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Role of Coronin 1B in PDGF-induced Migration of Vascular Smooth Muscle Cells

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Abstract

Rationale—The type I subclass of Coronins, a family of actin binding proteins, regulates 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.

Objective—The goal of the present study was to define the mechanism by which coronins regulate platelet-derived growth factor (PDGF)-induced VSMC migration.

Methods and Results—Coronin 1B (Coro1B) and 1C (Coro1C) were both found to be expressed in VSMCs at the mRNA and protein levels. Down regulation of Coro1B by siRNA increases PDGF-induced migration, while down regulation of Coro1C has no effect. We confirmed through kymograph analysis that the Coro1B-downregulation-mediated increase in migration is directly linked to increased lamellipodial protraction rate and protrusion distance in VSMC. In other cell types, coronins exert their effects on lamellipodia dynamics by an inhibitory interaction with the ARP2/3 complex, which is disrupted by the phosphorylation of Coro1B. We found that 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 was increased in response to vessel injury in vivo.

Conclusions—Our data support that phosphorylation of Coro1B and the subsequent reduced interaction with ARP2/3 complex participate in PDGF-induced VSMC migration, an important step in vascular lesion formation.

Keywords

vascular smooth muscle; coronin 1B; migration; platelet-derived growth factor

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Disclosures
None

Introduction

The migration of cells is important in multiple facets of normal and aberrant cell behavior. In the vasculature, the migration of vascular smooth muscle cells (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 percutaneous coronary intervention (PCI).[1, 2] In these latter diseases, one of the major characteristic changes is the accumulation of VSMCs within the intimal layer of the blood vessel.[3, 4] 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. Genetic manipulation of vascular cells combined with multiple inhibitory strategies have provided strong evidence that platelet-derived growth factor (PDGF) plays an important role in the migration of VSMCs into the neointima following acute injury and in atherosclerosis.[5] Although PDGF has been shown to be the primary regulator of VSMC migration *in vivo*, little is understood about the molecular mechanisms and intracellular signaling pathways that contribute to this migration.

Migration is a dynamic and cyclic process that generally begins with the extension of actin rich protrusions called lamellipodia.[6] 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.[7] Lamellipodia formation is regulated by the actin related protein 2/3 complex (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 distinctive 70° angle, leading to the formation of branched filament networks[8] that are required for efficient cell migration. For full activation, the ARP2/3 complex must bind with actin filaments and activator proteins such as Wiskott–Aldrich Syndrome protein (WASp) and suppressor of cAR /WASP family Verprolin-homologous protein (SCAR/WAVE).[9] 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.[10, 11]

Coronins are a family of evolutionarily conserved WD-repeat actin-binding proteins known to control a variety of cellular processes involving actin dynamics.[12] The coronin protein family includes 7 proteins in mammals, separated into three subclasses (type I, II, and III) based on phylogenetic similarity.[13, 1415] The type I coronins consist of coronin 1A (Coro1A), 1B (Coro1B), and 1C (Coro1C) and are the most studied coronin subfamily. 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.[16] On the other hand Coro1B and Coro1C are more ubiquitously expressed at higher levels in most tissues. [11] Coro1B localizes to the plasma membrane, regulates lamellipodia formation, and when phosphorylated is no longer able to bind ARP2/3 and inhibit its actin nucleation abilities.[11] In contrast, in epithelial cells, Coro1C was demonstrated to regulate focal adhesion dynamics and increase wound closure.[17] Thus, Coro1B 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 there have been significant advances in identifying the functions of 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, the goal of the present study was to determine if Coro1B and 1C are present in VSMCs and to explore the mechanism by which they might regulate PDGF-induced VSMC migration. Here, we provide data that demonstrate that Coro1B and 1C are expressed in VSMCs, and that Coro1B is highly phosphorylated after stimulation by the physiological agonist PDGF

in VSMC. The consequence of this phosphorylation is a decreased interaction of Coro1B with the ARP2/3 complex, leading to an increase in PDGF-induced VSMC migration. We also found that Coro1B phosphorylation is induced in the rat carotid balloon injury and mouse carotid wire injury model of neointimal formation. These data suggest that PDGF signals to Coro1B to coordinate lamellipodia formation and thus migration in VSMCs, and suggests a new therapeutic target for vasculopathies with a significant migratory component.

Methods

Materials

Recombinant human PDGF-BB was purchased from R&D Systems Inc. Rö-32-0423 and Gö-6796 PKC inhibitors were purchased from Calbiochem. Primary antibodies were purchased from Sigma (α-smooth muscle actin and β-actin), 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)), Cell Signaling Technologies (Myc (9B11 and 71D10) and PKCε)and Millipore (ArpC2). Mouse monoclonal antibody against coronin 1c was created in the laboratory of Dr. James Bear as previously described.[11] Streptadividin conjugated Quantum dots with a 603 fluorescent label were purchased from Invitrogen. siGlo Red transfection indicator was purchased from Dharmacon RNAI Technologies.

Cell Culture

Vascular smooth muscle cells (VSMC) were isolated from rat thoracic aorta by enzymatic digestion as previously described.[18] Isolated VSMCs were grown in Dulbecco's Modified Eagle's Media supplemented with 10% calf serum. 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.

Reverse Transcriptase PCR (RT-PCR)

RNA was purified from rat lung tissue, and VSMCs using the RNeasy kit (Qiagen), following the manufacturer's instructions. The purified RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) using random nanomers. The resultingcDNA 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: 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′), and 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′). 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′)

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 × 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.

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 × *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.

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 by Quick Change site-directed mutagenesis kit (Strategene) using 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′).

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 1.5×10^6 cells, or with 3 μg of annealed siRNA duplexes for Coro1B, Coro1C, PKCε or nonsilencing control sequence no. 1 from Qiagen per 1.5×10^6 cells. siRNA target sequences were as follows: Coro1b (5′-CAG CAC CTT CTG CGC AGT CAA -3′), Coro1c (5′-ACG AGA GAA AGT GTG AAC CTA -3′) and PKCε (5′-CCC GGG AAG AGC CAA TAC TTA -3′). The cells were transfected, allowed to attach and recover for 24 hours and then serum starved for 24–48 hours. Cells that were used for single cell tracking and kymography experiments were also co-transfected with 1 μg siGlo to visually detect siRNA transfection.

Modified Boyden Chamber Assay

Migration was measured using a modified Boyden chamber assay as previously described.[19, 20] Briefly, cells were grown to 60%confluence and then made quiescent in serum-free media for 48hours before migration. Membrane inserts were coated with 5 μg/cm^2 of type I rat tail collagen (BD Bioscience). VSMC were added at a density of 5×10^4 cells/well to the upper chamber of a Transwell dish with a 6.5-mm polycarbonate membrane insert containing 8-μm pores (Costar). VSMC were then exposed to PDGF (10 ng/mL) in the lower chamber and allowed to migrate for 4 hours. Nonmigrated cellswere 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 from three independent experiments. Images were quantified using ImageJ software.

Single Cell Tracking

Cells 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. Cells 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. Cells were transfected and migration was observed in two independent experiments.

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 4s for 4 min at 80x on the 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.[21, 22]

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 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.[23] 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.

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, 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.

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 then 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.

Statistics

Results are expressed as means ± SEM. Differences amonggroups 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.

Results

Expression Pattern and Role of Type I Coronins in VSMC Migration

There have been no studies to date examining coronin expression or function in VSMCs. To elucidate if Coro1B and Coro1C are expressed in VSMCs, we used reverse transcription PCR. Using RNA from rat lung tissue homogenates as a positive control, we discovered that both proteins are expressed in VSMCs (Figure 1A.) 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 Coro1B and 1C mRNA (Online Figure I A). Both, Coro1B and 1C proteins were detectable by western blot, and we were able to specifically knock down each protein (Online Figure II and Figure 1B). On average Coro1B was down regulated by 87 ± 3% and Coro1C by 68 ± 5%.

As previously stated, coronins have been shown to serve a significant function in actin dependent processes such as migration. To determine if either Coro1B or 1C plays a role in VSMC motility stimulated by PDGF, we used siRNA against either 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 1C and Online Figure III). Cells in which Coro1B was down regulated, showed increased PDGF-induced VSMC motility compared to siNegative control (siNeg Ctrl) transfected samples (210.3 ± 8.2 μm v. 166.6 ± 8.2 μm, $p< 0.001$) (Figure 1C, Video File I, Video File II and Online Figure IV). However, the down-regulation of 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, $p>0.05$) (Figure 1C). These data suggest that Coro1B plays a negative regulatory role in VSMC motility and that Coro1C does not modulate PDGF-induced motility in these cells.

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

To gain insight into how Coro1B might regulate migration, we examined its localization in VSMCs. We observed specific immunofluorescent staining at the cell periphery, in the cytosol and in the peri-nuclear region (Figure 2A). Coro1B also co-localizes with actin stress fibers when over expressed (Figure 2B).

Because of its membrane localization and the previous demonstration that Coro1B modulates lamellipodia dynamics in fibroblasts,[21] we next examined the effects of siRNA against Coro1B on PDGF-induced VSMC lamellipodia dynamics using Kymography (Figure 3A). siCoro1B transfected samples showed increased PDGF-induced lamellipodia protrusion rate (4.9 ± 0.2 μm/min v. 3.7 ± 0.2 μm/min, $p<0.0001$) (Figure 3B), and protrusion distance (1.4 ± 0.04 μm v. 1.1 ± 0.03 μm, $p< 0.0001$) (Figure 3C) when compared with siNeg Ctrl transfected samples. However, inhibition of Coro1B expression did not significantly affect PDGF-induced changes in lamellipodia persistence (Figure 3D). These changes in lamellipodia dynamics correspond to the changes that were observed in VSMC distance traveled and velocity (Figure 1C and Online Figure IV), supporting the notion that Coro1B may normally play an inhibitory role in VSMC migration.

PDGF Stimulation of VSMCs Induces Coronin 1B Serine 2 Phosphorylation via PKCε

To begin to understand how PDGF might regulate Coro1B activity, we focused on phosphorylation. Data presented by Cai *et al*. and others[11, 24] demonstrate that phosphorylation of Coro1B on Ser-2 can be stimulated by phorbol esters, although whether this occurs in response to a physiological agonist is not known. We hypothesized that PDGF would stimulate Coro1B phosphorylation on Ser-2 (p-Ser2 Coro1B) in a protein kinase C (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 4A). We also examined Coro1b phosphorylation in HCoASMC and found a similar pattern of phosphorylation (Online Figure IB). Next we examined the localization of phosphorylated Coro1B. When cells were stimulated with PDGF for 15 minutes there was an increase in p-Ser2 Coro1B staining when compared to unstimulated cells (Figure 4B). When siCoro1B transfected cells were stimulated with PDGF, p-Ser2 Coro1B detection was decreased by western blot and immunofluorescence (Online Figure V A and B). Ser-2 phosphorylated Coro1B stained in a stress fiber pattern as well as the cell periphery and lamellipodia. This pattern of staining was similar to that of total Coro1B (Figure 2A and B).

To determine if the phosphorylation of Coro1B is mediated by PKC, we used the pan-PKC inhibitor Rö-32-0432 (0.5 μmol/L). Rö-32-0432 reduced PDGF- induced Coro1B phosphorylation by 88 ± 10% compared to control treated samples (Figure 5A). Next we employed the use of classical PKC inhibitor Gö-6976 (0.5 μmol/L) and specific PKC siRNAs to narrow down the identity of possible PKC isoforms. Use of Gö-6976 and siRNA against PKCα had no effect on PDGF-induced Coro1B phosphorylation (Online Figure VI A and B). The lack of an effect of these reagents suggested that classical PKC isoforms were not responsible for Coro1B Ser-2 phosphorylation. Thus, we examined novel PKCs that had known cytoskeletal substrates and found PKCε to be a viable candidate.[25, 26] When siRNA against PKCε was transfected into VSMCs there was a significant decrease in PDGF-induced Coro1B phosphorylation (40 ± 3.7%) compared to PDGF stimulated siNeg Ctrl (Figure 5B). These data strongly suggest that PKCε phosphorylates Coro1B in response to PDGF stimulation in VSMCs.

Coronin Over Expression and Phosphorylation Deficient Mutant Decrease Migration

Next we assessed the importance of Coro1B over expression and phosphorylation state on PDGF-induced VSMC migration. We transfected wild type Coro1B (WT) or the phospho-deficient S2A mutant into VSMCs and measured their effects on PDGF-induced cell migration. Using the modified Boyden chamber assay, we observed a decrease in cell migration when cells were transfected with Coro1B WT (44 ± 11%), this decrease was exacerbated in the S2A mutant (71 ± 0.8%) when compared to empty control vector transfected cells (Figure 6A). To confirm expression and correct targeting and localization of Coro1B WT and mutant Coro1B S2A, we used western analysis and immunocytochemistry. We observed that Coro1B WT and S2A were expressed at or above endogenous Coro1B levels (Figure 6B) and Coro1b S2A had a similar localization pattern as endogenous Coro1B (compare Figure 6C and Figure 2), suggesting that mutant Coro1B S2A was being targeted to similar areas of the cell as the endogenous protein. Together, these data suggest that the phosphorylation state and expression of Coro1B is an important factor in the induction of PDGF mediated VSMC migration.

PDGF Stimulation Disrupts Coronin1B and ARP2/3 Complex Interaction

Previous data[11, 21] suggest that Coro1B and the ARP2/3 complex interact and that this complex can be dissociated by Coro1B phosphorylation.[11, 16] The interaction of Coro1B with the ARP2/3 complex inhibits ARP2/3 actin nucleation activity[21] which can possibly negatively affect lamellipodia formation and migration. We therefore 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 7A). This inhibition of interaction occurred during the time of peak PDGF induced Coro1B phosphorylation (Figure 4). 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 less co-immunoprecipitation with the ARP 2/3 complex subunit ARPC2, than the phospho-deficient mutant S2A by 48 ± 7% (Figure 7B). Over expression 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 over expressed protein (Online Figure VII). 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

Coronin 1B is Phosphorylated in the Neointima After Vascular 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. In Figure 8A, 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 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. However, at 14 days post surgery, there was a significant 63±11% increase in p-Ser2 Coro1B in injured vessels. 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, at which time VSMC are actively migrating.[27] In the mouse,

migration and proliferation begin 7 to 14 days post injury and are generally complete at 21 days.[23] In the rat, neointimal formation is generally complete at 14 days. We chose 10 days in the rat to capture a similar condition. P-Ser2 Coro1B was observed at low levels in the media of uninjured vessels (Figure 8B, panel a). In vessels from injured animals, there was significant staining in the neointima (Figure 8B, panel b). This staining pattern correlated closely to that of α-smooth muscle actin (Figure 8B panels c and d). These data suggest that Coro1B phosphorylation *in vivo* occurs in cells that are phenotypically modulated to migrate and proliferate.

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.[7] 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.[28, 29] 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.[21] SSH1L has been shown to dephosphorylate Coro1B as well.[21] 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*[21, 28] found that Coro1B expression actually promotes migration, and that cells deficient in Coro1B showed impaired migratory responses. 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 7), 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 (Online Figure VIII). It should be noted that Rat-2 cells are an immortalized cell line with

limited tumorgenicity,[30] 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. 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 stimulate 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 3). The amplitude of these changes were 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,[10, 11] 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 4). We have also shown that Coro1B phosphorylation and expression is regulated *in vivo* (Figure 8). 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.[31] 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,[32] 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,[25] cytokeratins 8 and 18, [26] and diacylglycerol kinase.[33] 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.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations and Acronyms

VSMC	Vascular smooth muscle
cell Coro1A	coronin 1a
Coro1B	coronin 1b
Coro1C	coronin 1c
PDGF	platelet-derived growth factor
PKCε	protein kinase C ε
ARP2/3 complex	actin related protein 2 and 3 complex
PCI	percutaneous coronary intervention
WASp	Wiskott–Aldrich Syndrome protein
SCAR	suppressor of cAR
WAVE	WASP family Verprolin homologous protein
DAPI	4′,6-diamidino-2-phenylindole
NegCtrl	Negative Control
and SSH1L	slingshot phosphatase 1L

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Novelty and Significance

What is Known?

- Coronins are a family of evolutionarily conserved WD-repeat actin-binding proteins known to control a variety of cellular processes involved in actin dynamics.
- Coronin 1B (Coro1B) and 1C (Coro1C) and are the most widely expressed members of the coronin family.
- Coro1B localizes to the plasma membrane, regulates lamellipodia formation, and when phosphorylated is no longer able to bind ARP2/3 and inhibit its actin nucleation abilities in transformed cells. In contrast, Coro1C regulates focal adhesion dynamics and increases wound closure.
- The expression and the role of coronins in vascular smooth muscle cell (VSMC) migration has yet to be determined.

What New Information Does This Article Contribute?

- Coro1B and 1C are expressed in VSMCs
- Downregulation of Coro1B increases PDGF-induced VSMC migration, while downregulation of Coro1C has no effect.
- Downregulation of Coro1B is directly linked to an increase in lamellipodial protrusion rate and protrusion distance
- PDGF induces the phosphorylation of Coro1B in a PKCε-dependent manner. This event decreases the interaction of Coro1B with the Arp2/3 complex, promoting PDGF-induced migration.
- Vessel injury increases Coro1B phosphorylation in vivo

Although several function of coronins have been identified, little is known about the physiological mechanisms that regulate activation of coronins and their role in VSMCs has not been examined. Here, we show that Coro1B and 1C are expressed in VSMCs, and that Coro1B downregulation increases PDGF-induced VSMC migration. This increase in migration is in direct contrast to what is observed in transformed cells, where migration is decreased when Coro1B is downregulated. We also demonstrate for the first time that in VSMCs Coro1B is highly phosphorylated after stimulation by PDGF. This phosphorylation decreases the interaction of Coro1B with the ARP2/3 complex, leading to an increase in PDGF-induced VSMC migration. Coro1B phosphorylation is stimulated in the rat carotid balloon injury and mouse carotid wire injury model of neointimal formation, suggesting that pathways that lead to Coro1B phosphorylation are active in vivo. These data demonstrate a novel role for Coro1B in PDGF-induced VSMC migration. Further understanding of the intracellular signaling mechanisms by which PDGF induces migration can expand therapeutic options to combat lesion formation.

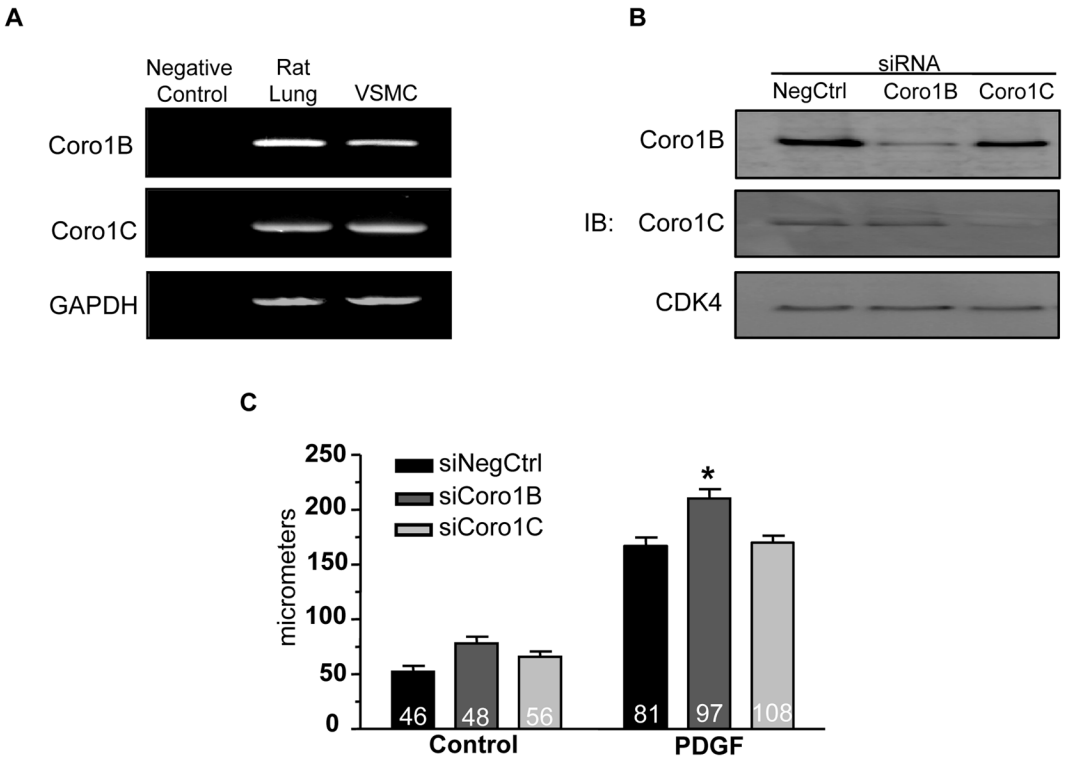

Figure 1. Coro1B and 1C are Expressed in VSMCs, but Only Coro1B Modulates PDGF-induced Changes in Cell Velocity

(A) VSMCs were grown in serum for 48 hours and then harvested for RNA. Coro1B,1C, and GAPDH mRNA was detected using sequence specific PCR primers. Rat lung cDNA was used as a positive control. **(B**) VSMCs were transfected with siNegCtrl, siCoro1B or siCoro1C with the Amaxa electroporation system. 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% (N= 10) and Coro1C by 68 ± 5% (N=5). **(C)** Live cell imaging was used to measure the distance traveled by VSMCs transfected with siNegCtrl, siCoro1B, or Coro 1C, cells were serum starved for 24hrs and then stimulated with 10ng/ml of PDGF for 12hrs. The graph represents the mean ± SEM of the distance traveled by each cell. The N (number of cells) is indicated within each column. (*p< 0.001, PDGF treated siNegCtrl compared to siCoro1B).

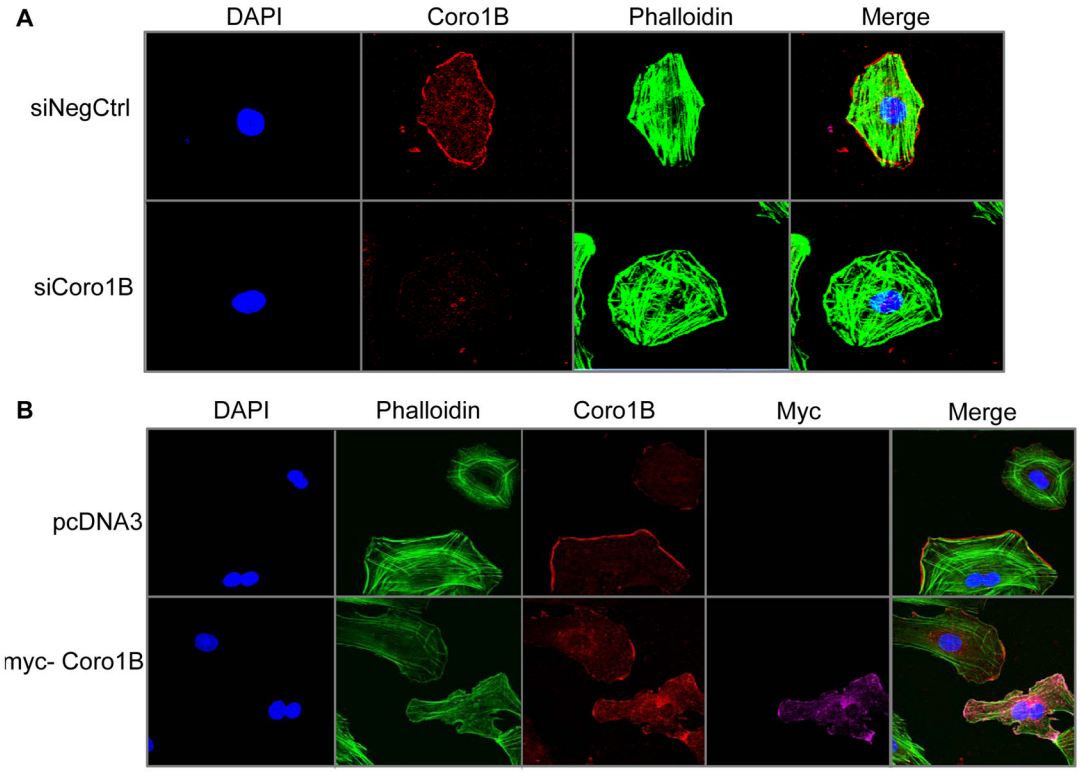

Figure 2. Coro1B Localizes to the Periphery of VSMCs

(A) 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 System. **(B**) VSMCs were transfected with empty vector or Myc- Coro1B with the Amaxa electroporation system, serum starved for 48 hrs and then fixed and permeabilized. Immunofluorescent images using DAPI (blue), phalloidin (green), Coro1B (red) and Myc (magenta) antibodies were then acquired.

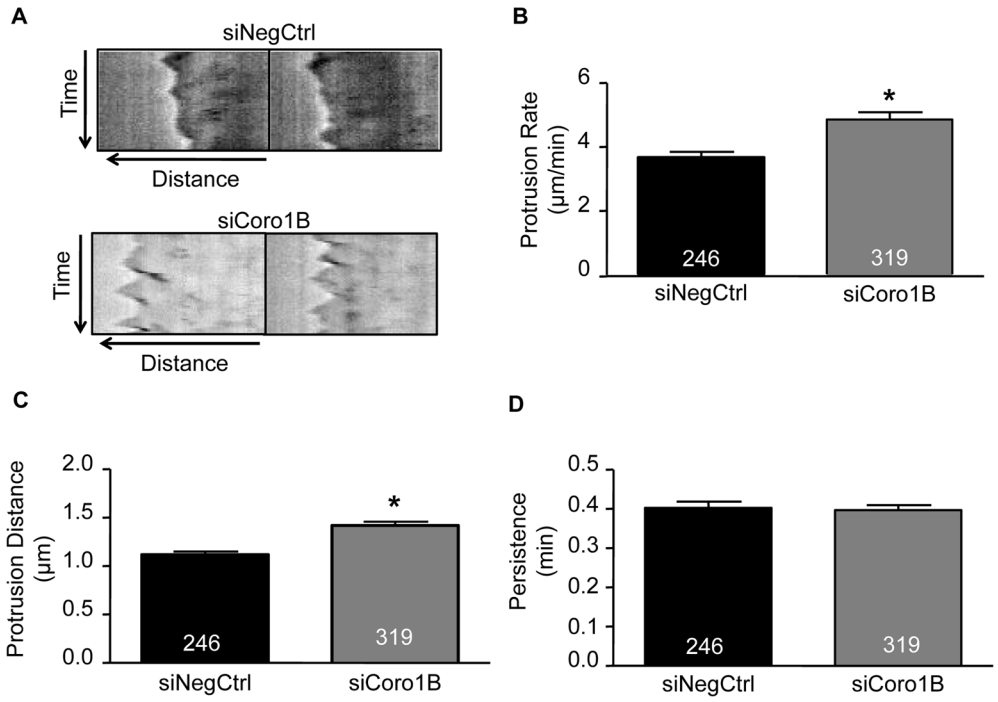

Figure 3. Down Regulation of Coro1B Increases Lamellipodia Protrusion Rate and Distance in VSMCs

(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).

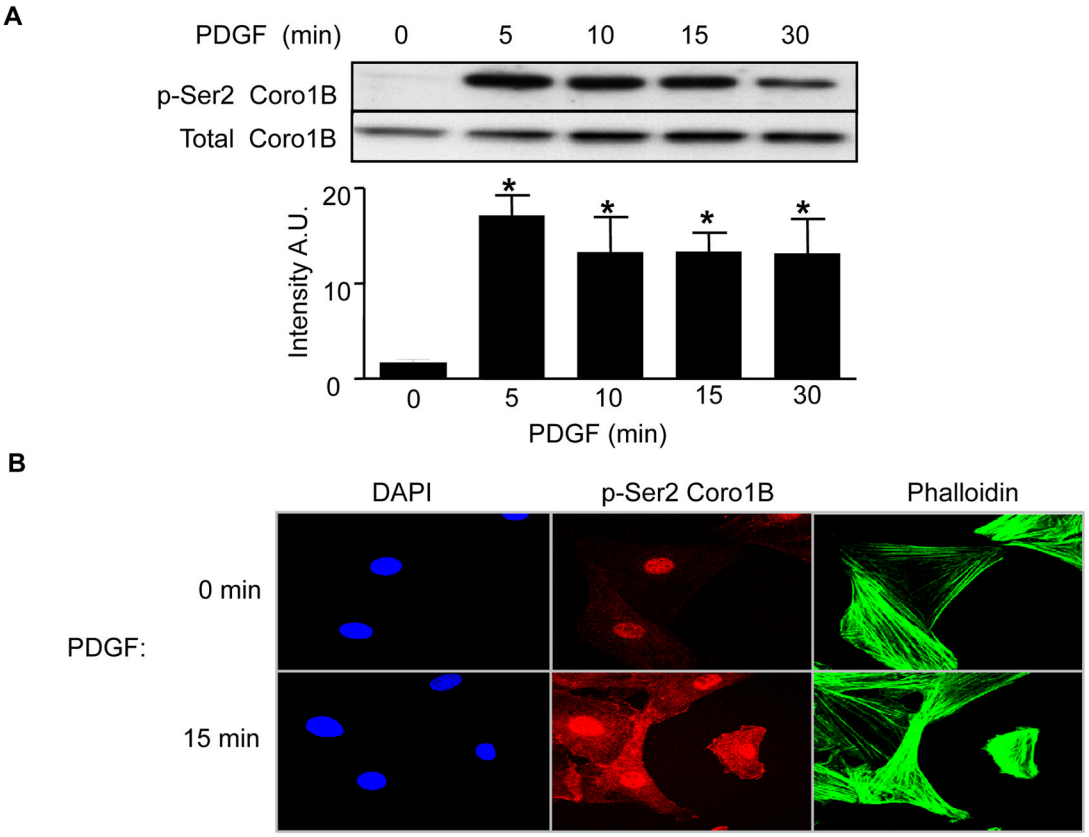

Figure 4. PDGF Stimulates Coro1B Ser-2 Phosphorylation in VSMCs

(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 densiometric analysis from 3–5 independent experiments. (*p< 0.01, compared to control). **(B)** 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 acquired using the Zeiss LSM 510 META Laser Scanning Confocal Microscope System.

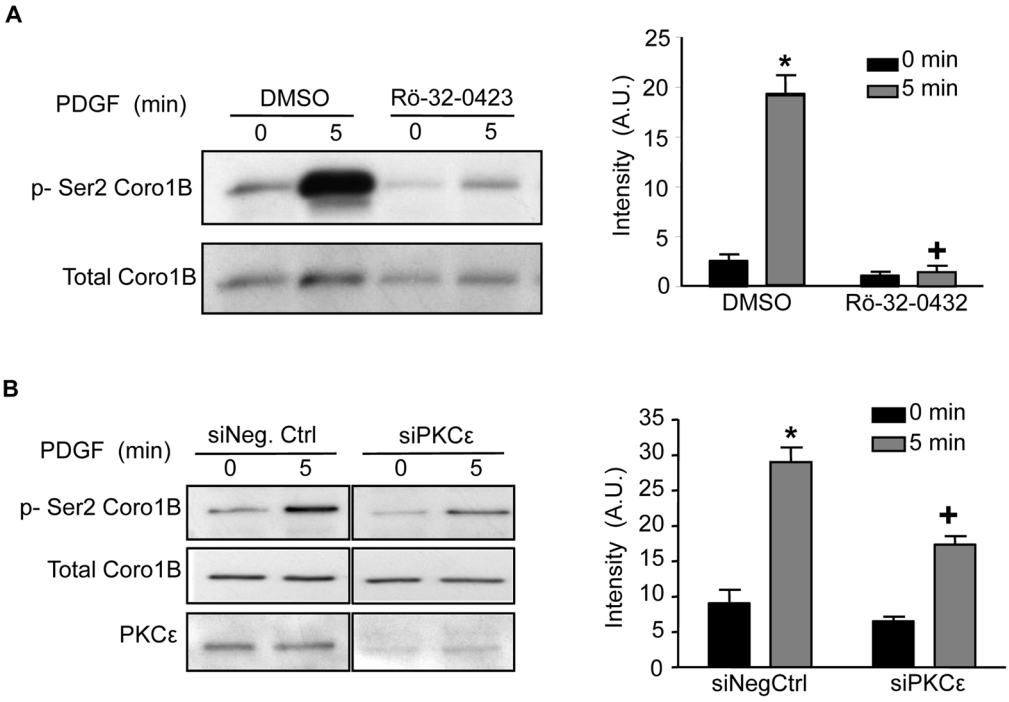

Figure 5. siRNA Against PKCε Attenuates Coro1B Five Minute Phosphorylation

(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 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 antibody. The graph represents the mean ± SEM of densiometric analysis from 4 independent experiments. (*$p < 0.01$ compared to control, = $p<0.0001$ compared to control PDGF treated). **(B**) VSMCs were transfected with 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 densiometric 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).

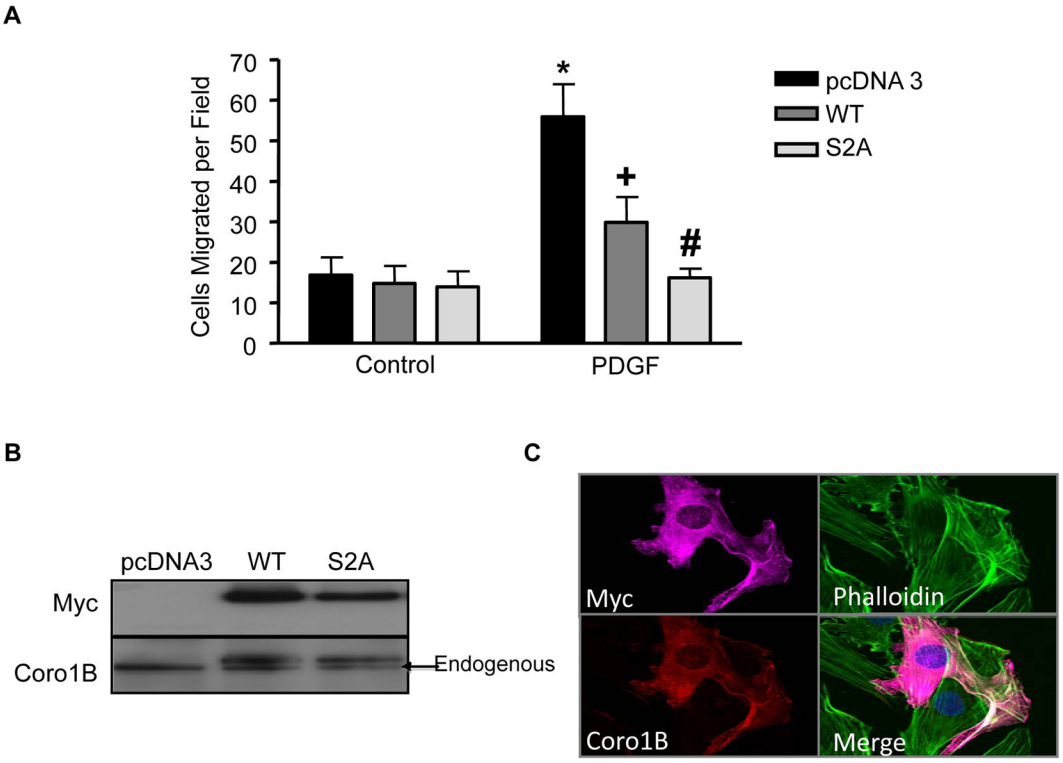

Figure 6. Coronin Over Expression and Phosphorylation Deficient Mutant Decrease Migration

VSMCs were transfected with the Myc tagged Coro1B WT or S2A phospho-deficient mutant using the Amaxa electroporation system and then serum starved for 24hrs. Cells were harvested and migration was assessed via modified Boyden chamber assay **(A).** This graph represents the mean ± SEM of the number of cells migrated per field (*p< 0.001, pcDNA3 vs pcDNA3 with PDGF stimulation, = p < 0.01, PDGF stimulated pcDNA3 vs. PDGF stimulated Coro1B WT transfected cells, and # p < 0.001, PDGF stimulated pcDNA3 vs. PDGF stimulated Coro1B S2A mutant transfected cells N=4). **(B)** Protein lysates from excess cells not used in the migration assay were harvested and immunoblotted with Myc and Coro1B to verify Coro1B WT and S2A protein expression. **(C)** VSMCs were transfected with Myc tagged 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 acquired using the Zeiss LSM 510 META Laser Scanning Confocal Microscope System.

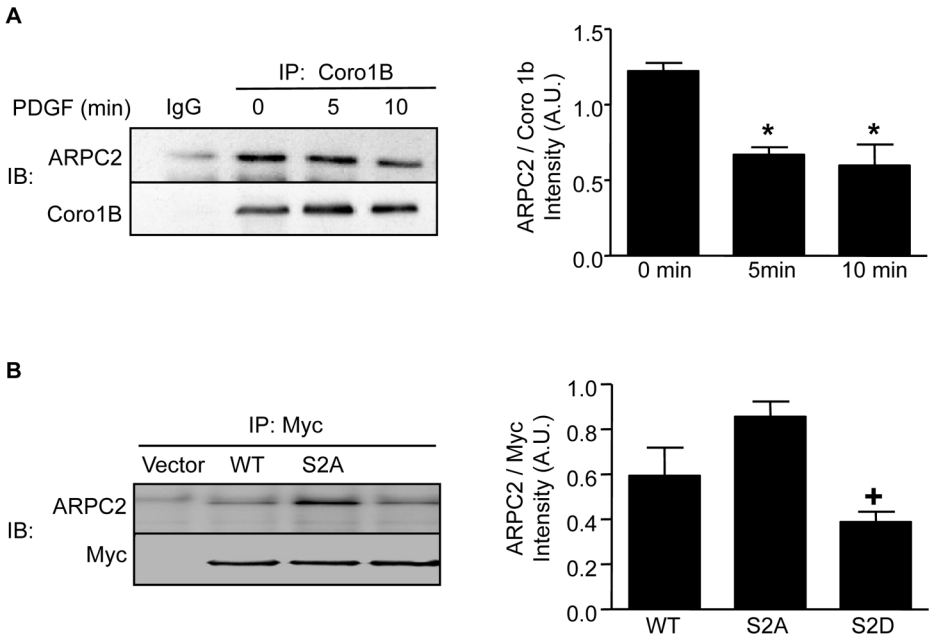

Figure 7. ARP2/3 subunit ARPC2 and Coro1B Interactions are Negatively Regulated by PDGF and are Dependent on the Phosphorylation State of Coro1B in VSMCs

(A) VSMCs were serum starved for 24 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 densiometric analysis from 3–4 independent experiments. Data are plotted as total ARPC2/ Coro1B. (*p< 0.05 compared to control). **(B)** 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 densiometric analysis from 4 independent experiments. Data are plotted as total ARPC2/Myc. (+ p< 0.05 compared to S2A).

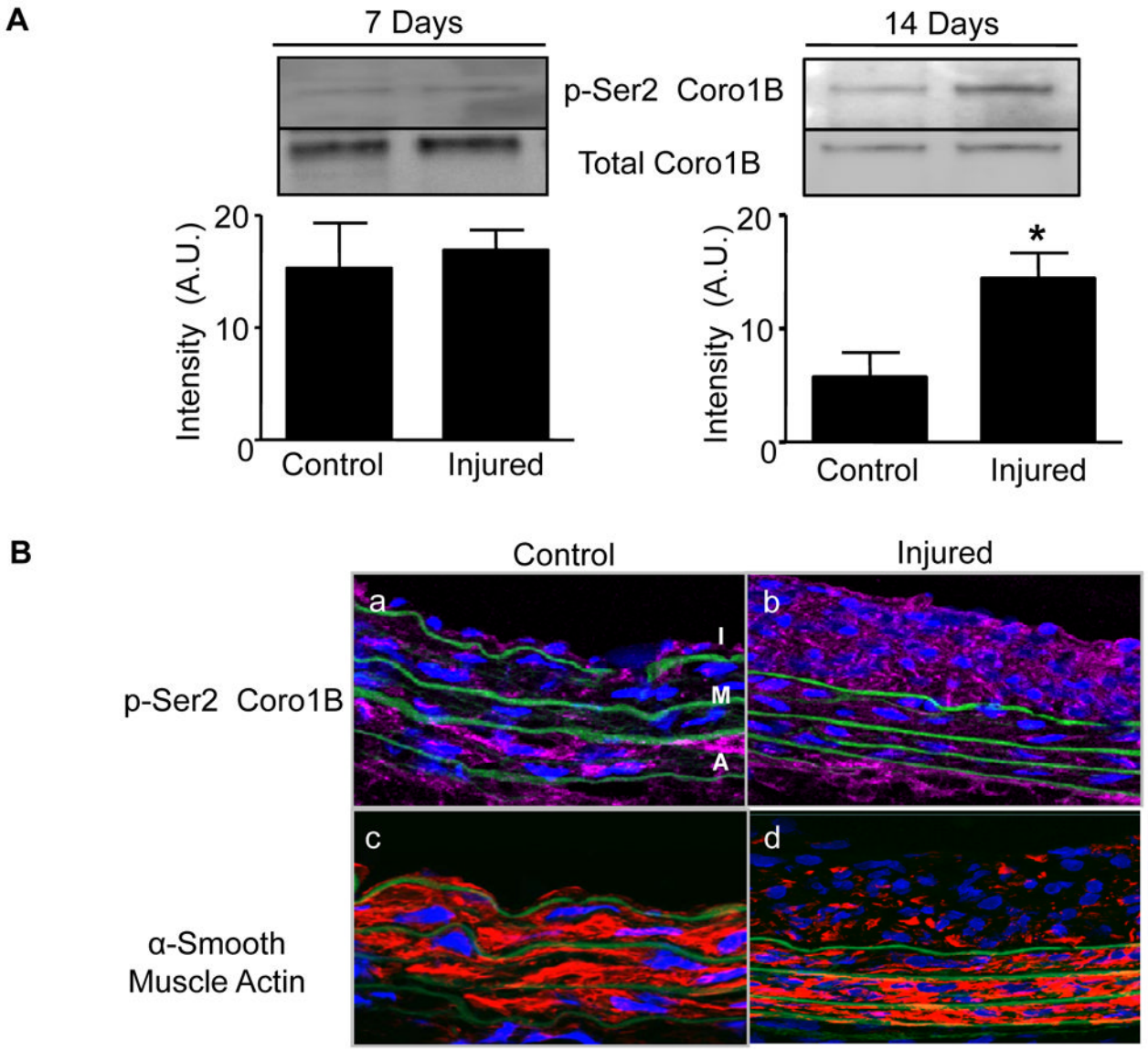

Figure 8. Coro1B is Phosphorylated in Response to Wire Injury in the Mouse Carotid Artery and in the Neointima After Rat Carotid Balloon Injury

(A) 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). **(B)** 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, panels a and b) or α smooth muscle actin (red, panels c and d) 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.