydrogen peroxide, thereby creating another mechanism by which Coro1B can modulate PDGF-induced VSMC migration (Figure 4.1).

4.1 VSMC Coronins

Type I coronins regulate many actin dependent processes such as proliferation, phagocytosis, migration and endocytosis in multiple cell types. In VSMCs, Coro1B and 1C are the predominate type I coronins that are expressed. Both Coro1B and Coro 1C share the highest sequence similarity among the type I coronins and have been demonstrated to regulate similar processes. However only the inhibition of Coro1B expression modulated PDGF-induced VSMC migration. Coro1B and Coro1C co-

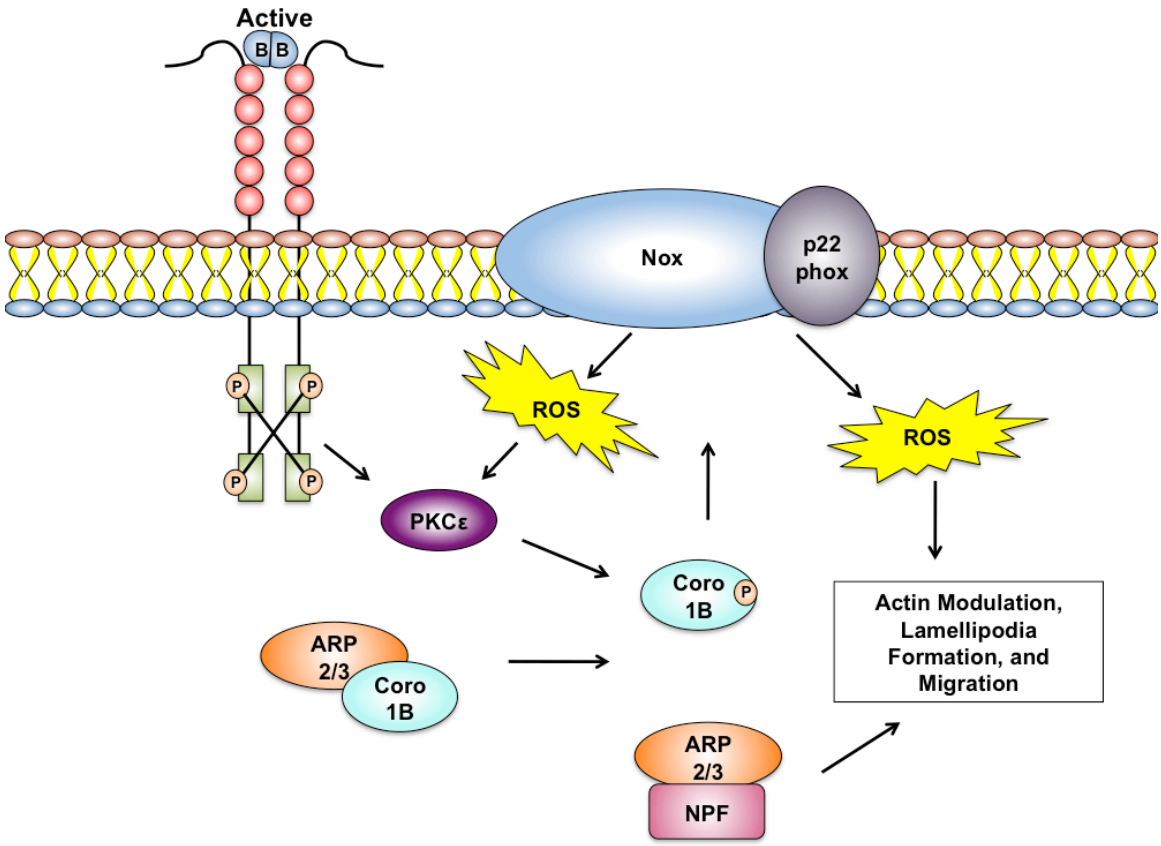


Figure 4.1 Model: The Role of Coro1B in PDGF-induced VSMC Migration. We describe a mechanism by which PDGF, acting through PKCε, regulates the phosphorylation of the actin binding protein Coro1B and its interaction with the Arp2/3 complex to modulate lamellipodia formation and migration. Additionally Coro1B knockdown increases PDGF-induced ROS production in VSMC migration, thus providing another mechanism by which Coro1B can regulate PDGF-induced VSMC migration.

immunoprecipitate in a PDGF-independent manner. Additionally, stimulation with PDGF leads to the PKC-mediated phosphorylation of Coro1B, whereas PDGF does not stimulate serine phosphorylation in PKC target sequences on Coro1C. These data suggest that although Coro1B and 1C are structurally similar, their roles in PDGF-induced VSMC migration are clearly different.

4.1.1 Coronin 1B Functions and Localization

4.1.1.1 Coronin 1B regulation of the Arp2/3 complex and SSH1L

In VSMCs we have demonstrated that Coro1B negatively regulates PDGF-induced migration and changes in lamellipodia dynamics. PDGF stimulation of these cells leads to an increase in Coro1B phosphorylation and a reduction in Coro1B/Arp2/3 complex interactions thereby promoting migration. Similarly, Coro1B interactions with Arp2/3 are phosphorylation state dependent in Rat-2 fibroblasts. Yet in Rat-2 fibroblasts, Coro1B phosphorylation negatively regulates migration (140), and knockdown of Coro1B inhibits migration and negatively regulates lamellipodia dynamics in this cell line (194). Consequently, in Rat-2 cells Coro1B is a positive regulator of migration.

Although Coro1B interactions with the Arp2/3 complex are phosphorylation dependent in both Rat-2 fibroblasts and VSMCs, the effects of Coro1B expression have opposing results in the two cell types. This dichotomy can possibly be explained by the differences in additional Coro1B binding partners. In Rat-2 fibroblasts, Coro1B interacts with a GFP-tagged SSH1L and localizes the phosphatase to the lamellipodia where it dephosphorylates and activates cofilin (194). Rat-2 fibroblasts that were Coro1B deficient also displayed increased levels of cofilin phosphorylation (194), thereby supporting a positive role for Coro1B in this cell type. However, in VSMCs an interaction between SSH1L and Coro1B was not detectable. Furthermore, knockdown of Coro1B did not significantly affect cofilin phosphorylation in VSMCs. Rat-2 fibroblasts and

VSMCs also have conflicting responses to chemotactic stimuli. Rat-2 fibroblasts demonstrate a negative chemotactic response to PMA whereas some VSMCs respond positively to PMA stimulation (81). Unlike VSMCs, Rat-2 fibroblasts do not respond chemotactically to PDGF stimulation and have higher basal ERK activity, thereby underlining other differences between the two cell types. Such differences as these could account for the diverging roles of Coro1B in the two cell types.

We have attributed many of the differences in the effect of siCoro1B on Rat-2 fibroblasts and VSMC migration to cell type specificity. To further explore and confirm differences in Coro1B cell type specificity, multiple chemotactic agents should be used as well as cells from different lineages in cell migration assays under the previously mentioned conditions. Also, examination of endogenous Coro1B and SSH1L interactions should be evaluated in these cell types. The effects of Coro1B deficiency on lamellipodia dynamics should also be assessed. Based on our present observations, in cells where Coro1B and SSH1L interact, Coro1B knockdown should inhibit migration. On the other hand, in cells where Coro1B and SSH1L do not interact then Coro1B knockdown should have a positive effect on migration.

4.1.1.2 Coronin 1B regulation of ROCK

Recently, in the breast adenocarcinoma cell line MCF-7, Coro1B was shown to immunoprecipitate with the pleckstrin homology domain of rho-associated coiled coil containing protein kinase 2 (ROCK 2) and regulate ROCK2 dependent signaling through a SSH1L-dependent mechanism (259). ROCK is a kinase belonging to AGC family of serine/threonine kinases that is activated by the binding of the small GTPase Rho (260). Activation of ROCK leads to the phosphorylation and inhibition of myosin phosphatase as well as the phosphorylation of myosin light chain, calponin, LIM domain containing protein kinase (LIMK), PTEN, and protein kinase C-potentiated inhibitor protein of 17

kDa (CPI-17) (261-266). Coro1B downregulation was demonstrated to increase ROCK dependent phosphorylation and inhibition of myosin phosphatase and subsequently enhance myosin light chain and cofilin phosphorylation (259). Coro1B overexpression in MCF-7 cells attenuates these processes via binding to ROCK (259). Hence, Coro1B can modulate migration via another mechanism that includes negative regulation of pathways downstream of ROCK.

ROCK is a major regulator of migration, contractility, and gene expression in VSMCs, which has been associated with various cardiovascular pathological processes such as atherosclerosis, hypertension, and ischaemia-reperfusion injury (267). In VSMCs inhibition of ROCK has been demonstrated to decrease PDGF-induced migration (268, 269). Thus, it is probable that ROCK inhibition by Coro1B would have a negative effect on PDGF-induced VSMC migration. As shown in Chapter 2, overexpression of WT Coro1B decreases migration. This is consistent with the concept that overexpression of Coro1B might inhibit ROCK activity and that inhibition of this activity decreases PDGF-induced migration. In addition to migration, the regulation of ROCK activity by Coro1B may influence VSMC contraction and gene expression. In VSMCs ROCK phosphorylates myosin light chain and myosin phosphatase to stimulate contraction (264). Furthermore, ROCK is also known to regulate VSMC gene expression and differentiation markers, such as smooth muscle α actin, calponin, and SM22α (270, 271). The use of the ROCK inhibitor Y-27632 reduces VSMC specific promoter activity of these differentiation genes by about 50% (270). Therefore, the knockdown of Coro1B should increase the expression of these genes and increase VSMC differentiation. Indeed, in preliminary studies we have observed an increase in smooth muscle α actin expression in HASMs transfected with siCoro1B (unpublished observations). This suggests that Coro1B may indeed regulate this process through a

ROCK mediated mechanism; however, further studies are required to validate the observation.

Currently our data are consistent with a role for Coro1B in ROCK signaling in VSMCs. However to prove this, an interaction between ROCK and Coro1B should be established in VSMCs. Co-immunoprecipitation experiments aimed at determining if and how PDGF can regulate this interaction should be designed (e.g. creation of domain mutations and mutation of posttranslational modification sites). Assays to determine ROCK kinase activity in the presence of Coro1B mutants should also be performed to verify a specific effect on the kinase activity of the protein. To determine if the interaction of these proteins regulates migration a mutant Coro1B and/or ROCK that does not bind the endogenous protein or each other should be created and the effects of their overexpression on PDGF-induced VSMC migration should be assessed. Additionally, Coro1B has the potential to regulate other functional endpoints such as contraction and gene expression. To determine if Coro1B plays a role in VSMC contraction, knockdown, overexpression and rescue studies should be done using gel contraction assays with pro-contraction stimuli such as angiotensin II, endothelin, vasopressin and/or norepinephrine. Negative regulation of ROCK by Coro1B overexpression would suggest that VSMC contraction would be decreased in these conditions. Furthermore, use of these vasoconstrictors should induce the dissociation of ROCK and Coro1B in co-immunoprecipitation experiments. To confirm a role for Coro1B in VSMC gene expression via modulation of ROCK dependent signaling, experiments aimed at monitoring smooth muscle specific gene expression under conditions in which Coro1B can not interact with ROCK and when Coro1B is overexpressed should been examined. Also studies that measure smooth muscle gene expression under conditions when both Coro1B and ROCK are knocked down should be done to verify that the Coro1B affects gene expression via a ROCK mediated mechanism. Continued exploration into the effect

of Coro1B on ROCK activity would not only provide information on another mechanism by which Coro1B regulates VSMC migration, but would also provide insight on how Coro1B might regulate other vital processes in VSMCs.

4.1.1.3 Coro1B Localization Suggests Other Possible Functions

Coro1B has been demonstrated to localize to F-actin structures in multiple cell types. In VSMCs, Coro1B was observed at the cell periphery, at the leading edge of the cell and lightly along stress fibers. Additionally, Coro1B is enriched in cell fractions from the membrane, cytosol, and cytoskeleton. Antibodies against phosphorylated Coro1B stained stress fibers, diffusely throughout the cytosol, the nucleus and around the cell periphery. In Rat-2 fibroblasts, Coro1B is observed at the leading edge of the cell and has a diffuse pattern in the cytosol (140). In electron micrographs of mouse embryonic fibroblast lamellipodia, Coro1B is observed at actin branches (194). Like VSMCs and Rat-2 cells, dorsal root ganglion neurons exhibit cytoplasmic and cortical actin staining for Coro1B (199). Moreover, Coro1B is localized to sites of active actin turnover known as growth cones in these neurons (199). In addition to colocalizing with F-actin structures, Coro1B also localized to intracellular canalicular regions of the rabbit parietal cell that are known to regulate HCl secretion (193). Researchers postulated that Coro1B could possibly direct membrane/cytoskeletal rearrangements in the gastric parietal cell due to its localization and based on the fact that the *Dictyostelium discoidium* coronin counterpart regulates endocytosis (193); however, experiments to prove this hypothesis are lacking. Interestingly, cholinergic stimulation of H+-secretion is partially facilitated by PKCε activation in rabbit parietal cells (272). These two pieces of data suggest an additional function for Coro1B, endocytosis/exocytosis, which has yet to be explored.

Historically, coronins have been associated with vesicular trafficking. In the slime mold *D. discoidium,* coronin localizes to the phagocytic cup and phagocytosis is reduced

by a third in coronin null cells (273). In monocyte/macrophages and neutrophils, Coro1A regulates phagocytosis and phagosome formation (177, 179). The maintenance of Coro1A at the phagocytic cup also regulates lipid uptake and degradation in macrophages. Knockdown of Coro1A increases acetylated-LDL uptake and degradation (144). Moreover, the human, mouse, and rat Coro1B genes contain binding sites for multiple sterol response element binding proteins (SREBPs) and peroxisome proliferator-activated receptor gamma (PPARγ) (274). Taken together, these data support a potential role for Coro1B in cholesterol homeostasis. In VSMCs, the uptake of lipids is important in the conversion of VSMCs to foam cells (19). To determine if Coro1B regulates cholesterol homeostasis, LDL-uptake and degradation should be measured in VSMCs deficient in Coro1B. Also Coro1B expression should be measured to ascertain if its expression can be regulated by cholesterol.

Interestingly, phosphorylated Coro1B was also detected in the nucleus of VSMCs. The localization of Coro1B to the nucleus has a precedent. In primary cultured hippocampal neurons and Hela cells, overexpressed GFP-Coro1B localized to the cytoplasm and nucleus (275). Additionally, in *in vitro* assays Coro1B immunoprecipitated with α-importin and in Hela cells was transported into the nucleus by an importin α5/β- and RAN-dependent mechanism (275). Therefore, it is possible that Coro1B can participate in gene transcription and/or cell cycle regulation.

Actin mediated regulation of gene expression and transcription is an interesting and emerging field. Recent data have shown that nuclear actin can regulate transcription via direct interaction with transcription factors, RNA polymerases and chromatin remodeling complexes (276). Proteins that regulate and bind actin in the cytosol such as profilin, cofilin, myosin I, and gesolin are present in the nucleus (276, 277). In particular, Arp 2/3 and the NPF N-WASP were demonstrated to regulate RNA polymerase II activity via their actin nucleation activity (278, 279). Consequently,

proteins that regulate Arp2/3 activity such as Coro1B can potentially regulate RNA polymerase II activity. For these reasons, RNA polymerase II- dependent transcription activity assays should be designed to examine a potential role for Coro1B in the regulation of transcription in VSMCs.

4.1.2 Coronin 1C Functions and Localization

Like other type I coronins, Coro1C has been shown to interact with actin and modulate many of its processes. Coro1C modulates migration through an Arp2/3 dependent mechanism in HEK, intestinal epithelial cells, and mouse embryonic fibroblasts (MEFs) and regulates neuronal outgrowth in PC-12 cells (173, 205, 206). In VSMCs stimulated with PDGF, however, siRNA against Coro1C had no effect on VSMC migration. The fact that Coro1C does not specifically bind Arp2/3 or cofilin in VSMCs may partially explain why Coro1C knockdown does not modulate PDGF-induced VSMC migration. In intestinal epithelial cells, Coro1C knockdown increases basal cell migration through increased focal adhesion kinase (FAK) phosphorylation and activity (206). FAK is activated at sites of integrin engagement, as well as in response to contractile and growth factor stimulation (280). Coro1C knockdown had no significant effect on basal or PDGF-induced VSMC migration, and yet PDGF stimulation of VSMCs increases FAK phosphorylation and FAK is required for efficient VSMC migration (281). This suggests that Coro1C does not regulate FAK phosphorylation and signaling in VSMCs. However, measuring the effects of Coro1C downregulation on focal adhesion dynamics such as number, size and protein composition should be performed to rule out this possibility.

While not measured in VSMCs, Coro1C WT expression had no effect on HEK cells wound closure (205). In spite of this, Coro1C mutants lacking the WD domains, beta propeller region and/or the coiled coil region, induced a significant reduction in migration in HEK cells (205). Although Coro1C knockdown does not have an effect on

PDGF-induced VSMC migration, it is not known if overexpression of the protein would alter migration. PDGF and growth factor stimulation have been shown to upregulate Coro1C expression (202), and perhaps increased expression of Coro1C would increase migration. The overexpression of Coro1C is associated with the malignancy and metastasis of such cancers as primary effusion lymphoma, hepatocellular carcinoma and diffuse gliomas (207, 210, 282). Before Coro1C can be totally excluded as a participant in PDGF-induced VSMC migration, future studies will have to examine the effects of long term PDGF exposure on Coro1C expression and the effects of Coro1C upregulation/overexpression on VSMC migration.

The role of Coro1C in PDGF-induced VSMC migration is quite uncertain at the present time; however, Coro1C may regulate other actin dependent processes in VSMCs. Although the role of Coro1C in vesicular trafficking was not studied here, Coro1C has also been associated with vesicular trafficking in other cell types. Removal of Coro1C coiled-coil or β-propeller regions reduces macropinocytosis and secretion in Hek293 cells (205). In the MIN6 insulin secreting β cell line, Coro1C interacts with and is a Rab27a-GDP effector protein (208). Rabs are a family of small GTPases that primarily function by regulating receptor mediated vesicular trafficking (283). In MIN6 cells, knockdown of Coro1C inhibits insulin-induced endocytosis (208). Rab27a expression has also been associated with Coro1A regulated phagosome maturation. Rab27a directly regulates the nascent process of phagocytosis by prolonging the stage of actin coating by suppressing Coronin 1A (284). In macrophages, the NADPH oxidase is localized to a Rab27A/B-regulated secretory compartment (285). Based on the fact that Coro1C can act as a Rab27a effector protein, one could speculate that Coro1C might regulate vesicular trafficking of NADPH oxidases.

4.1.3 Coronin 1B and Coronin 1C Complex Formation

Coro1C was shown to co-immunoprecipitate with Coro1B while not specifically immunoprecipitating with its previously described interacting partners Arp2/3 or cofilin in VSMCs. These data suggest for the first time that coronins can form heterodimers or that Coro1B and Coro1C have a common binding partner. Moreover, this interaction with Coro1B was not regulated in a PDGF-dependent manner. Although this interaction does not interfere with PDGF induced migration, coronins may be important in other actin dependent processes. As with Coro1B, Coro1C has been associated with endocytosis/exocytosis. Additionally, both Coro1B and 1C have been associated with Rab proteins (199, 208). Collectively, these data support a role for Coro1B and Coro1C in vesicular trafficking and their interaction may regulate this process in VSMCs. As stated previously, vesicular trafficking in VSMCs is important in lipid uptake in during foam cell formation, as well as matrix metalloproteinase and growth factor secretion. Because vesicular trafficking plays a significant role in VSMC pathologies, the importance of Coro1C and 1B interactions in vesicular trafficking should be evaluated.

4.2 Consequences of Coronin Phosphorylation

Phosphorylation is a very common post-translational modification that occurs on a serine, threonine or tyrosine residue by kinase proteins. The phosphorylation of a protein can regulate its interaction with other proteins, change its localization, and alter its expression level in many cell types. The phosphorylation of proteins is a common mode of intracellular communication that activates signal transduction pathways that lead to multiple outcomes such as migration, proliferation, phagocytosis and survival.

4.2.1 Coronin 1B

4.2.1.1 Coronin 1B Serine 2 Phosphorylation

The phosphorylation of Coro1B at serine 2 culminates with the dissociation of Coro1B from the Arp2/3 complex. The dissociation of Coro1B from this complex leads to an increase in VSMC migration. Mutation of this serine 2 to a non-phosphorylatable alanine leads to a marked reduction in VSMC migration. Data presented in Chapter 2 demonstrate that PDGF-induced Coro1B phosphorylation at serine 2 is partially mediated by PKCε. Phosphorylation or mutation of this site did not visually alter Coro1B localization to the cell periphery under the conditions that were tested. This suggests that although this site is important for its interaction with the Arp2/3 complex, it is not a major regulator of its localization. Furthermore, the importance of Coro1B serine 2 phosphorylation in regulating its interactions with other proteins is largely unknown. For example, in other cell types where Coro1B was shown to interact with SSH1L, it is not known whether Coro1B serine 2 phosphorylation can regulate their interaction. Moreover, although the arginine 30 residue has been shown to regulate the interaction of Coro1B with F-actin, the effects of Coro1B phosphorylation at serine 2 on the interaction of Coro1B with F-actin filaments have yet to be determined. Additionally, it is still unknown if Coro1B serine 2 phosphorylation can regulate the interaction of Coro1B with ROCK. Consequently, Coro1B serine 2 phosphorylation can possibly regulate multiple interactions beyond its ability to negatively regulate the interaction of the Arp2/3 complex and Coro1B.

To gain more insight on how Coro1B regulates PDGF-induced VSMC migration, it would be very worthwhile to study how the PDGF stimulation of Coro1B serine 2 phosphorylation may regulate interactions with other cytoskeletal proteins. This process should begin with validating the interactions of Coro1B with ROCK, SSH1L, and F-actin in VSMCs. Although we have not explored the possible interactions of Coro1B with ROCK and F-actin in VSMCs, we have immunoprecipitated both Coro1B and SSH1L separately and we were unable to detect a specific interaction between the two proteins

in this cell type. Next, with the use of phosphorylation mutants, changes in protein binding based on Coro1B phosphorylation state should be assessed. Mapping the regions of ROCK, SSH1L, and F-actin that bind to Coro1B using truncated constructs would be informative as well. The opposite experiment will also be necessary to determine what portion of Coro1B binds to the other proteins. The significance of these PDGF-regulated phosphorylation interactions will also have to be determined using functional assays designed to measure migration, lamellipodia dynamics, focal adhesion dynamics, vesicular trafficking and contraction.

4.2.1.2 Predicted Coronin 1B Phosphorylation Sites

The regulation of Coro1B activity may also be regulated by yet undefined phosphorylation modifications. Prediction software has suggested that Coro1B can be phosphorylated at multiples sites by various kinases. Using multiple software programs, Coro1B was predicted to be phosphorylated on at least 25 serine, threonine or tyrosine residues by various kinases that include Src family member tyrosine kinases, CK2, Ca^{2+}/camodulin-dependent protein kinase 2 (CamK2), protein kinase A (PKA), Aurora Kinase, Janus kinase 2 (JAK2), AKT and receptor tyrosine kinases such as anaplastic lymphoma kinase (ALK), EGFR and PDGFR (243-246, 286). These sites were conserved in human, mouse and rat and many of these phosphorylation sites have been validated by mass spectrometry (Table 4.1) (286). However the regulation, function and cell type specificity of these phosphorylations have yet to be determined.

The tyrosine kinase Src and its family members lymphocyte specific protein kinase (LCK), Gardner-Rasheed feline sarcoma viral oncogene homolog (FGR) and FYN kinase are known to regulate and phosphorylate many signaling pathways important in proliferation and migration in various cell types including VSMCs (287-291). Jak2 has been shown to regulate proliferation, contraction and migration in VSMCs

(292-294). Src, its family members and Jak2 have been predicted to phosphorylate Coro1B at tyrosine residue 26. This is in very close proximity to the arginine 30 residue that is responsible for Coro1B binding to actin (165). Perhaps this phosphorylation site may regulate the interaction of Coro1B and actin.

Predicted phosphorylation was also identified in the WD repeat coding regions that form the β-propeller. At serine 199, threonine 209, tyrosine 309, and serine 330 in Coro1B, kinases such as PKA, PKC, CamK2, Aurora K, and PAK2, as well as receptor tyrosine kinases, were suggested to mediate these phosphorylations (Table 4.1). Proteins with tertiary β-propeller structures are known to act as scaffolds due to the protein-protein interactions mediated by these structures. As a result, phosphorylation of Coro1B in this region may regulate is interaction with multiple proteins. PKA, also known as c-AMP protein kinase, traditionally inhibits VSMC migration and migration initiated by growth factor stimulation in VSMCs (295). Conversely, CK2 activity regulates proliferation and adhesion in VSMCs (296, 297). CamK2 regulates proliferation, actin dynamics and migration in VSMCs (298-301). Aurora kinase plays a role in mitosis in cells of various lineages (302, 303). Lastly, AKT positively regulates a myriad of processes that include survival, proliferation and migration in VSMCs (304-307). Upstream of AKT, PKC, CamK2, and PAK2 are PDGF and EGF receptor signaling. Both receptors, which promote growth and migration in VSMCs, are predicted to phosphorylate Coro1B in the β-propeller region as well. Phosphorylation of Coro1B by these growth factor receptors could possibly regulate their endocytosis. The predicted phosphorylation of Coro1B at these sites might regulate the interaction of Coro1B with proteins that are important for any of the aforementioned processes.

The possible phosphorylation of Coro1B by these kinases leaves a plethora of options to be explored in the field of coronins. To determine if any of these kinases can phosphorylate Coro1B, the use of *in vitro* kinase assays would be the best first

Conserved Phosphorylation Site	Predicted Kinase
Y-26	Src, Lck, Fgr, Fyn, ALK, JAK2
S-199	CamK2, PKA, PKC
T-209	AKT, Aurora K, PKA, CamK2, PKC, PAK2
Y-303	EGFR, PDGFR, ALK
S-330	CK2
S-413	CK2, GCRK-1

Table 4.1. Predicted Coro1B Phosphorylation Sites and Kinases. Potential Coro1B phosphorylation sites were identified by comparing conserved predicted phosphorylation sites of rat, human, and mouse using the Human Protein Reference Database Phosphosite Prediction program and NetPhosK 1.0 server. Sites were filtered by comparing them to known phosphorylation sites identified through Cell Signaling Phosphosite database.

approach. The creation and use of phosphodeficient mutants in the *in vitro* kinase assays would help determine if these sites were specifically phosphorylated by the proposed kinases. Additionally, confirmation that phosphorylation of Coro1B occurs in a kinase-dependent manner *in vivo* is required and the consequences of these phosphorylations on known Coro1B protein interactions should also be addressed. Lastly, these kinases function in various pathways that are required for migration, cell cycle regulation and proliferation, contraction, and hypertrophy in VSMCs; therefore, functional assays that measure these endpoints should be assessed.

4.2.1.3 Coronin 1B Dephosphorylation

Data provided in Chapter 3 demonstrate that in VSMCs the dephosphorylation of Coro1B is under the control of an okadaic acid-sensitive phosphatase. In Rat-2 fibroblasts, 100 nM of okadaic acid did not increase Coro1B serine phosphorylation and therefore researchers speculated that an okadaic acid-sensitive phosphatase does not regulate Coro1B phosphorylation in this cell type (194). Furthermore, overexpression of SSH1L led to the inhibition of PMA-induced Coro1B phosphorylation and overexpression of a mutant SSH1L extended Coro1B serine phosphorylation (194). In Nox1 knockout cells where SSH1L activity is decreased, an increase in Coro1B phosphorylation was observed. This observation suggested that Coro1B could be dephosphorylated by SSH1L. However, in VSMCs siRNA against SSH1L did not increase basal or PDGF-induced Coro1B phosphorylation. These data suggest that SSH1L does not regulate Coro1B phosphorylation in this cell type. Differences in phosphatase activity toward Coro1B could be cell type or agonist specific, and further work is required to identify the Coro1B phosphatase in VSMCs.

4.2.2 Coronin 1C

In initial studies, we set out to determine if Coro1C could be regulated in a similar manner as Coro1B in response to PDGF in VSMCs. Computational analysis of the Coro1C sequence predicted multiple PKC phosphorylation sites with in the protein. Yet unlike Coro1B, Coro1C was not phosphorylated in p-Ser-PKC mediated manner by PDGF in VSMCs. However, a recent survey of the literature has demonstrated that Coro1C can be phosphorylated by CK2 at serine 463. CK2 is constitutively active tetramer that is regulated by expression and assembly. Specifically, in VSMCs CK2 regulates cell adhesion, mitogenesis, and force of contraction (296, 297, 308). Coro1C has been demonstrated to regulate cell spreading and possibly adhesion in intestinal epithelial cells. CK2 has also been shown to regulate cell adhesion; thus, Coro1C could be a CK2 substrate that regulates this process. To determine if this serine phosphorylation occurs in VSMCs, endogenous Coro1C should be immunoprecipitated and protein analyzed by mass spectrometry. Additionally, Coro1C phosphorylation should be assessed in the presence of a CK2 inhibitor and activator.

Coro1C also contains other predicted phosphorylation sites (Table 4.2). Serine 299 and tyrosine 301 are found in the β-propeller region of the protein. ERK, glycogen synthase kinase 3 (GSK-3), CDK4, and G protein-coupled receptor kinase 1 (GRK-1) are predicted to phosphorylate this residue. Prediction data suggest that tyrosine phosphorylation of the 301 residue is mediated by the PDGFR. Phosphorylation in this region could possibly regulate Coro1C protein interactions. Additionally, domain deletion mutations have demonstrated that these regions are important for controlling secretion, macropinocytosis, and neuronal outgrowth (208), further supporting the idea that phosphorylation of this region may regulate the previously described processes. Consequently, the study of these sites could contribute vastly to understanding VSMC vesicular trafficking. Initial studies should be aimed at determining if these sites are

Conserved Phosphorylation Site	Predicted Kinase
S-299	CK, ERK, GSK-3, CDK4, GRK-1
Y-301	PDGFR
Y-304	Unknown

Table 4.2. Predicted Coro1B Phosphorylation Sites and Kinases. Potential Coro1C phosphorylation sites were identified by comparing conserved predicted phosphorylation sites of rat, human, and mouse using the Human Protein Reference Database Phosphosite Prediction program and NetPhosK 1.0 server. Sites were filtered by comparing them to known phosphorylation sites identified through Cell Signaling Phosphosite database.

phosphorylated *in vivo*, and by which kinases. The creation of phosphorylation mutants is also necessary to determine the functional importance of the phosphorylation events. Most importantly, the significance of the phosphorylations should be determined with assays measuring secretion and endocytosis.

4.3 Coronin as an Effector of PKC

PKC expression and activity is associated with many physiological and pathological processes throughout the body. Malicious PKC signaling has been associated with many disease processes such as tumor progression, hypertension, atherosclerosis, and diabetes (220, 309, 310). Recently, due to their role in many diseases, PKCs have been the target of many small molecule inhibitors created by pharmaceutical companies. However, to make specific inhibitors that have minimal side effects, thorough investigation of PKC signaling pathways are required.

The PKC kinase family consists of ten isotypes subdivided into 3 families. It is not uncommon that PKC isotypes have disparate functions depending on the cell type and the cellular environment in which they are expressed. Therefore pan-PKC inhibitors could have deleterious side effects in a given system. For example, PKCε and δ have opposite roles in the preconditioning process in the heart, with PKCε having a positive role and PKCδ having an inhibitory role (223). Under these conditions having an inhibitor that negatively regulated the activity of both enzymes would be of no advantage. Hence, inhibitors of specific isozymes are necessary. Above isozyme specificity, inhibitors that obstruct direct PKC substrate interaction and phosphorylation may limit side effects.

This work has identified Coro1B as a PKCε substrate and has shown that phosphorylation of Coro1B positively regulates PDGF-induced migration. Increased PKCε activity increases neointimal hyperplasia in the rat model of balloon injury with stenting (228). While PKCε has been shown to play a pivotal role in migration of VSMCs

(251), how it does so remains to be fully explored. Here we suggest that Coro1B mediates some of the effects of PKCε on PDGF-induced VSMC migration. Of relevance, PKCε has been demonstrated to interact with or phosphorylate a diverse subset of proteins such as cytoskeletal proteins, kinases, ion channels, transcription factors and binding/anchoring proteins (311). Unnecessarily inhibiting the phosphorylation of these other proteins could lead to unwanted secondary effects, suggesting that a better strategy would be to target PKCε-Coro1B interactions.

4.4 Coronin and ROS

Vast amounts of data have shown that ROS are not only a by-product of cellular respiration but also act as signaling molecules (90, 312). Overproduction of ROS can lead to harmful signaling and disease progression. Specifically, in the vasculature deviant ROS production is linked to hypertension, atherosclerosis, and restenosis (90, 313). A common source for this ROS in the vasculature is the NADPH oxidase family. PDGF stimulation of VSMCs activates Nox1 in VSMCs to increase ROS production, which is required for full PDGFR activation (101, 111). Nox1 signaling is associated with the progression of neointimal formation and Ang II induced-hypertension (111, 314, 315). In VSMCs, Nox1 activity is regulated by cytosolic subunits p47phox and NoxA1 along with the small GTPase Rac. Recent data also demonstrate that scaffold proteins tyrosine kinas substrate 4 (Tks4) and Tks5 can also activate Nox1 in lieu of p47phox in colon cancer cells (316). Tsk5 is expressed in VSMCs and regulates the ROS dependent formation of actin rich structures known as podosomes/invadipodia other cell types (317, 318).

Data collected by our lab and others have attributed PDGF-induced Nox-derived superoxide production to activation to Nox1. In chapter 3, we present data demonstrating that Coro1B knockdown in VSMCs increases PDGF-induced superoxide

and hydrogen peroxide production. Collectively, these data suggest under ordinary conditions Coro1B negatively regulates PDGF-induced Nox-derived superoxide and hydrogen peroxide production in VSMCs. These data are consistent with a negative role for Coro1B in migration, because ROS derived from Nox1 are required for PDGF-induced migratory signaling. Due to the multifaceted properties of Coro1B, the pathways by which it could mediate its effects on ROS production are numerous.

Coro1B regulation of PDGF-induced superoxide production could be mediated by sequestering known cytosolic subunits p47phox and/or NoxA1 (p67phox homologue) in the cytosol. Coro1A can be found in a complex with p40phox and p67phox in phagocytes (176). Due to sequence similarity with Coro1A, Coro1B could possibly interact with these proteins in a similar manner. Phosphorylation of p47phox by PKC is required for its translocation and activation in VSMCs (319). Because both p47phox and Coro1B are known substrates for PKC, the possibility exists that coordinate phosphorylation of these proteins may disrupt a putative interaction between them. Additionally, Src mediated tyrosine phosphorylation of residue 110 in NoxA1 and 508 in Tks4 regulate Nox1 activity (320). Additionally, prediction software suggests that Coro1B can be phosphorylated by Src. Src also indirectly regulates p47phox serine phosphorylation, so a similar interaction dependent upon Src-mediated phosphorylation could also be postulated. However, for this hypothesis to be true, Coro1B would have to bind to or co-immunoprecipitate with p47phox, NoxA1, Tks4 and/or Tks5.

A role for coronins in vesicular trafficking has been described. Coro1A has long been associated with the NADPH oxidase in macrophages. Coro1A coats the phagosome and regulates the fusion of the phagosome with the lysosome. NADPH oxidase vesicular trafficking is regulated by multiple Rab proteins (285, 321, 322). Rab27 regulates NADPH oxidase vesicular recycling in macrophages (285). Moreover, Coro1B and Rab13 expression are jointly regulated in neuronal outgrowth. Therefore, Coro1B

may regulate PDGF-induced Nox-mediated ROS production by modulating Rab activity and vesicular recycling.

The study of the role of Coro1B in PDGF-induced Nox-derived ROS production is currently in its infant stages. To truly define the function of Coro1B in PDGF-induced ROS production, overexpression experiments should also be completed. Considering that downregulation of Coro1B increases ROS production, overexpression of the protein could possibly decrease ROS production. Additionally, knockdown and add back experiments would confirm the role of Coro1B in this process. Subsequently, the interaction of Coro1B with Nox1 cytosolic subunits should be determined. If a positive interaction is discovered, the regulation of this interaction by the agonist PDGF should be investigated. Phosphorylation is a common mechanism by which interactions are regulated in cells. Therefore, the role of Coro1B phosphorylation (known and predicted) on PDGF-induced ROS and protein interactions should be assessed. Coro1B may also modulate ROS production via regulation of vesicular trafficking and recycling. Therefore, measuring changes or alterations in NADPH trafficking and recycling is also required.

Downregulation of Coro1B also unilaterally increased hydrogen peroxide production in response to PDGF. In siNegCtrl transfected samples, Coro1B downregulation by PDGF did not stimulate NADPH-derived hydrogen peroxide production. However, PDGF stimulation of siCoro1B transfected samples significantly increased NADPH derived ROS production. Under normal conditions, PDGF stimulation of the NADPH oxidase primarily produces superoxide with little measurable hydrogen peroxide. Data collected here suggests that along with an increase in superoxide, there is also an increase in the conversion of superoxide to hydrogen peroxide in siCoro1B transfected PDGF-stimulated samples. Increases the conversion of superoxide to hydrogen peroxide could be mediated by an increase in superoxide dismutase (SOD) expression and/or localization to the endosome. SOD is an enzyme that converts

superoxide to hydrogen peroxide. Future experiments should also include measuring possible changes in SOD expression and localization in VSMCs deficient in Coro1B.

4.5 Coronin and Disease

Due to their cellular functions, coronins can and do function in a host of diseases throughout the body. Data provided in this dissertation support a role for Coro1B in cardiovascular disease progression. Furthermore, the literature also confirms a role for coronins in cancer, immune responses, neurological diseases and disorders. Consequently, studies into the mechanisms of type I coronin regulation and expression are warranted to combat a host of pathological diseases.

4.5.1 Coronins and Cardiovascular Disease

In VSMCs, the increased phosphorylation of Coro1B by PDGF is a positive stimulus for migration by decreasing its inhibitory interaction with the Arp2/3 complex. The Arp2/3 complex is relieved of Coro1B inhibition and it is free to interact with NPFs and promote actin nucleation, positive changes in lamellipodia dynamics, as well as migration. Based on our preliminary *in vivo* data, Coro1B is phosphorylated in response to carotid wire injury in mice and balloon injury in rat. Our data support the idea that Coro1B phosphorylation may be necessary for VSMC migration and neointimal formation. However, to prove this hypothesis the creation of smooth muscle specific knock out and transgenic animals would be required. Transgenic animals would include an overexpression mouse model as well mice containing a non-phosphorylatable point mutation at serine 2. All animals would be subjected to both femoral and carotid wire injury and restenosis would be measured in these animals through morphometric analysis. Both the overexpression as well as the point mutation models would be expected to reduce neointima formation in injured vessels. Coro1B knockouts should

have increased neointimal formation. Changes in extracellular matrix deposition and proliferation should also be measured.

Coro1B expression and signaling may also regulate other cardiovascular diseases such as hypertension and atherosclerosis. Data from the literature also suggest that Coro1B could regulate VSMC contraction through an inhibitory interaction with the kinase ROCK. The contraction of VSMCs is not only important in migration and restenosis, but in hypertension as well. To evaluate a role for Coro1B in agonist-induced hypertension, the effects of AngII infusion on blood pressure in knock out and transgenic animals should determined. The effects of Coro1B on hypertensive states of genetically bred hypertensive mice would also be measured by crossing transgenic and knock out animals with the inbred strain. Coro1B and 1C may also participate in VSMC vesicular trafficking. Vesicular trafficking is required for lipid homeostasis and growth factor and extracellular matrix secretion. VSMC migration, lipid homeostasis, proliferation, and matrix secretion culminate to promote atherosclerosis under certain conditions. To determine the role of Coro1B in this process, Coro1B knockout and transgenic animals should be crossed with ApoE knockout animals and fed a high fat diet to promote atherogenesis in mouse animal models. Since Coro1B has the potential to regulate all the pathways described above, it could in fact be a pivotal regulator of vascular lesion formation.

4.5.2 Coronins and Cancer

Cancer is a general classification for a group of diseases in which the hallmark characteristic is uncontrolled cell growth (2, 128). The cause of this uncontrolled growth is generally due to mutation or abnormal activation of genes and ultimately proteins that control growth. Cancerous cells become exceedingly malignant as they gain the ability to metastasize or migrate from their point of origin. Hence, mutations that enhance cell

migration are advantageous to this process. The upregulation of Coro1C in many aggressive cancers has been documented. In nonendometriod endometrial carcinoma, head and neck squamous cell carcinomas, melanoma, hepatocellular carcinoma, primary effusion lymphoma and diffuse gliomas, Coro1C expression is upregulated and corresponds with an increase in cancer malignancy (210, 211, 282). In gliomas, Coro1C upregulation increases proliferation, extracellular matrix invasion, and cell velocity linking Coro1C to an increase in invasion and metastasis (207).

As of yet Coro 1B has not been associated with cancer progression and disease. However, Coro 1A expression has been associated with increased invasiveness in SK-BR3 breast cancer cells (323). Additionally, both Coro1A and 1B are downstream of PKC signaling which suggests a possible role for the coronins as regulators of signaling that promote tumor progression. Specifically, PKCε signaling is associated with increases in cell motility and metastasis in human primary non-small cell lung cancer through regulation of the small GTPase Rac (324). Coro1A regulates Rac activity through an interaction with the Rac GEF β-PIX (145). Therefore, Coro1A could promote cancer malignancy via this mechanism. Additionally, Coro1B is a specific downstream effector of PKCε, making it a possible participant in cancer progression. Both Coro1A and Coro1B interact with ROCK, thereby providing another avenue to control carcinogenesis. ROCK participation in carcinogenesis is fairly cell type specific. In melanoma cells ROCK activation promotes invasiveness (325). On the other hand, in human colon carcinoma cells ROCK activity attenuates cell migration and matrix invasion (326). These data suggest that the role of Coro1A or 1B could be cell type specific in cancer as well.

4.5.3 Coronins and the Immune Response

Coronins have long been studied in the immune system. The most widely studied coronin, Coro1A, was initially identified as a phagosome coat protein in phagocytic cells of the immune system (174). Although it is not completely understood if Coro1A is required for phagocytic function, it does regulate calcium dependent signaling in lymphocytes and regulates apoptosis in thymocytes as well (185, 186). Of pharmacological interest is the recruitment of Coro1A to phagoctic vesicles containing mycobacteria. Recruitment of these proteins halts the fusion of the phagosome and the lysosome, prolonging the life of the mycobacteria (327). This pathway is used by a host of bacteria evading destruction. These data support a role for Coro1A in bacterial evasion. Importantly this pathway is used by the pathogen *M. tuberculosis* to evade host defense (328). The creation of drugs that inhibit this recruitment and modulate host responses to these bacteria would be beneficial for hard to treat and persistent infections. Additionally, Coro1A is upregulated in the mouse model of cardiac allograft rejection making it a possible diagnostic maker for monitoring alloimmune responses (329). Although expressed in immune cells, Coro1B is dispensable for mast cell functions. Coro1C is also expressed in cells of the immune system; however, data suggest that it plays a pathophysiological role due to its upregulation in lymphoma and peripheral blood mononuclear cells from human immunodeficiency virus (HIV) positive patients (330). Although the functions of Coro1A have been highly explored in the immune system, the roles of Coro1B and 1C have largely been ignored. Data from multiple cell types have demonstrated that although some coronin functions are redundant, not all are, and therefore the roles of Coro1B and 1C in immune function should be evaluated.

4.5.4 Coronins, Neuronal Plasticity, and Schizophrenia

In the brain modulation of the actin cytoskeleton is required for lamellipodia and filopodia formation; both are a prerequisite for neuronal outgrowth. All type I coronins are expressed in the brain. Both Coro1B and 1C have been shown to directly regulate neuronal outgrowth. In PC-12, neuroblastoma and dorsal root ganglion neurons the overexpression of Coro1B and Rab13 promotes neuronal outgrowth and siRNA against Coro1B or Rab13 reduces neuronal outgrowth in PC12 cells (199). In *in vivo* models of spinal injury, both Coro1B and Rab 13 expression are upregulated and *in vitro* models of injury inhibition of Coro1B and Rab expression inhibits healing. However, to completely establish a role for Coro1B in regeneration in response spinal injury the use of knockout animals should be included in future studies. Nevertheless, this suggests that actin regulation and vesicular trafficking are required for neuronal plasticity and outgrowth. Neuronal outgrowth in these cells has been directly linked to upregulation of p53-mediated transcriptional regulation of Rab 13 and Coronin1B (331). This p53 activity is regulated by the acetylation of both cytoskeletal protein promoters (332). The schizophrenia related protein dysbindin-1 also regulates neurite outgrowth, p53 activity, and therefore Coro1B and Rab13 expression through recruiting the protein nectin to the cytosol and releasing its repressive effects on p53 (333). Dysbindin is a protein associated with vesicular trafficking and is shown to regulate neuronal outgrowth and dendritic spine formation (334). Since Coro1B is transcriptionally regulated by dysbindin, it would not be surprising if Coro1B played a role in the pathology associated with schizophrenia.

Although Coro1B works to modulate migration in both spinal cord injury and restenosis, it does so by opposite means. Activation of p53 is inhibitory for VSMC migration in restenosis (335, 336), whereas it is required for neuronal outgrowth in

neuronal systems (331). Although p53 oppositely regulates these events, its regulation of Coro1B can be the same. Overexpression of Coro1B inhibits PDGF-induced migration. This supports the ideas that p53 regulates the transcription of Coro1B and that activation of p53 transcriptional activity can inhibit VSMC migration. This again suggests that the actions of Coro1B can indeed be cell type specific.

In neurons, Coro1C is expressed throughout the brain and is required for neurite outgrowth and differentiation. Coro1C also regulates the secretion of epinephrine in these cells as well (205). Appropriate vesicular transport is also a necessity for proper axonal guidance and growth. In other cell types Coro1C mediated vesicular trafficking is regulated by Rab27 (146). Vesicular trafficking in neurons is important in neurotransmitter release and uptake. Therefore, Coro1C may play a large role in neuronal communication and outgrowth. CK2, a known Coro1C kinase, regulates neuronal outgrowth through phosphorylation of cytoskeletal proteins such as GAP-43 and the Nogo receptor in neuronal cells (337, 338). With the discovery of CK2 as a regulator of Coro1C interactions, it is possible that CK2 modulates neuronal cells via Coro1C.

4.6 Summary and Future Directions

We describe a mechanism by which PDGF, acting through PKCε, regulates the phosphorylation of the actin binding protein Coro1B and its interaction with the Arp2/3 complex to modulate lamellipodia formation and migration. Additionally, Coro1B knockdown increases PDGF-induced ROS production in VSMC migration, thus providing another mechanism by which Coro1B can regulate PDGF-induced VSMC migration. The Coro1B phosphorylation response occurs *in vivo* as a reaction to vessel injury, suggesting a role for Coro1B in the restenotic response. Future experiments with knockout and transgenic animals will definitively determine the role of Coro1B in vessel

remodeling caused by restenosis and atherosclerotic lesion formation. Furthermore, the ubiquitous nature and the multifunctional properties of the protein make it a possible participant in a multitude of cellular processes that regulate numerous physiological and pathophysiological states.

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