Protein kinase C δ regulates the release of collagen type I from vascular smooth muscle cells via regulation of Cdc42

Justin Lengfeld[a,*], Qiwei Wang[a,*], Andrew Zohlman[b,*,†], Susana Salvarezza[c], Stephanie Morgan[a], Jun Ren[a], Kaori Kato[a], Enrique Rodriguez-Boulan[c], and Bo Liu[a]

[a]Division of Peripheral Vascular Surgery, Department of Surgery, University of Wisconsin–Madison, Madison, WI 53705; [b]Department of Surgery, Weill Cornell Medical College, New York, NY 10065; [c]Department of Ophthalmology, Dyson Vision Research Institute, and Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY 10065

ABSTRACT **Collagen type I is the most abundant component of extracellular matrix in the arterial wall. Mice knocked out for the protein kinase C δ gene (PKCδ KO) show a marked reduction of collagen I in the arterial wall. The lack of PKCδ diminished the ability of arterial smooth muscle cells (SMCs) to secrete collagen I without significantly altering the intracellular collagen content. Moreover, the unsecreted collagen I molecules accumulate in large perinuclear puncta. These perinuclear structures colocalize with the *trans*-Golgi network (TGN) marker TGN38 and to a lesser degree with *cis*-Golgi marker (GM130) but not with early endosomal marker (EEA1). Associated with diminished collagen I secretion, PKCδ KO SMCs exhibit a significant reduction in levels of cell division cycle 42 (Cdc42) protein and mRNA. Restoring PKCδ expression partially rescues Cdc42 expression and collagen I secretion in PKCδ KO SMCs. Inhibition of Cdc42 expression or activity with small interfering RNA or secramine A in PKCδ WT SMCs eliminates collagen I secretion. Conversely, restoring Cdc42 expression in PKCδ KO SMCs enables collagen I secretion. Taken together, our data demonstrate that PKCδ mediates collagen I secretion from SMCs, likely through a Cdc42-dependent mechanism.**

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INTRODUCTION

Type I collagen—the most abundant collagen in the blood vessel wall and other tissues in the human body—is a critical structural and functional component of a healthy artery. Collagen in the arterial wall is produced by fibroblasts and smooth muscle cells (SMCs); either too much or too little collagen can contribute to the pathogenesis of vascular disease (Rudijanto, 2007). The presence of excessive collagen in the atherosclerotic plaque is believed to be an important element of occlusive disease by expanding the plaque mass (Libby *et al.*, 2010). Furthermore, collagen and other matrix proteins are not inert bystanders; rather, they contribute to arterial homeostasis and pathogenesis by influencing the proliferative behavior of SMCs (Hollenbeck *et al.*, 2004). Too little collagen or thinning and weakening of collagen in the fibrous cap region of atherosclerotic plaques is believed to contribute to plaque rupture (Rekhter, 1999), a complex pathological event that is frequently responsible for heart attacks and strokes.

Because of all the aforementioned factors, understanding the process by which collagen is synthesized, trafficked, and secreted in SMCs provides opportunities for the treatment of arterial diseases, as well as systemic fibrotic states.

Collagen type I, along with types II, III, V, and IX, forms into fibrils. Type I is a heterotrimer composed of two α1 chains and one α2 chain. Collagen chains are translated and initially processed in the rough endoplasmic reticulum (ER), where the heterotrimer is formed with the aid of protein disulfide isomerase. The newly synthesized collagen molecules are then transported through the cisternae of the Golgi complex through a cisternal maturation mechanism (Bonfanti *et al.*, 1998). Finally, the collagen molecules are packaged in

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*These authors contributed equally to the manuscript.

†Present address: Division of Cardiovascular Medicine, Boston Medical Center, Boston, MA 02118.

Address correspondence to: Bo Liu (liub@surgery.wisc.edu).

Abbreviations used: Cdc42, cell division cycle 42; PKCδ, protein kinase C δ; SMC, smooth muscle cell; TGN, *trans*-Golgi network.

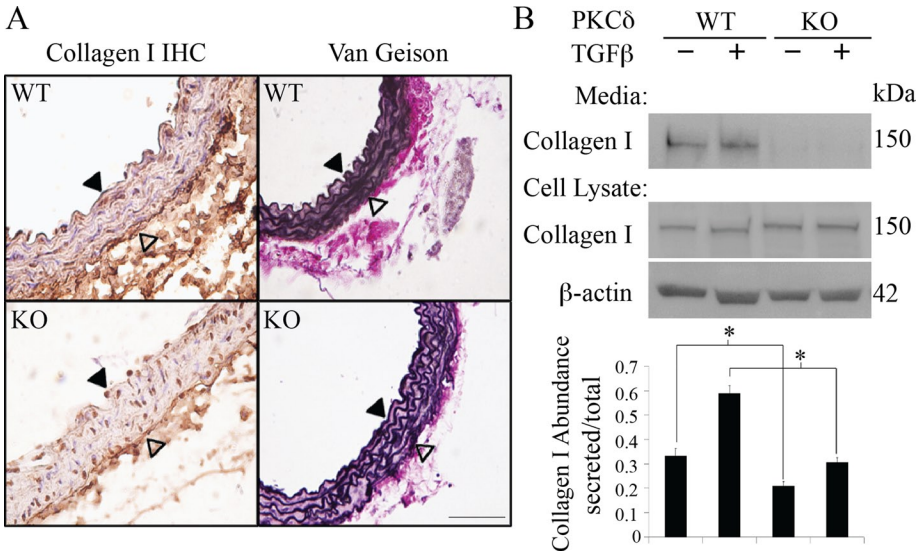

FIGURE 1: PKCδ is necessary for efficient collagen I secretion. (A) Representative mouse aortic sections immunostained for collagen I (left) or van Geison stains (right). Scale bar, 100 μm. (B) Primary mouse aortic SMCs isolated from PKCδ KO mice or their WT littermates were starved for 48 h and then treated for 48 h with or without 5 ng/ml TGFβ. Extracellular (media) and intracellular (cell lysate) collagen I contents were analyzed by Western blot. Representative blots and quantifications are shown. Optical density of secreted collagen I was normalized to the total collagen I density (secreted plus intracellular). Equal loading of cell lysate was confirmed by reprobing with an anti–β-actin antibody. Values are expressed as mean ± SEM (n = 3). *p < 0.05.

post-Golgi vesicular carriers at the *trans*-Golgi network (TGN) and secreted (Canty *et al.*, 2004). Although much work has been performed on collagen transcription, the signaling mechanisms that control collagen trafficking and secretion have yet to be fully elucidated.

Protein kinase C δ (PKCδ) is a 78-kDa member of the novel PKC family of serine/threonine kinases. PKCδ regulates multiple cellular processes, including proliferation (Fukumoto *et al.*, 1997) and apoptosis (Leitges *et al.*, 2001; Ryer *et al.*, 2005) of vascular SMCs. More recently, PKCδ has been found to play an important role in the synthesis of extracellular matrix proteins. In vascular SMCs, PKCδ activity is necessary for induction of fibronectin production by transforming growth factor β (TGFβ; Ryer *et al.*, 2006). Studies of PKCδ knockout (KO) mice reveal that mice lacking PKCδ develop normally but exhibit an antiapoptotic phenotype when subjected to models of vascular injury such as vein graft or carotid artery ligation (Leitges *et al.*, 2001; Bai *et al.*, 2010; Yamanouchi *et al.*, 2010). More recently, PKCδ was reported to be involved in the regulation of chemokine expression and consequently proinflammatory signaling (Liu *et al.*, 2010).

In human fetal lung fibroblasts, PKCδ stimulates elastin expression by stabilizing its mRNA (Kucich *et al.*, 2002). In renal mesangial cells, PKCδ was found to be important for TGFβ-dependent transcription of the gene that encodes collagen α2 chain COLIA2 (Runyan *et al.*, 2003). However, the potential roles of PKCδ in other important steps of collagen synthesis besides gene transcription, such as translation, posttranslational modifications, and trafficking, remain undetermined. It has been shown that PKCδ localizes to the Golgi complex in fibroblasts (Goodnight *et al.*, 1995; Kajimoto *et al.*, 2001; Schultz *et al.*, 2004) and that it regulates the retrograde transport of Shiga toxin from early endosomes to the Golgi complex. However, a role of PKCδ in anterograde transport has not been reported.

During trafficking through the Golgi apparatus, secreted proteins are subject to posttranslational processing and are incorporated into post-Golgi transport carriers (PGTCs) for secretion. These processes are regulated by multiple factors, including a recently described Golgi-based signaling system (Pulvirenti *et al.*, 2008). Pro–collagen I aggregates (300–400 nm) and small, cargo-like vesicular stomatitis virus glycoprotein (60–100 nm) are transported in the same PGTC, suggesting that both use the same mechanism to exit the Golgi (Polishchuk *et al.*, 2003). Assembly and exit of PGTC carrying cargo from the Golgi complex to the plasma membrane take place from the TGN and are mediated by a variety of mechanisms, including clathrin and clathrin adaptors, lipid rafts, and microtubule and actin motors (De Matteis and Luini, 2008). Work in the past decade implicated the actin cytoskeleton, including actin filaments, motors, and regulators, as important participants in Golgi exit of plasma membrane proteins (Musch *et al.*, 1997; Stamnes, 2002; Carreno *et al.*, 2004; Rosso *et al.*, 2004; Cao *et al.*, 2005; McNiven and Thompson, 2006; Au *et al.*, 2007; Lazaro-Dieguez *et al.*, 2007; Salvarezza *et al.*, 2009; von Blume *et al.*, 2009; Miserey-Lenkei *et al.*, 2010).

It has been shown that incubation with cytochalasin B to disassemble actin filaments reduces the number of intracellular collagen fibril carriers in tendon cells, although it does not prevent collagen secretion (Canty *et al.*, 2006). The best molecular candidate to regulate actin dynamics is the Rho GTPase Cdc42, known to associate with the Golgi complex in an ADP-ribosylation factor– and brefeldin A–dependent manner (Erickson *et al.*, 1996) and to regulate the post-Golgi transport of several membrane and secretory proteins (Musch *et al.*, 2001; Pelish *et al.*, 2006). More recently, it has been shown that reduction of Cdc42 activation and its recruitment to the Golgi by the guanine nucleotide exchange factor faciogenital dysplasia protein 1 small interfering RNA (siRNA) blocks the pro–collagen I trafficking at the Golgi complex (Egorov *et al.*, 2009).

In the present study, we investigated the role of PKCδ in collagen secretion from vascular SMCs. Our results suggest that PKCδ regulates trafficking of collagen I by controlling its exit from the *trans*-Golgi network through a mechanism involving Cdc42.

RESULTS

PKCδ is a critical mediator of collagen I secretion in vascular smooth muscle cells

Aortas were harvested from PKCδ KO mice or their wild-type (WT) littermates and analyzed for collagen I content by immunohistochemistry. WT mice revealed considerable collagen I staining within both the media (Figure 1A, ▲) and adventitia of the arterial wall (Figure 1A, Δ). In contrast, PKCδ KO mice showed a significantly diminished amount of collagen I. Van Geison staining revealed similar elastin contents in both genotypes (Figure 1A).

We next tested the ability of aortic SMCs isolated from PKCδ KO mice to synthesize and secrete collagen I in vitro. PKCδ gene deficiency dramatically reduced the level of collagen I detected in the media without significantly altering its content in the cell lysate (Figure 1B). Using real-time (RT)–PCR technology, we assessed mRNA levels of the α1 and α2 chains of the collagen I molecule (COL1α1 and COL1α2, respectively). PKCδ gene deficiency reduced mRNA levels of COL1α1 by ~20% as compared with WT, whereas COL1α2 remained largely unchanged (Supplemental Figure S1).

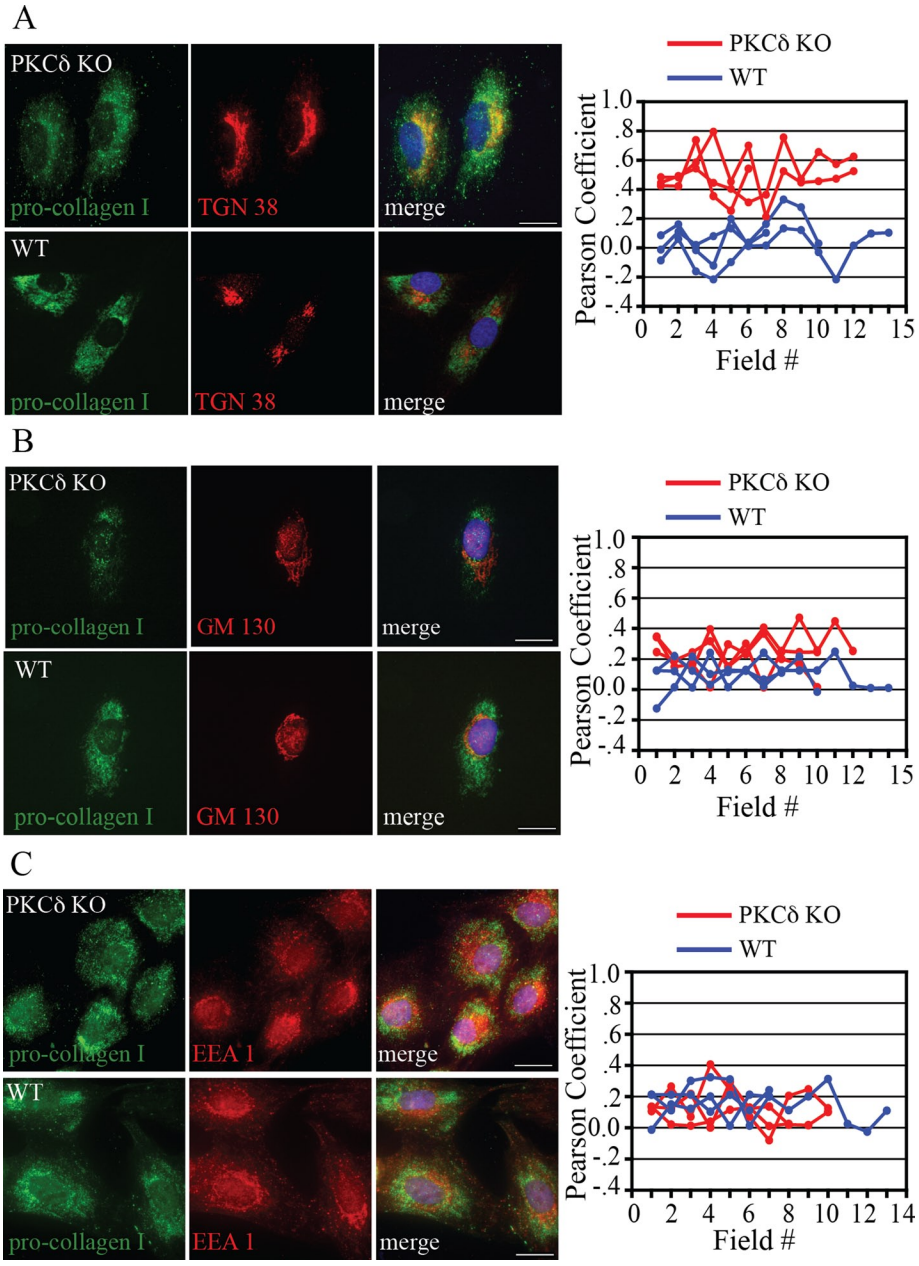

FIGURE 2: Intracellular accumulation of pro–collagen I in the *trans*-Golgi complex of PKCδ-knockout SMCs. Primary mouse aortic SMCs isolated from PKCδ KO mice or their WT littermates were fixed and coimmunostained using antibodies specific to pro–collagen I (left and green in merged images) and TGN38, a marker for *trans*-Golgi; GM130, a marker for *cis*-Golgi; or EEA1, a marker for sorting endosomes (middle and red in merged images). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue in merged images). Confocal fluorescence images were acquired with a BD Biosciences pathway confocal microscope using a 60×/1.42 objective. (A) Representative optical sections of PKCδ KO cells (top) show that pro–collagen I is accumulated at the perinuclear region, showing higher colocalization with TGN38 (Pearson coefficient, right graph) than in WT cells (bottom). Pearson coefficient values range from −1 to 1, where 1 means complete colocalization (right). Each point in the graph represents a Pearson colocalization coefficient for each field of a slide. Each line represents an independent experiment. Note the positive Pearson values in all the optical sections through the PKCδ KO cells (red). (B, C) Cells were coimmunostained using antibodies specific to pro–collagen I and GM130 (B) or EEA1 (C). Note that PKCδ deficiency induces the perinuclear localization of procollagen I, but it does not colocalize with *cis*-Golgi (B, right) or endosomes (C, right). Scale bar, 20 μm.

Because SMCs are often exposed to various profibrotic factors such as TGFβ in a disease state such as occlusive vascular disease, we then repeated the experiment in the presence of 5 ng/ml TGFβ. Similarly, PKCδ gene deficiency selectively diminished collagen I in the extracellular compartment (Figure 1B) in the presence of TGFβ. To test whether a similar collagen I phenotype can be produced by transient inhibition of PKCδ, we acutely inhibited PKCδ activity in A10 cells—a rat aortic smooth muscle cell line—with the chemical inhibitor rottlerin. As seen in Supplemental Figure S2A, rottlerin reduced the amount of secreted collagen in the presence or absence of TGFβ. In contrast, intracellular collagen content was not significantly altered by the rottlerin treatment (Supplemental Figure S2A). In comparison, PKCδ gene deficiency produced different effects on collagen type III and tropoelastin. As shown in Supplemental Figure S3, PKCδ KO SMCs displayed greatly reduced levels of collagen III as compared with WT cells in both extracellular and intracellular compartments. However, PKCδ gene deficiency slightly increased levels of tropoelastin detected in the culture media without altering the level of this matrix protein inside the cell (Supplemental Figure S3). Taken together, these data suggest a role of PKCδ in regulation of the basic collagen secretion mechanism.

PKCδ inhibition causes intracellular accumulation of pro–collagen I in *trans*-Golgi

We next studied the intracellular localization of the collagen molecules that were retained in cells lacking PKCδ activity. Inhibition of PKCδ with rottlerin in A10 cells caused similar perinuclear clusters of collagen I (Supplemental Figure S2B, arrowhead).

To determine the organelle in which collagen I and pro–collagen I were trapped, we double stained SMCs with antibodies specific to pro–collagen I and markers for *cis*- and *trans*-Golgi network (GM130 and TGN38) or early endosomes (EEA1). Immunofluorescence was performed in aortic SMCs isolated from PKCδ KO mice or their WT littermates. WT SMCs showed the TGN arranged symmetrically around the nuclei and procollagen distributed evenly throughout the cytoplasm (Figure 2A, WT). KO SMCs displayed focal dilations in the TGN that colocalized with perinuclear clumps of procollagen I (Figure 2A, PKCδ KO). Compared to the WT cells, PKCδ KO cells showed a slight accumulation of procollagen I in *cis*-Golgi (Figure 2B). Comparison of EEA1 staining with that of procollagen I failed to show any significant colocalization of procollagen I clusters and endosomes (Figure 2C). Similar colocalization patterns were observed in rottlerin-treated A10 cells (Supplemental Figure S4). Taken together, these data suggest that in the

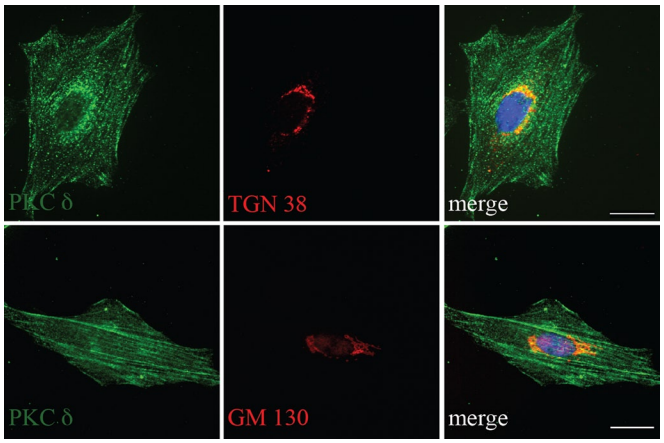

FIGURE 3: PKCδ resides in the *trans*-Golgi network. WT primary mouse aortic SMCs were coimmunostained using antibodies specific to PKCδ (green) and TGN38 or GM130 (red). Nuclei were counterstained with DAPI (blue). Confocal fluorescence images were acquired with a BD Biosciences pathway confocal microscope using a 60×/1.42 objective. Scale bar, 20 μm.

absence of PKCδ, collagen I is transported across the Golgi to reach the TGN but does not exit the TGN toward the cell surface.

High PKCδ protein expression is localized to the *trans*-Golgi

To further explore the role of PKCδ in collagen trafficking, we next examined the intracellular localization of PKCδ in relation to the *cis* and *trans* portions of the Golgi stack. As seen in Figure 3, PKCδ co-localized with both TGN38 and GM130 markers, with high abundance in the *trans*-Golgi networks. Such an intracellular distribution pattern supports the involvement of PKCδ in regulation of Golgi exit of post-Golgi transport carriers.

Cdc42 is impaired in PKCδ KO SMCs

Because the Rho GTPase Cdc42 has been implicated in post-Golgi protein trafficking (Kroschewski *et al.*, 1999; Musch *et al.*, 2001; Pelish *et al.*, 2006), we evaluated the effect of PKCδ gene deficiency on Cdc42. Primary mouse aortic SMCs isolated from PKCδ KO mice or their WT littermates were analyzed for Cdc42 activity by measuring GTP-bound Cdc42. As shown in Figure 4A, PKCδ gene deficiency led to a ~50% reduction of GTP-bound Cdc42, indicating decreased levels of total and active Cdc42 in PKCδ KO cells. Western blotting and RT-PCR analysis revealed a similar reduction in the level of Cdc42 protein and mRNA in PKCδ KO SMCs when compared with wild-type SMCs (Figure 4, B and C). To test whether the lack of PKCδ affects the Golgi localization of Cdc42, we performed coimmunostaining of Cdc42 and TGN38 in PKCδ WT and KO SMCs. Whereas the intensity of Cdc42 was less in KO cells, cells of both genotypes displayed similar distribution patterns of Cdc42 within the Golgi stack (Figure 4D).

Western blot analysis of PKCδ WT and KO SMCs revealed that other members of the Rho GTPase family—specifically RhoA, RhoB, and RhoC—were also down-regulated in PKCδ gene–deficient SMCs. Rac1/2/3 did not appear to be altered by PKCδ gene deficiency (Supplemental Figure S5).

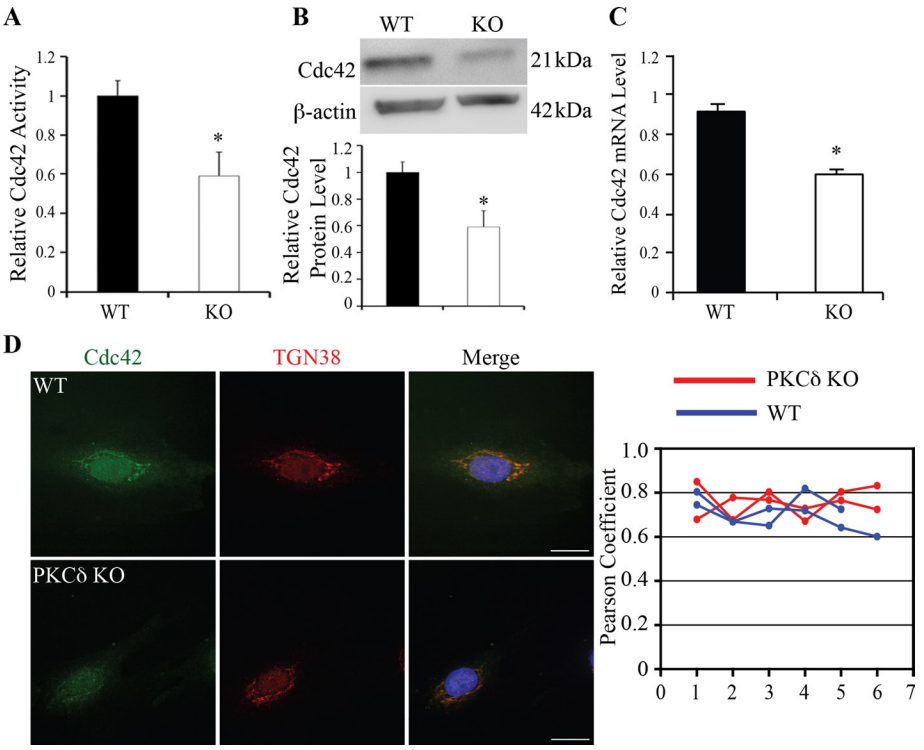

FIGURE 4: Reduction of Cdc42 mRNA and protein expression in PKCδ-knockout SMCs. Primary mouse aortic SMCs isolated from PKCδ KO mice or their WT littermates were starved for 48 h. (A) Activation of Cdc42 was measured by the level of GTP-bound Cdc42 protein in cell lysates. (B) Levels of Cdc42 protein were analyzed by Western blot. Representative blots and quantifications are shown. Equal loading of cell lysate was confirmed by reprobing with an anti–β-actin antibody. (C) Levels of Cdc42 mRNA, normalized to GAPDH mRNA expression, were assessed by real-time PCR. Values are expressed as mean ± SEM (n = 3). *$p < 0.05$ as compared with WT. (D) PKCδ WT or KO SMCs were coimmunostained using antibodies specific to Cdc42 (green) and TGN38 (red). Nuclei were counterstained with DAPI (blue). Colocalization of Cdc42 and TGN38 was quantified by Pearson coefficient analysis (right). Pearson coefficient values range from −1 to 1, where 1 means complete colocalization. Each point in the graph represents a Pearson colocalization coefficient for each field of a slide. Each line represents an independent experiment. Confocal fluorescence images were acquired with a BD Biosciences pathway confocal microscope using a 60×/1.42 objective. Scale bar, 20 μm.

Cdc42 is an important effector necessary for PKCδ-mediated collagen I secretion

If Cdc42 is a critical coeffector with PKCδ, inhibition of this GTPase should mimic the collagen phenotype we observed with PKCδ gene deficiency. To test this, we treated A10 cells with the Cdc42-specific inhibitor secramine A or solvent (dimethyl sulfoxide) for 48 h. As shown in Figure 5A, secramine A eliminated extracellular collagen I but did not significantly affect the amount of collagen detected in cell lysate. Furthermore, immunocytochemistry performed on A10 cells treated with secramine A displayed perinuclear clumping of pro–collagen I co-localized with TGN38, similar to results seen in PKCδ gene–deficient SMCs (Figure 5B).

To confirm the results obtained with secramine A, we inhibited Cdc42 by silencing

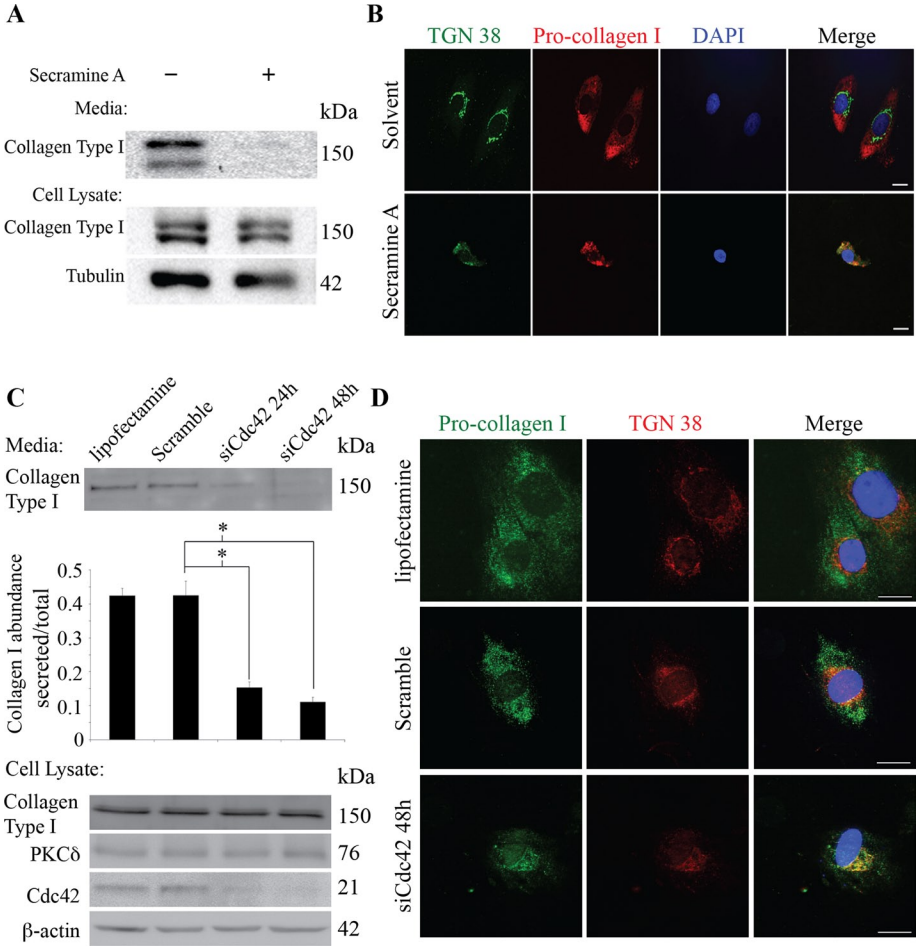

FIGURE 5: Cdc42 is necessary for collagen I trafficking. (A, B) A10 SMCs were treated with secramine A or solvent (control) for 48 h. (A) Media conditioned by SMCs or cell lysate were harvested and analyzed for collagen I by Western blotting. Equal loading was confirmed by reprobing for tubulin. (B) Cells were coimmunostained using antibodies specific to pro–collagen I (red) and TGN38 (green). Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm. (C, D) Primary mouse aortic SMCs were transfected in Opti-MEM I medium with 20 nM of siRNA for mouse Cdc42 or scramble siRNA. (C) Cells and media were harvested 24 and 48 h posttransfection. Media were evaluated by Western blotting for collagen I content and quantified. Values are expressed as mean ± SEM (n = 3). *$p < 0.05$. Cell lysate was evaluated by Western blotting for collagen I, PKCδ, Cdc42, and β-actin. (D) Cells were coimmunostained using antibodies specific to procollagen I (green) or TGN38 (red). Nuclei were counterstained with DAPI (blue). Confocal fluorescence images were acquired with a BD Biosciences pathway confocal microscope using a 60×/1.42 objective. Scale bar, 20 μm.

its expression with siRNA. The wild-type mouse aortic SMCs were transfected with either siRNA to Cdc42 or a control siRNA. At 24 and 48 h posttransfection, cell lysate and media were collected for Western blot analysis. At both time points, siRNA to Cdc42 was able to significantly ablate collagen trafficking to the media with no significant effects on the intracellular contents of collagen I or PKCδ, despite the marked reduction in Cdc42 protein levels (Figure 5C). Similar to secramine A, siRNA to Cdc42 caused PKCδ WT cells to display perinuclear clumping of pro–collagen I colocalized with TGN38 (Figure 5D). Collectively, our results suggest that Cdc42 is a critical mediator in collagen I trafficking out of the SMCs.

Restoration of PKCδ expression rescues collagen I secretion and Cdc42 expression

To further support the role of PKCδ in collagen trafficking and Cdc42 expression, we attempted to restore PKCδ expression in the knockout SMCs with an adenovirus vector that expresses the wild-type PKCδ (AdPKCδ). Primary mouse aortic SMCs from PKCδ KO mice were infected with AdPKCδ or a control vector AdLacZ. Forty-eight hours after viral infection, cells were lysed for Western analysis. AdPKCδ but not AdLacZ significantly restored PKCδ expression in PKCδ KO SMCs (Figure 6A). Furthermore, SMCs infected with AdPKCδ but not AdLacZ produced extracellular collagen I at a level that was comparable to the wild-type SMCs (Figure 6A). As shown in Figure 6B, intracellular Cdc42 levels were also partially restored by AdPKCδ, further implying a relationship between these two signaling proteins.

Restoration of Cdc42 expression partially rescues collagen I secretion

If Cdc42 is downstream of PKCδ in the collagen-trafficking pathway, rescue of Cdc42 levels in primary mouse aortic SMCs from PKCδ KO mice should result in the restoration of collagen trafficking. Plasmids encoding the wild-type Cdc42 (Wills *et al.*, 1996) or a constitutively active form (V12) (Musch *et al.*, 2001) were introduced to PKCδ KO SMCs using a Nucleofector. Compared to the GFP plasmid control, transfection with either Cdc42 WT or Cdc42 V12 increased Cdc42 protein contents in PKCδ KO SMCs without altering the expression of PKCδ or collagen I (Figure 7A). Western blotting of the extracellular proteins in the media demonstrated that ectopic expression of either Cdc42 construct was able to partially rescue the collagen-trafficking phenotype in the absence of PKCδ (Figure 7A). Furthermore, immunocytochemistry performed on PKCδ KO SMCs transfected with Cdc42 WT or Cdc42 V12 for 48 h displayed even distribution of pro–collagen I throughout the cytoplasm, similar to results seen in WT SMCs (Figure 7B).

DISCUSSION

Tight regulation of collagen is critical to homeostasis of the arterial wall, as well as to stability of atherosclerotic plaque. Malfunction in any of the regulatory steps, that is, synthesis, secretion, and degradation, can potentially lead to abnormal structure of the blood vessel or plaque rupture. By using a combination of pharmacological, molecular, and genetic approaches, we provided evidence that suggests a novel role of PKCδ in the regulation of collagen secretion. The requirement of PKCδ activity in normal trafficking of type I collagen through the Golgi apparatus is demonstrated by our observation that pro–collagen I molecules were trapped in the TGN in arterial SMCs that lack PKCδ activity or expression. To our knowledge, this is the first report on a regulatory role of PKCδ in collagen trafficking.

We did not observe significant collagen accumulation in *cis*-Golgi or the EEA1–positive endosomes in PKCδ-null or rottlerin-treated cells, suggesting this PKC isoform may be specifically required for

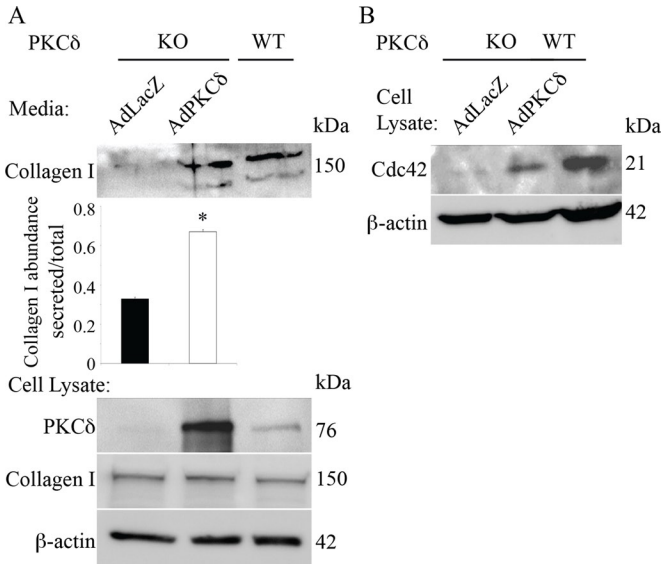

FIGURE 6: Restoration of PKCδ expression rescues collagen I secretion and Cdc42 expression in PKCδ-knockout SMCs. Primary mouse aortic SMCs from PKCδ KO mice were infected with AdPKCδ or a control vector AdLacZ. (A) Representative Western blot of media conditioned by SMCs analyzed for collagen I. Quantification of secreted collagen is expressed as relative band density. Values are expressed as mean ± SEM (n = 3). *p < 0.05. Cell lysates were blotted with the antibodies specific to PKCδ, collagen I, or β-actin. (B) Representative Western blot of cell lysate analyzed for Cdc42.

collagen I secretion vesicles to bud off the TGN. A study by Tisdale (2003) showed that inhibition of PKCλ in HeLa cells resulted in newly synthesized reporter protein being trapped in the ER (it is possible that different PKC isoforms differentially regulate distinct steps of protein secretion). It has been shown that PKCδ localizes at the Golgi complex in fibroblasts (Goodnight *et al.*, 1995; Kajimoto *et al.*, 2001; Schultz *et al.*, 2004). Our immunohistochemistry analysis confirmed a similar localization of PKCδ in vascular SMCs, particularly rich in *trans*-Golgi stacks. Furthermore, PKCδ is involved in the endosomes to Golgi trafficking of Shiga toxin in HeLa cells (Torgersen *et al.*, 2007), raising the possibility that PKCδ might also affect anterograde traffic at the Golgi level.

As an essential player in controlling protein secretion (Kroschewski *et al.*, 1999) and post-Golgi protein trafficking (Pelish *et al.*, 2006; Egorov *et al.*, 2009), the involvement of Cdc42 in collagen I trafficking is not unexpected. Secramine A, a small chemical inhibitor, has been found to inhibit the activation of Cdc42 by inhibiting its binding to membranes, GTP, and effectors in a Rho GDP dissociation inhibitor–dependent manner (Pelish *et al.*, 2006). Consistent with the prior reported effect of secramine A on Golgi-mediated protein trafficking, we found that secramine A eliminated secretion of collagen I by vascular SMCs, an observation that was duplicated with Cdc42 mRNA knockdown. The striking similarity between the effect of PKCδ inhibition and Cdc42 inhibition on collagen secretion and trafficking led us to hypothesize that PKCδ regulates Golgi-mediated protein trafficking through Cdc42-dependent mechanisms. Indeed, we found a substantial reduction in the level of GTP-bound Cdc42 in PKCδ-null cells.

Activation of Cdc42 involves conversion from an inactive, GDP-bound form to the active, GTP-bound form, catalyzed by guanine nucleotide exchange factors (Sinha and Yang, 2008). It is unclear whether PKCδ gene deficiency affected this conversion and led to a lower level of GTP-bound active form of Cdc42. Because a similar reduction was also observed in the level of Cdc42 mRNA and total Cdc42 protein, we speculate that PKCδ functions to maintain Cdc42 levels, presumably through regulation of its gene expression. This notion is further supported by our finding that transfection of Cdc42 expression vectors encoding either the wild-type or constitutively active form enabled collagen secretion in the absence of PKCδ. A prominent role of Cdc42 has also been reported in other PKCδ-mediated functions, such as podosome assembly of endothelial cells (Tatin *et al.*, 2006) and neurite outgrowth and stress fiber dismantling (Troller and Larsson, 2006). Further studies are needed to determine how the Cdc42 gene is directly or indirectly regulated by PKCδ.

We noticed that levels of collagen I secreted by Cdc42-transfected, PKCδ-null SMCs were still significantly lower than what was secreted by the wild-type cells despite their comparable levels of Cdc42 expression. This result suggests that PKCδ may regulate collagen secretion through additional mechanisms. Ser-71, located within a putative Akt phosphorylation site (64ydRIRplSYp73), is conserved among members of the Rho GTPase family, including Rac1, Cdc42, Rho A, Rho B, and Rho C (Kwon *et al.*, 2000). By using a recombinant Akt, Kwon and colleagues showed that this sequence can be phosphorylated by Akt at Ser-71, at least in vitro. However, how phosphorylation influences the activation of these small GTPases remains unclear. Although we observed a significant decrease in the level of phosphorylated Cdc42 (Ser-71) caused by PKCδ gene deficiency (unpublished data), we do not believe that PKCδ has a direct role in regulation of Cdc42 activation, since both the wild-type Cdc42 plasmid and its constitutive form showed a similar capacity in rescuing collagen secretion in the absence of PKCδ. Alternatively, other members of the Rho GTPase family could contribute to collagen I secretion. As we showed, RhoA, RhoB, and RhoC were reduced by ~50% in PKCδ KO SMCs as compared with WT. The fact that exogenous Cdc42 was able to eliminate the accumulation of collagen I in TGN argues for the critical role of this particular GTPase in regulation of Golgi exit. Perhaps other steps involved in the post-Golgi transport of secretion vesicles require RhoA, RhoB, or RhoC, which were not designed to be restored by Cdc42 transfection.

PKCδ has been reported to stimulate elastin expression by stabilizing its mRNA in human fetal lung fibroblasts (Kucich *et al.*, 2002). We did not observe any significant effect of PKCδ gene deletion on elastin accumulation in the arterial wall. It is possible that the role of PKCδ in regulation of extracellular matrix (ECM) proteins is cell type specific; however, we cannot rule out the possibility that technical limitations might have prevented us from detecting subtle changes in elastin expression. Of note, PKCδ KO mice are viable and do not spontaneously develop aortic aneurysm or aortic rupture as seen in mice with ECM mutations such as mice deficient in lysyl oxidase, which are unable to cross-link elastin and collagen (Maki *et al.*, 2002).

In summary, we showed that PKCδ is a crucial factor in the movement of collagen type I past the TGN on its path to secretion. We further showed that PKCδ exerts its effect in part through Cdc42. This is the first evidence that PKCδ has a role in collagen type I trafficking and a beginning of understanding the mechanisms of collagen secretion after it leaves the endoplasmic reticulum. Our finding that PKCδ is necessary for Cdc42 expression provides a new avenue for further elucidation of the collagen secretion process and regulatory mechanisms involved in atherosclerotic plaque formation and stability.

MATERIALS AND METHODS

General materials and reagents

Rottlerin was obtained from Sigma-Aldrich (St. Louis, MO). DMEM and other tissue culture reagents were from Invitrogen (San Diego,

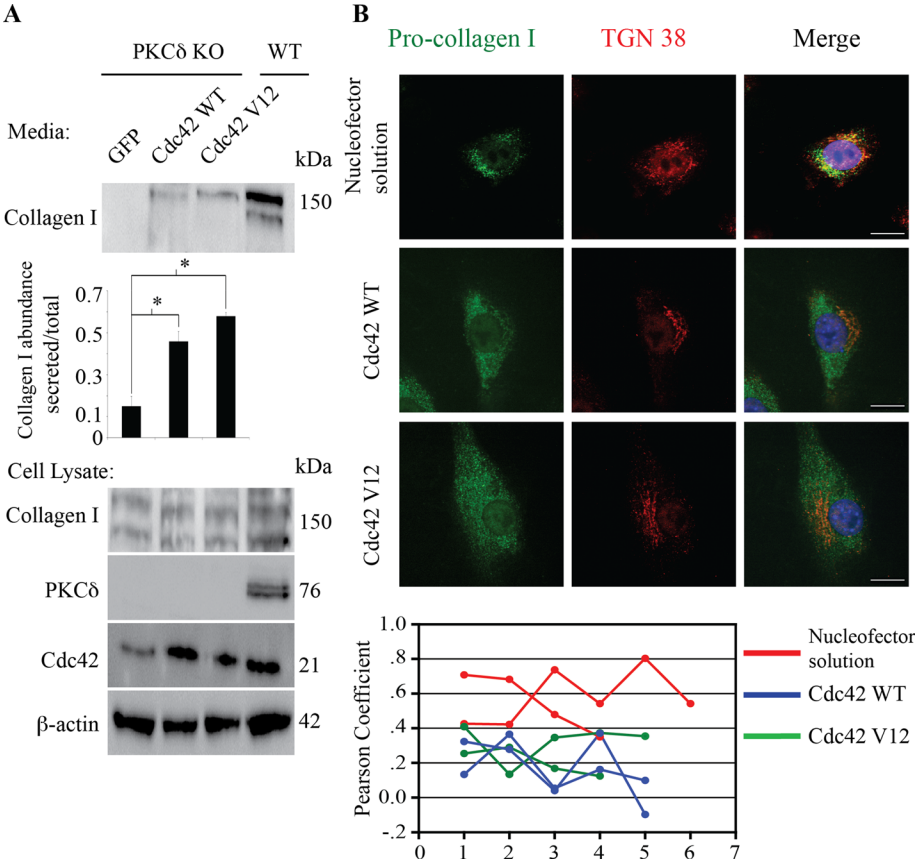

FIGURE 7: Restoration of Cdc42 expression enhances collagen I secretion by PKCδ-knockout SMCs. Primary mouse aortic SMCs from PKCδ KO were transfected with WT Cdc42 DNA plasmid, V12 Cdc42 DNA plasmid, or a control GFP plasmid. (A) At 48 h posttransfection, media or cell lysate were harvested and analyzed by Western blotting. Representative Western blot of media conditioned by SMCs was analyzed for collagen I. Quantification of secreted collagen is expressed as relative band density. Values are expressed as mean ± SEM (n = 3). *p < 0.05. Cell lysates were blotted with the antibodies specific to PKCδ, collagen I, Cdc42, or β-actin. (B) At 48 h posttransfection, cells were coimmunostained using antibodies specific to pro–collagen I (green) and TGN38 (red). Nuclei were counterstained with DAPI (blue). Colocalization of pro–collagen I and TGN38 was quantified by Pearson coefficient analysis (bottom graph). Pearson coefficient values range from −1 to 1, where 1 means complete colocalization. Each point in the graph represents a Pearson colocalization coefficient for each field of a slide. Each line represents an independent experiment. Confocal fluorescence images were acquired with a BD Biosciences pathway confocal microscope using a 60×/1.42 objective. Scale bar, 20 μm.

CA). Antibodies used include collagen I, collagen III (Fitzgerald Industries, Acton, MA), Cdc42, Rho GTPase antibody sampler kit (Cell Signaling Technologies, Danvers, MA), PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA), TGN38 (rat antibody), EEA1, β-actin (Sigma-Aldrich, St. Louis), TGN38 (mouse antibody), GM130, tropoelastin, α-tubulin (Abcam, Cambridge, MA), and pro–collagen I (Developmental Studies Hybridoma Bank, Iowa City, IA). Other chemicals and reagents if not specified were purchased from Sigma-Aldrich.

Cell culture

The mouse aortic SMCs were isolated from the thoracic and abdominal aorta based on a protocol described by Clowes *et al.* (1994). Primary SMCs were grown at 37°C in 5% CO_2 in DMEM modified to contain 1 g/l D-glucose, L-glutamine, 110 mg/l sodium pyruvate supplemented with 10% fetal bovine serum (FBS; Gemini, Woodland, CA), and antibiotics. Cells between three and seven passages were used for all experiments. The generation of mice with targeted deletion of PKCδ was described elsewhere (Miyamoto *et al.*, 2002). Rat aortic A10 SMCs were obtained from American Type Culture Collection (Manassas, VA) and grown as recommended in DMEM modified to contain 4 mM L-glutamine, 4.5 g/l glucose, 1 mM sodium pyruvate, and 1.5 g/l sodium bicarbonate supplemented with 10% FBS and antibiotics.

Transfection

Plasmid DNAs encoding Cdc42 wild-type or constitutive mutant V12 have been described previously (Musch *et al.*, 2001). siRNA to Cdc42, UUUGGGUCCCAACAAGCAAGAAAGG, or its scrambled control was obtained from Invitrogen (Grand Island, NY). Transfection of plasmid DNA was carried out using Nucleofector technology (Amaxa Biosystems, Lonza, Cologne, Germany). For each treatment group, one million cells were suspended in 100 μl of Nucleofector Solution R, mixed with 5 μg of plasmid DNA, and electroporated using the proprietary program D-033. Cells were then plated onto six-well plates or seeded on chamber slides (BD Biosciences, San Jose, CA) containing 10% FBS DMEM. For siRNA transfection, primary mouse SMCs were plated onto six-well plates or seeded on chamber slides at 50–60% confluence and incubated for 24 h. Cells were then transfected in Opti-MEM I medium with 20 nM of siRNA for mouse Cdc42 or control siRNA using Lipofectamine RNAiMAX transfection reagent as described by the manufacturer's protocol (Invitrogen). At 12 h posttransfection Opti-MEM I medium was replaced with DMEM containing 10% FBS.

Adenoviral infection

The construction of the PKCδ-expressing adenoviral vector has been previously described (Ryer *et al.*, 2006). Infection was carried out by incubating SMCs with adenovirus (30,000 particles/cell) in DMEM/2% FBS for 4 h. After the removal of adenovirus, cells were cultured containing 10% FBS and then in DMEM/0.5% FBS for 48 h.

Immunoblotting

The same number of PKCδ WT and KO SMCs was seeded to each plate and cultured simultaneously. Cells were made quiescent at the same time by incubation in medium containing 0.5% FBS for 48 h and then collected. SMCs were lysed in radioimmunoprecipitation assay buffer consisting of 50 mM Trizma HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium dioxycholate, and 0.1% SDS (all reagents from Sigma-Aldrich). Then equal amounts of protein extract were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes as described previously. To analyze secreted proteins, media were collected from treated cell culture and concentrated using Ultracel 10K Centrifugal Filters (Millipore, Billerica, MA). Each media sample was centrifuged at 2000 RCF for 10 min, ultimately yielding a 40× concentration.

For Western blotting, the membranes were incubated with rabbit polyclonal antibodies to collagen I, collagen III, tropoelastin, Cdc42, RhoA, RhoB, RhoC, Rac1/2/3, and mouse monoclonal antibodies to β-actin and pro–collagen I, followed by horseradish peroxidase–labeled goat anti–rabbit or anti–mouse immunoglobulin G (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (PerkinElmer-Cetus, Boston, MA). For quantification of secreted proteins, optical density of secreted proteins, determined by ImageJ (National Institutes of Health, Bethesda, MD), was normalized to the total protein density (secreted plus intracellular).

Immunohistochemistry and immunocytochemistry

Mouse aortas were embedded in OTC compound, frozen, and then cut into 7-μm sections. For immunohistochemical analysis, protocols were supplied by the diaminobenzidine (DAB) kit (DakoCytomation, Carpinteria, CA), and collagen I and pro–collagen I antibodies were selected based on institutional experience. Antigen retrieval was performed in a citrate buffer water bath for 10 min. Endogenous peroxidase was quenched by submerging slides in 3% hydrogen peroxide for 5 min, followed by two washes in phosphate-buffered saline (PBS). Sections were then incubated for 30 min in 2% bovine serum albumin to minimize nonspecific binding and then for 1 h at room temperature with either anti–collagen I at 1:50 or anti–pro-collagen I (for human tissue) at 1:1000. Sections were washed three times in PBS and incubated with horseradish peroxidase–labeled anti–primary antibody (1:1000) for 30, min followed by incubation in freshly prepared EnVision+ solution and developed with DAB. All sections were then counterstained with hematoxylin and mounted.

For immunofluorescence, SMCs were seeded on chamber slides (BD Biosciences) and fixed in 2% paraformaldehyde at room temperature for 10 min. After permeabilization in 0.5% bovine serum albumen, 1% goat serum, and 0.1% saponin, the cells were decorated with primary antibodies against pro–collagen I, PKCδ, Cdc42, TGN38, GM130, and EEA-1, followed by secondary antibodies tagged with Alexa 488 and 568. Images were generated with a Zeiss Axio-Observer spinning disk confocal microscope (Carl Zeiss, Jena, Germany) or a BD Biosciences pathway confocal microscope. Colocalization between TGN38, GM130, or EEA-1 and pro–collagen I or Cdc42 was quantified by Pearson coefficient analysis with ImageJ. For Pearson coefficient analysis, measurements were performed on ≥10 cells in multiple fields and repeated in two or three independent experiments.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from WT or PKCδ KO mice aortic SMCs using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Two micrograms of total RNA was used for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems). Each cDNA template was amplified in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers for Cdc42, Col1α1, Col1α2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were purchased from Qiagen. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized by the level of GAPDH.

Cdc42 activation assay

Primary SMCs were grown to 70% confluence and serum starved for 48 h and then lysed according to the protocol supplied by the Cdc42 Activation Assay Biochem Kit (Cytoskeleton, Denver, CO). Briefly, cell lysates were incubated in a Cdc42-GTP affinity plate for 15 min. The wells were probed with anti-Cdc42 monoclonal antibody and a secondary antibody. Finally, the plate was developed with a colorimetric substrate, and the absorbance was read at 490 nm.

Statistical analysis

Unpaired Student's *t* test was used to evaluate the statistical differences between control and treated groups. Differences with $p < 0.05$ were considered significant. All experiments were repeated at least three times. Error bars on the graphs represent SEM. Values were expressed as mean ± SEM.

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REFERENCES

Au JS, Puri C, Ihrke G, Kendrick-Jones J, Buss F (2007). Myosin VI is required for sorting of AP-1B-dependent cargo to the basolateral domain in polarized MDCK cells. J Cell Biol 177, 103–114.

Bai X, Margariti A, Hu Y, Sato Y, Zeng L, Ivetic A, Habi O, Mason JC, Wang X, Xu Q (2010). Protein kinase Cdelta deficiency accelerates neointimal lesions of mouse injured artery involving delayed reendothelialization and vasohibin-1 accumulation. Arterioscler Thromb Vasc Biol 30, 2467–2474.

Bonfanti L, Mironov AA Jr, Martinez-Menarguez JA, Martella O, Fusella A, Baldassarre M, Buccione R, Geuze HJ, Mironov AA, Luini A (1998). Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. Cell 95, 993–1003.

Canty EG, Lu Y, Meadows RS, Shaw MK, Holmes DF, Kadler KE (2004). Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. J Cell Biol 165, 553–563.

Canty EG, Starborg T, Lu Y, Humphries SM, Holmes DF, Meadows RS, Huffman A, O'Toole ET, Kadler KE (2006). Actin filaments are required for fibripositor-mediated collagen fibril alignment in tendon. J Biol Chem 281, 38592–38598.

Cao H, Weller S, Orth JD, Chen J, Huang B, Chen JL, Stamnes M, McNiven MA (2005). Actin and Arf1-dependent recruitment of a cortactin-dynamin complex to the Golgi regulates post-Golgi transport. Nat Cell Biol 7, 483–492.

Carreno S, Engqvist-Goldstein AE, Zhang CX, McDonald KL, Drubin DG (2004). Actin dynamics coupled to clathrin-coated vesicle formation at the *trans*-Golgi network. J Cell Biol 165, 781–788.

Clowes MM, Lynch CM, Miller AD, Miller DG, Osborne WR, Clowes AW (1994). Long-term biological response of injured rat carotid artery seeded with smooth muscle cells expressing retrovirally introduced human genes. J Clin Invest 93, 644–651.

De Matteis MA, Luini A (2008). Exiting the Golgi complex. Nat Rev Mol Cell Biol 9, 273–284.

Egorov MV *et al.* (2009). Faciogenital dysplasia protein (FGD1) regulates export of cargo proteins from the Golgi complex via Cdc42 activation. Mol Biol Cell 20, 2413–2427.

Erickson JW, Zhang C, Kahn RA, Evans T, Cerione RA (1996). Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. J Biol Chem 271, 26850–26854.

Fukumoto S, Nishizawa Y, Hosoi M, Koyama H, Yamakawa K, Ohno S, Morii H (1997). Protein kinase C delta inhibits the proliferation of vascular smooth muscle cells by suppressing G1 cyclin expression. J Biol Chem 272, 13816–13822.

Goodnight JA, Mischak H, Kolch W, Mushinski JF (1995). Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. J Biol Chem 270, 9991–10001.

Hollenbeck ST, Itoh H, Louie O, Faries PL, Liu B, Kent KC (2004). Type I collagen synergistically enhances PDGF-induced smooth muscle cell proliferation through pp60src-dependent crosstalk between the alpha-2beta1 integrin and PDGFbeta receptor. Biochem Biophys Res Commun 325, 328–337.

Kajimoto T, Ohmori S, Shirai Y, Sakai N, Saito N (2001). Subtype-specific translocation of the delta subtype of protein kinase C and its activation by tyrosine phosphorylation induced by ceramide in HeLa cells. Mol Cell Biol 21, 1769–1783.

Kroschewski RR, Hall AA, Mellman II (1999). Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat Cell Biol 1, 8–13.

Kucich U, Rosenbloom JC, Abrams WR, Rosenbloom J (2002). Transforming growth factor-beta stabilizes elastin mRNA by a pathway requiring active Smads, protein kinase C-delta, and p38. Am J Respir Cell Mol Biol 26, 183–188.

Kwon T, Kwon DY, Chun J, Kim JH, Kang SS (2000). Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. J Biol Chem 275, 423–428.

Lazaro-Dieguez F, Colonna C, Cortegano M, Calvo M, Martinez SE, Egea G (2007). Variable actin dynamics requirement for the exit of different cargo from the *trans*-Golgi network. FEBS Lett 581, 3875–3881.

Leitges M, Mayr M, Braun U, Mayr U, Li C, Pfister G, Ghaffari-Tabrizi N, Baier G, Hu Y, Xu Q (2001). Exacerbated vein graft arteriosclerosis in protein kinase Cδ null mice. J Clin Invest 108, 1505–1512.

Libby P, DiCarli M, Weissleder R (2010). The vascular biology of atherosclerosis and imaging targets. J Nucl Med 51(Suppl 1), 33S–37S.

Liu B, Dhawan L, Blaxall B, Taubman M (2010). Protein kinase Cδ mediates MCP-1 mRNA stabilization in vascular smooth muscle cells. Mol Cell Biochem 344, 73–79.

Maki JM, Rasanen J, Tikkanen H, Sormunen R, Makikallio K, Kivirikko KI, Soininen R (2002). Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. Circulation 106, 2503–2509.

McNiven MA, Thompson HM (2006). Vesicle formation at the plasma membrane and *trans*-Golgi network: the same but different. Science 313, 1591–1594.

Miserey-Lenkei S, Chalancon G, Bardin S, Formstecher E, Goud B, Echard A (2010). Rab and actomyosin-dependent fission of transport vesicles at the Golgi complex. Nat Cell Biol 12, 645–654.

Miyamoto A *et al.* (2002). Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cδ. Nature 416, 865–869.

Musch A, Cohen D, Kreitzer G, Rodriguez-Boulan E (2001). cdc42 regulates the exit of apical and basolateral proteins from the *trans*-Golgi network. EMBO J 20, 2171–2179.

Musch A, Cohen D, Rodriguez-Boulan E (1997). Myosin II is involved in the production of constitutive transport vesicles from the *trans*-Golgi Network. J Cell Biol 138, 291–306.

Pelish HE, Peterson JR, Salvarezza SB, Rodriguez-Boulan E, Chen JL, Stamnes M, Macia E, Feng Y, Shair MD, Kirchhausen T (2006). Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation in vitro. Nat Chem Biol 2, 39–46.

Polishchuk EV, Di Pentima A, Luini A, Polishchuk RS (2003). Mechanism of constitutive export from the Golgi: bulk flow via the formation, protrusion, and en bloc cleavage of large *trans*-Golgi network tubular domains. Mol Biol Cell 14, 4470–4485.

Pulvirenti T *et al.* (2008). A traffic-activated Golgi-based signalling circuit coordinates the secretory pathway. Nat Cell Biol 10, 912–922.

Rekhter M (1999). Collagen synthesis in atherosclerosis: too much and not enough. Cardiovasc Res 41, 376–384.

Rosso S, Bollati F, Bisbal M, Peretti D, Sumi T, Nakamura T, Quiroga S, Ferreira A, Caceres A (2004). LIMK1 regulates Golgi dynamics, traffic of Golgi-derived vesicles, and process extension in primary cultured neurons. Mol Biol Cell 15, 3433–3449.

Rudijanto A (2007). The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. Acta Med Indones 39, 86–93.

Runyan CE, Schnaper HW, Poncelet A-C (2003). Smad3 and PKCdelta mediate TGF-beta1-induced collagen I expression in human mesangial cells. Am J Physiol Renal Physiol 285, F413–F422.

Ryer EJ, Hom RP, Sakakibara K, Nakayama KI, Nakayama K, Faries PL, Liu B, Kent KC (2006). PKCdelta is necessary for Smad3 expression and transforming growth factor beta-induced fibronectin synthesis in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 26, 780–786.

Ryer EJ, Sakakibara K, Wang C, Sarkar D, Fisher PB, Faries PL, Kent KC, Liu B (2005). Protein kinase C delta induces apoptosis of vascular smooth muscle cells through induction of the tumor suppressor p53 by both p38-dependent and p38-independent mechanisms. J Biol Chem 280, 35310–35317.

Salvarezza SB, Deborde S, Schreiner R, Campagne F, Kessels MM, Qualmann B, Caceres A, Kreitzer G, Rodriguez-Boulan E (2009). LIM kinase 1 and cofilin regulate actin filament population required for dynamin-dependent apical carrier fission from the *trans*-Golgi network. Mol Biol Cell 20, 438–451.

Schultz A, Ling M, Larsson C (2004). Identification of an amino acid residue in the protein kinase C C1b domain crucial for its localization to the Golgi network. J Biol Chem 279, 31750–31760.

Sinha S, Yang W (2008). Cellular signaling for activation of Rho GTPase Cdc42. Cell Signal 20, 1927–1934.

Stamnes M (2002). Regulating the actin cytoskeleton during vesicular transport. Curr Opin Cell Biol 14, 428–433.

Tatin F, Varon C, Genot E, Moreau V (2006). A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester. J Cell Sci 119, 769–781.

Tisdale EJ (2003). Rab2 interacts directly with atypical protein kinase C (aPKC) iota/lambda and inhibits aPKCiota/lambda-dependent glyceraldehyde-3-phosphate dehydrogenase phosphorylation. J Biol Chem 278, 52524–52530.

Torgersen ML, Walchli S, Grimmer S, Skanland SS, Sandvig K (2007). Protein kinase Cδ is activated by Shiga toxin and regulates its transport. J Biol Chem 282, 16317–16328.

Troller U, Larsson C (2006). Cdc42 is involved in PKCepsilon- and delta-induced neurite outgrowth and stress fibre dismantling. Biochem Biophys Res Commun 349, 91–98.

von Blume J, Duran JM, Forlanelli E, Alleaume AM, Egorov M, Polishchuk R, Molina H, Malhotra V (2009). Actin remodeling by ADF/cofilin is required for cargo sorting at the *trans*-Golgi network. J Cell Biol 187, 1055–1069.

Wills A, Crowther MTM, Sayers R, Bell P (1996). Pathogenesis of abdominal aortic aneurysms—cellular and biochemical mechanisms. Eur J Vasc Endovasc Surg 12, 391–400.

Yamanouchi D, Kato K, Ryer EJ, Zhang F, Liu B (2010). Protein kinase C delta mediates arterial injury responses through regulation of vascular smooth muscle cell apoptosis. Cardiovasc Res 85, 434–443.