

A VE-cadherin–PAR3– α -catenin complex regulates the Golgi localization and activity of cytosolic phospholipase A₂ α in endothelial cells

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ABSTRACT Phospholipase A₂ enzymes hydrolyze phospholipids to liberate arachidonic acid for the biosynthesis of prostaglandins and leukotrienes. In the vascular endothelium, group IV phospholipase A₂ α (cPLA₂ α) enzyme activity is regulated by reversible association with the Golgi apparatus. Here we provide evidence for a plasma membrane cell adhesion complex that regulates endothelial cell confluence and simultaneously controls cPLA₂ α localization and enzymatic activity. Confluent endothelial cells display pronounced accumulation of vascular endothelial cadherin (VE-cadherin) at cell–cell junctions, and mechanical wounding of the monolayer stimulates VE-cadherin complex disassembly and cPLA₂ α release from the Golgi apparatus. VE-cadherin depletion inhibits both recruitment of cPLA₂ α to the Golgi and formation of tubules by endothelial cells. Perturbing VE-cadherin and increasing the soluble cPLA₂ α fraction also stimulated arachidonic acid and prostaglandin production. Of importance, reverse genetics shows that α -catenin and δ -catenin, but not β -catenin, regulates cPLA₂ α Golgi localization linked to cell confluence. Furthermore, cPLA₂ α Golgi localization also required partitioning defective protein 3 (PAR3) and annexin A1. Disruption of F-actin internalizes VE-cadherin and releases cPLA₂ α from the adhesion complex and Golgi apparatus. Finally, depletion of either PAR3 or α -catenin promotes cPLA₂ α -dependent endothelial tubule formation. Thus a VE-cadherin–PAR3– α -catenin adhesion complex regulates cPLA₂ α recruitment to the Golgi apparatus, with functional consequences for vascular physiology.

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INTRODUCTION

The phospholipase A₂ (PLA₂) family of esterases hydrolyzes the *sn*-2 group of glycerophospholipids to generate free fatty acid and lysophospholipid products (Dennis, 1997). The PLA₂ family can be divided into three major groups based on general structure and regulation mechanisms: group IV cytosolic PLA₂ (cPLA₂), group VI Ca²⁺-independent PLA₂, and secretory PLA₂ enzymes (Akiba and Sato, 2004). All PLA₂ members consist of a catalytic domain that

mediates binding and cleavage of phospholipids. The cPLA₂ group IV consists of at least six members (cPLA₂ α , β , γ , δ , ϵ , and ζ), of which cPLA₂ α is the most extensively characterized. This Ca²⁺-regulated protein binds intracellular membranes upon agonist stimulation and cytosolic Ca²⁺ elevation. Unique to this group, membrane binding enables cPLA₂ α to preferentially cleave phospholipids containing arachidonic acid (AA) at the *sn*-2 position to liberate the fatty acid for eicosanoid production (Dennis, 1997). Thus cPLA₂ α activity is a rate-limiting step in membrane receptor-mediated AA liberation and subsequent prostaglandin synthesis (Kramer and Sharp, 1997). These lipid derivatives regulate diverse functions, including cell proliferation, apoptosis, synaptic plasticity, and Ca²⁺ signaling (Mashimo *et al.*, 2008; Le *et al.*, 2010; Wang and Sun, 2010). Such regulation is particularly important in the vascular endothelium to control vascular tone, angiogenesis, hemostasis, and inflammation (Hurt-Camejo *et al.*, 2001; Herbert *et al.*, 2009; Alberghina, 2010; Tosato *et al.*, 2010).

The mature endothelium consists of confluent quiescent cell monolayers that are cell cycle arrested in the G₀ phase (Chen *et al.*,

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Abbreviations used: AA, arachidonic acid; AnxA1, annexin A1; cPLA₂, cytosolic phospholipase A₂; PAR, partitioning defective protein; PGE₂, prostaglandin E₂; siRNA, small interfering RNA; VE-cadherin, vascular endothelial cadherin; VEGFR2, vascular endothelial growth factor receptor 2.

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2000; Nosedá et al., 2004). This is largely due to contact-mediated inhibition of growth factor signaling and cell proliferation linked to adherens-based junction formation in the mature confluent endothelium (Lampugnani et al., 2003). Disruption of cell–cell contacts by soluble signals or mechanical wounding causes these cells to re-enter the cell cycle and undergo mitosis and cell migration to restore endothelial cell confluence, which, in turn, regulates vascular function. Both proliferative and migratory responses are also essential for new blood vessel sprouting, that is, angiogenesis (Carmeliet, 2000, 2005). Although angiogenesis is a complex, multifactorial process, components of phospholipase A₂ signaling have been implicated in its control, including arachidonic acid (Nie et al., 2000) and prostaglandin E₂ (PGE₂). The latter was shown to increase vessel sprouting in an endothelial nitric oxide synthase–dependent manner (Namkoong et al., 2005). In addition, proliferating nonconfluent cells produce more AA and prostaglandins than do quiescent confluent cells (Evans et al., 1984; Whatley et al., 1994), which has been attributed to elevated endothelial cPLA₂α activity (Herbert et al., 2005). Similar variation may exist in endothelial cells undergoing active vessel sprouting, where actively migrating cells (i.e., “tip cells”) may generate more AA. Uniquely, in quiescent confluent endothelial cells (i.e., those in undamaged, unstimulated vessels), endothelial cPLA₂α is inactivated upon sequestration at the Golgi apparatus; this membrane localization is annexin A1 dependent. However, in proliferating cells, cPLA₂α released from Golgi membranes displays elevated enzyme activity (Herbert et al., 2005, 2007). This reversible control of cPLA₂α is important for regulating the production of eicosanoids, controlling vessel tone, and influencing angiogenic responses. Further understanding of the processes regulating this relocation event may provide novel strategies for modulating endothelial function.

The formation of adherens junctions enriched with vascular endothelial cadherin (VE-cadherin) enables endothelial cells to perform their specialized cellular functions (Nelson and Nusse, 2004). Deletion of the VE-cadherin gene causes embryonic lethality to due severe vascular defects (Crosby et al., 2005). VE-cadherin forms homophilic complexes that recruit cytoplasmic regulators, including catenins, c-Src tyrosine kinase, partitioning defective proteins (PARs/ PARs), and several protein phosphatases (Conacci-Sorrell et al., 2002; Meng and Takeichi, 2009). The catenins contain actin-binding sites enabling cell surface integral membrane proteins to recruit the actin cytoskeleton to such multisubunit complexes (Meng and Takeichi, 2009). The assembly/disassembly of cadherin-based junctional complexes thus couples cell–cell adhesiveness to intracellular signaling and gene expression (Caveda et al., 1996; Carmeliet et al., 1999). Dissociation of these complexes is a prerequisite for endothelial re-entry into the cell cycle and cell migration (Lampugnani et al., 2003; Zanetta et al., 2005). VE-cadherin is also a regulator of vascular sprouting, stabilizing cell–cell interactions during stalk-tip cell polarization and protrusion (Herbert and Stainier, 2011). Regulation by vascular endothelial growth factor (VEGF) and other cytokines, dynamic turnover, and recruitment of intracellular complexes, including catenins, actin, and Raf-1, allows VE-cadherin to actively contribute to the angiogenic process (Vestweber et al., 2008; Abraham et al., 2009; Harris and Nelson, 2010; Wimmer et al., 2012).

Recently the biosynthesis and secretion of VE-cadherin complexes into cell–cell junctions was demonstrated to require cPLA₂α enzyme activity (Regan-Klapisz et al., 2009). Conversely, the establishment of cell–cell contacts has been shown to influence cPLA₂α localization (Herbert et al., 2005). One question that arises is whether VE-cadherin–mediated control of endothelial cell confluence and cell cycle progression in turn regulates cPLA₂α Golgi localization.

Furthermore, what is the consequence of this regulation in the wider context of endothelial function? We addressed these questions by testing the requirement for VE-cadherin and associated complex components (i.e., catenins) on cPLA₂α distribution, enzyme activity, and angiogenesis. This study lends support for a pathway leading from the VE-cadherin complex toward enzymatic intracellular localization, endothelial function, and vascular physiology.

RESULTS

VE-cadherin engagement regulates cPLA₂α Golgi localization

VE-cadherin is a major component of adherens junctions in endothelial cells, recruiting cytoplasmic signaling molecules such as β-catenin and actin to the cytosolic face of the plasma membrane (Vestweber, 2008). The formation of adherens and tight junctions requires cPLA₂α activity (Regan-Klapisz et al., 2009; Bechler et al., 2010). We thus asked whether any components of the VE-cadherin complex regulate cPLA₂α recruitment to the Golgi apparatus. We first analyzed VE-cadherin and cPLA₂α intracellular localization in confluent and semiconfluent primary human umbilical vein endothelial cells (HUVECs; Figure 1). Subconfluent proliferating endothelial cells displayed little VE-cadherin plasma membrane staining and residual Golgi apparatus–associated cPLA₂α (Figure 1A). In contrast, confluent HUVEC monolayers exhibited uniform plasma membrane localization for VE-cadherin at sites of cell–cell contact (Figure 1A). This distribution was similar to that seen for the platelet endothelial adhesion molecule PECAM-1 (CD31) but not the transferrin receptor, which recycles between the plasma membrane and endosomes (Supplemental Figure S1A). Of interest, elevated plasma membrane VE-cadherin levels correlated with enriched cPLA₂α Golgi staining (Figure 1A). However, cells at the scratched edge of a wounded endothelial monolayer showed reduced VE-cadherin plasma membrane staining and concomitantly reduced Golgi cPLA₂α levels (Figure 1A). Wound-border cells displayed a 45% reduction and subconfluent cells a 65% loss in cPLA₂α at the Golgi apparatus when compared with levels colocalizing with Golgi apparatus marker TGN46 in the confluent state (Figure 1B).

Immunoblotting showed that VE-cadherin and cPLA₂α levels were similar under these different cellular conditions, discounting altered protein turnover for the observed phenomenon (Figure 1C). Similarly, β-catenin, a key component of VE-cadherin complexes, TGN46, VEGF receptor 2 (VEGFR2; a receptor tyrosine kinase), nor α-tubulin was altered. However, key regulators of the cell cycle—cyclin A and phosphorylated cyclin D₁—were reduced in confluent HUVECs, reflecting the lowered proliferation status of such contact-inhibited cells (Figure 1C). When subconfluent, early-passage HUVECs were reseeded to confluence, VE-cadherin homophilic complexes began forming after 6 h. This correlated with the translocation of cPLA₂α to the Golgi apparatus (Figure 1D). However, when confluent cells were reseeded to subconfluence, it required between 12 and 16 h or adherent cell growth for Golgi cPLA₂α to redistribute within the cytoplasm (Supplemental Figure S1B). Thus a dynamic relationship exists among cell confluence, VE-cadherin engagement, and the Golgi apparatus localization of cPLA₂α.

What is the requirement for VE-cadherin in Golgi cPLA₂α localization? To test this, we applied a functional blocking antibody specific for VE-cadherin to confluent HUVECs for 18 h prior to analysis of cPLA₂α localization (Figure 2A). This triggered a reduction in cPLA₂α Golgi localization (Figure 2A, bottom), whereas the isotype control antibody had no effect (Figure 2A, top). This correlated with a 25% reduction in levels of cPLA₂α codistributing with TGN46 upon VE-cadherin antibody pretreatment relative to the isotype control

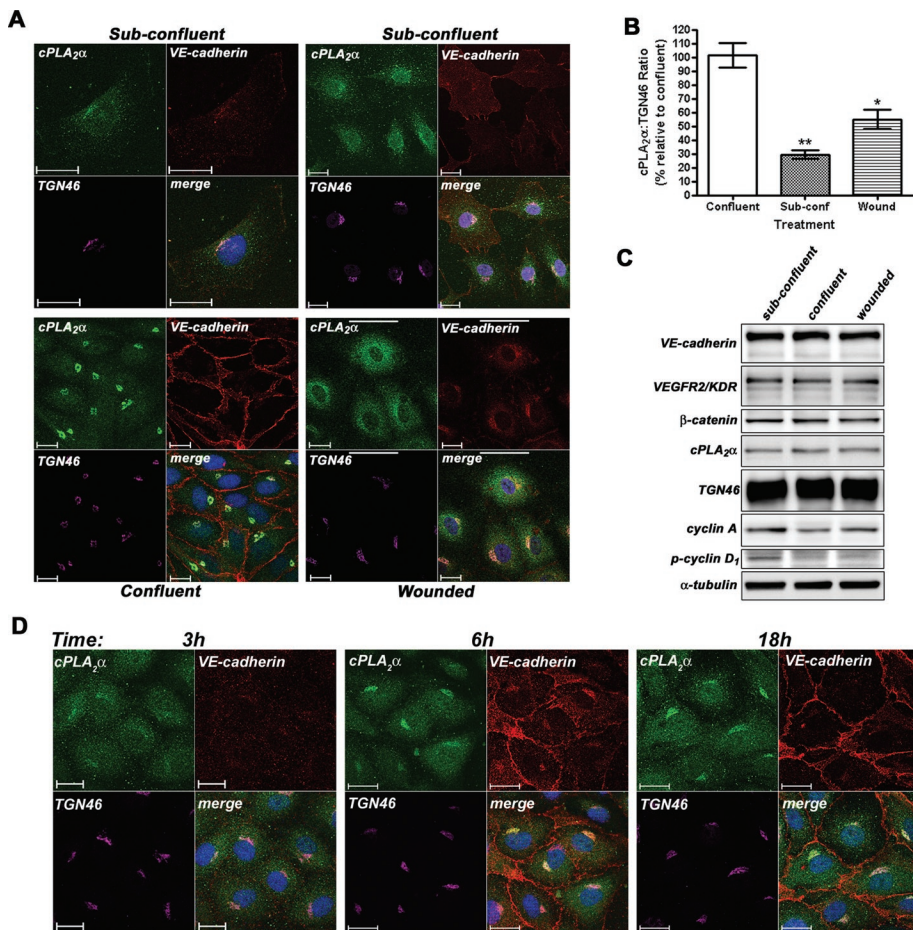


FIGURE 1: Localization of cPLA₂α at the Golgi apparatus coincides with VE-cadherin expression in confluent endothelial cells. (A) HUVECs grown to subconfluence (top), confluence, or confluence followed by scratch wounding and recovery for 18 h (direction indicated by line) were fixed and imaged by confocal microscopy for cPLA₂α (green), VE-cadherin (red), and TGN46 (a Golgi apparatus resident protein; purple). Scale bars, 20 μm. (B) Ratio of cPLA₂α to TGN46 staining at the Golgi apparatus was determined from >200 cells using LSM software and expressed as a percentage of ratio from confluent cells. * p < 0.05, **p < 0.01. (C) HUVEC lysates from subconfluent, confluent, or a multiple scratch-wounded monolayer recovered for 18 h were analyzed by SDS-PAGE and immunoblotted with antibodies against the indicated proteins. Blots are representative of four independent experiments. (D) Subconfluent HUVECs were reseeded to confluence and allowed to recover for the indicated times prior to fixation and analysis of cPLA₂α localization and VE-cadherin engagement.

(Figure 2B). To confirm the requirement for VE-cadherin, we next used small interfering RNA (siRNA) oligonucleotide duplexes to knock down VE-cadherin protein levels prior to analysis of cPLA₂α localization and activity (Figure 2C). In treated HUVECs, VE-cadherin protein levels were reduced by ~88%, but cPLA₂α, VEGFR2, TGN46, α-tubulin, and actin levels were not affected (Figure 2C). Surprisingly, β-catenin levels were also reduced by ~85% following VE-cadherin depletion (Figure 2C). VE-cadherin siRNA-targeted cells exhibited dramatically reduced VE-cadherin levels at cell-cell junctions (Figure 2D). Of importance, treated cells also displayed a significant decrease (~43%) in cells displaying Golgi cPLA₂α (Figure 2E), and overall levels of Golgi cPLA₂α decreased by ~60% relative to the TGN46 marker protein in VE-cadherin-depleted cells (Figure 2F). Furthermore, human coronary artery endothelial cells (HCAECs; Figure 2G) also displayed Golgi cPLA₂α localization, correlating with cell-cell junction distribution of VE-cadherin and β-catenin. Taken together, these results suggest that VE-cadherin is required for cPLA₂α Golgi sequestration.

confluent endothelial cells (Supplemental Figure S2C). These findings suggest growth factor-receptor tyrosine kinase signaling is not required for VE-cadherin-mediated regulation of Golgi cPLA₂α localization.

VE-cadherin regulation of cPLA₂α Golgi localization requires the coordinated activity of α-catenin and δ-catenin

The catenins (α, β, γ [plakoglobin], and δ [p120]) are major signaling effectors linking cadherin engagement to intracellular responses ranging from modulating actin dynamics to altering gene expression (Nelson and Nusse, 2004; Cavallaro et al., 2006; Gavert and Ben-Ze'ev, 2007; Dejana et al., 2008). Given that VE-cadherin knockdown in HUVECs also reduced cellular β-catenin levels (see Figure 2C), is β-catenin responsible for regulating cPLA₂α localization to the Golgi apparatus? Consistent with β-catenin protein down-regulation (see Figure 2C), VE-cadherin depletion in HUVECs also resulted in loss of β-catenin from the cell periphery with no evidence of increased β-catenin accumulation within the

We previously showed that wounded endothelial cells display increased cPLA₂α activity with greater arachidonic acid (AA) synthesis (Herbert et al., 2009). Do VE-cadherin levels thus regulate cPLA₂α enzymatic activity in confluent or wounded endothelial cells? To answer this, we used siRNA to knock down VE-cadherin levels and analyzed AA release as a measure of cPLA₂α enzymatic activity. VE-cadherin-depleted endothelial cells produced ~30% more [³H] AA than controls upon stimulation with the calcium ionophore A23187 (Figure 2H). This effect was further enhanced by HUVEC monolayer wounding and recovery, for which an additional ~25% increase in [³H] AA could be detected in cells treated with siRNA against VE-cadherin (Figure 2H). Basal AA released during cell growth was also assessed, where HUVEC monolayers subjected to VE-cadherin knockdown displayed a significant increase (~20%) in [³H] AA release compared with controls; this effect was further enhanced by endothelial cell monolayer wounding (Figure 2H). Thus VE-cadherin regulates both cPLA₂α localization and activity in confluent endothelial cells. Of importance, reconstitution of this pathway in nonendothelial (epithelial HeLa) cells by cotransfection of VE-cadherin and green fluorescent protein (GFP)-cPLA₂α demonstrated that the formation of VE-cadherin complexes is sufficient to promote the Golgi accumulation of cPLA₂α (Supplemental Figure S2A, arrowheads). In addition, initiation of the Golgi localization signal in HUVECs does not appear to require the growth factor receptor VEGFR2 (Wheeler-Jones et al., 1997; Neagoe et al., 2005). Signaling through this pathway following VEGF-A treatment was unaffected by VE-cadherin depletion (Supplemental Figure S2B), and pharmaceutical inhibition of VEGFR2 with SU5416 did not alter cPLA₂α distribution in

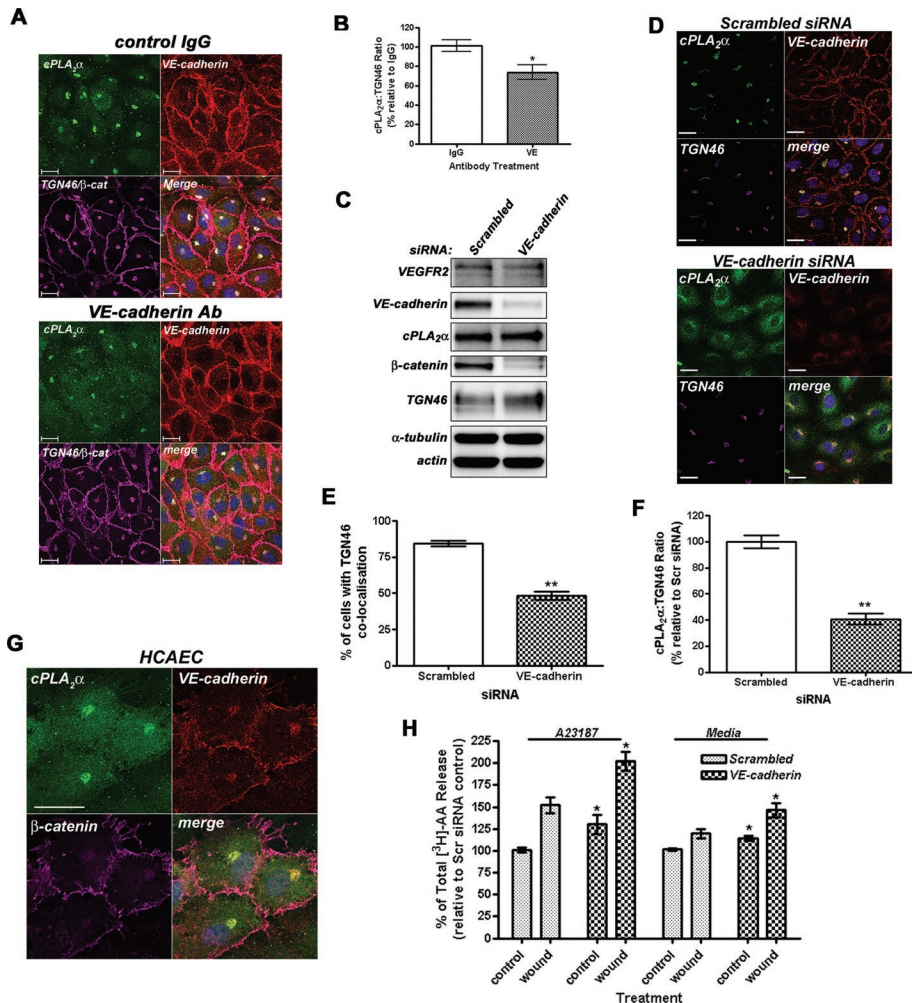


FIGURE 2: VE-cadherin regulates the localization and activity of cPLA₂α. (A) Confluent HUVEC monolayers treated for 18 h with either anti-VE-cadherin BV9 or mouse IgG1 antibody at 5 μg/ml prior to fixation, staining, and analysis by confocal microscopy. (B) The ratio cPLA₂α:TGN46 was determined and expressed relative to isotype control levels. *p < 0.05; n = 4 independent experiments analyzing 120 random cells. (C) VE-cadherin or scrambled siRNA-treated HUVECs were analyzed by immunoblotting for the indicated proteins. (D) HUVECs were treated with siRNA against VE-cadherin or a scrambled sequence for 48 h prior to fixation and immunostaining for cPLA₂α (green), VE-cadherin (red), and TGN46 (purple). Scale bar, 20 μm. Cells from D were analyzed for percentage displaying colocalization between TGN46 and cPLA₂α (E) and ratio of cPLA₂α to TGN46 expression (F) at the Golgi apparatus. **p < 0.01; results compiled from >500 cells across six independent experiments. (G) HCAECs were grown to confluence, fixed, and analyzed by immunostaining for cPLA₂α and VE-cadherin expression. Scale bar, 20 μm. (H) VE-cadherin or scrambled siRNA-treated HUVECs were grown to confluence prior to scratch wounding as indicated and [³H]AA release measured following A23187 (5 μM for 15min) stimulation (left) or basal release into the media during overnight recovery following multiple scratch wounding (right). AA release was calculated relative to total cellular radioactivity and expressed as a percentage of control scrambled siRNA levels. *p < 0.05 compared with matched scrambled siRNA-treated conditions from three compiled independent experiments performed in triplicate.

nucleus (Supplemental Figure S3A). However, siRNA-mediated depletion of β-catenin did not alter cPLA₂α Golgi distribution despite a dramatic loss of β-catenin from cell-cell borders (Supplemental Figures S3B and S4). Depletion of β-catenin did not affect total levels of VEGFR2, VE-cadherin, cPLA₂α, or TGN46 (Supplemental Figure S3C). The loss of β-catenin did not affect growth factor-stimulated intracellular signaling via VEGFR2, ERK1/2, and AKT over a 30-min stimulation period (Supplemental Figure S3D). Similarly, depletion of γ-catenin also failed to alter the Golgi

apparatus localization of cPLA₂α in confluent HUVECs (Supplemental Figure S3E). Furthermore, depletion of β-catenin did not affect the colocalization of α-, γ-, or δ-catenin with VE-cadherin at cell-cell junctions (Supplemental Figure S4). Thus neither β-catenin nor γ-catenin appears to be required for the VE-cadherin-mediated regulation of cPLA₂α Golgi localization.

To further investigate the role of catenin-related proteins in Golgi cPLA₂α localization, we subjected α-catenin, β-catenin, γ-catenin, and δ-catenin to systematic siRNA-mediated protein knock-down (Figure 3A). Depletion of either α-catenin or β-catenin had no significant effect on levels of VE-cadherin or downstream effectors of the VEGFR2 signaling pathway (Figure 3A). However, γ-catenin depletion resulted in significant up-regulation of both α-catenin and VE-cadherin, with β- and δ-catenin levels also elevated (Figure 3, A–C). In contrast, δ-catenin knockdown triggered a significant reduction in levels of VE-cadherin and the remaining catenins, although a substantial pool of α- and γ-catenin remained (Figure 3, A–C). This effect was also detected using microscopy (Figure 3E; and unpublished data) and was specific to the VE-cadherin adhesion complex, as neither VEGFR2 protein levels nor downstream signaling was perturbed (Figure 3A and Supplemental Figure S4).

Do α-catenin or δ-catenin affect cPLA₂α localization via VE-cadherin? We assessed this by depleting α- and δ-catenin and examining Golgi cPLA₂α distribution using microscopy. Surprisingly, knockdown of α-catenin decreased the incidence of cells displaying Golgi cPLA₂α staining (Figure 3D) and caused a reduction in the ratio of cPLA₂α:TGN46 (by 38%), indicating release of cPLA₂α into the cytoplasm or soluble fraction. This was also accompanied by an overall decrease in VE-cadherin at the cell periphery and less-defined cell-cell borders (Figure 3D). Similarly, upon δ-catenin depletion, endothelial cells displayed altered morphology and exhibited fewer cell-cell contacts, associated with an apparent decrease in VE-cadherin at intercellular junction sites (Figure 3E). However, δ-catenin-depleted cells still contained Golgi cPLA₂α despite displaying fewer adherens junctions (Figure 3E). This effect was most evident in wounded endothelial monolayers, where cells migrating into the wound area failed to redistribute cPLA₂α from the Golgi apparatus to the cytoplasm (Figure 3F). A summary of changes in the ratio of cPLA₂α to TGN46 following catenin knockdown is shown in Figure 3G. Taken together, these results indicate the presence of a dynamic complex initiated by VE-cadherin engagement, where α-catenin acts to promote cPLA₂α Golgi localization, with this action opposed by δ-catenin.

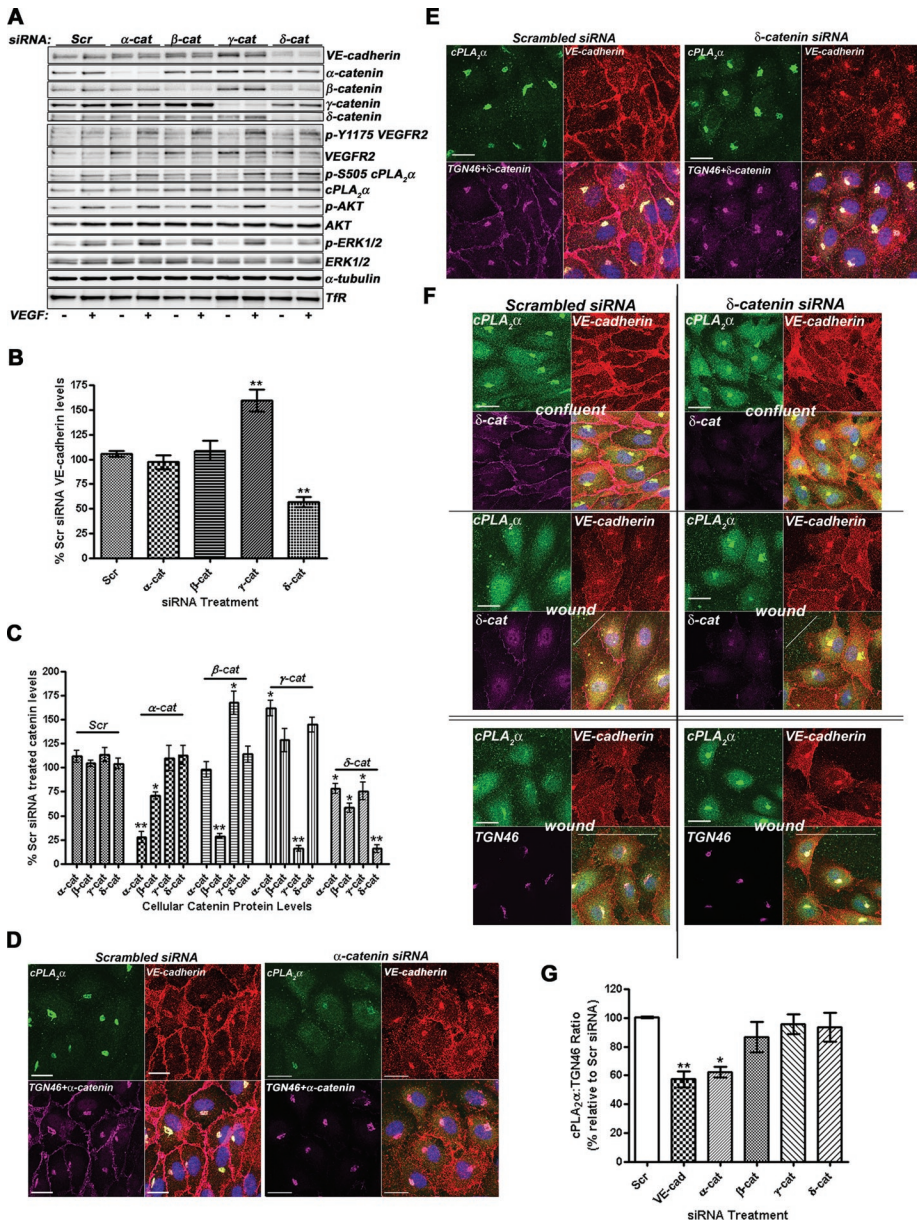


FIGURE 3: α -Catenin regulates the relocalization of cPLA₂ α to the Golgi apparatus upon cell confluence. (A) HUVECs treated with siRNA against α -catenin, β -catenin, γ -catenin, δ -catenin, or scrambled control siRNA for 48 h were serum starved (4 h) and stimulated with VEGF-A (25 ng/ml, 30 min). Lysates were analyzed by immunoblotting as indicated. Shown are representative blots from four independent experiments. (B) Cells treated with siRNA as in A were analyzed for VE-cadherin protein expression, quantified, and expressed as a percentage of scrambled siRNA-treated levels. Results represent five independent experiments. (C) Cells treated with siRNA as in A were analyzed by immunoblotting for catenin expression. Results quantified from five independent experiments are expressed as a percentage of scrambled siRNA-treated levels. (D) HUVECs treated with siRNA against either α -catenin or a scrambled sequence were fixed and processed by immunostaining with anti-cPLA₂ α , anti-VE-cadherin, and a combination of anti-TGN46/anti- α -catenin antibodies. Scale bar, 20 μ m. (E) HUVECs transfected with either scrambled siRNA or siRNA against δ -catenin were grown to confluence, fixed, and immunostained with antibodies against cPLA₂ α (green), VE-cadherin (red), and a combination of TGN46 and δ -catenin (purple). Scale bar, 20 μ m. (F) HUVECs treated with siRNA against δ -catenin or scrambled control were left confluent (upper) or scratch wounded and recovered for 18 h (middle, bottom) prior to fixation and analysis by immunostaining as indicated. Wound directions are indicated by white line. (G) HUVECs transfected with indicated siRNA were processed as outlined in D and E. The ratio of cPLA₂ α to TGN46 fluorescence intensity at the Golgi apparatus was determined and expressed as a percentage of scrambled siRNA-treated cells. Quantified results represent >200 cells from at least four independent experiments. * $p < 0.05$, ** $p < 0.01$.

To determine whether signaling through either α -catenin or δ -catenin was dominant in regulating cPLA₂ α localization, we simultaneously knocked down both components by siRNA in confluent HUVECs. Surprisingly, loss of δ -catenin was the most robust phenotype, with cells depleted of both α -catenin and δ -catenin continuing to display Golgi-associated cPLA₂ α (Figure 4A). The distribution was indistinguishable from that of both scrambled and δ -catenin siRNA-treated cells. When the foregoing cells were mechanically wounded and allowed to recover, wound border cells from dual-catenin-targeted cells (α -catenin + δ -catenin) failed to redistribute from the Golgi apparatus (Figure 4B). This effect was similar to what was found in δ -catenin-knockdown cells, where there was no significant change in cPLA₂ α levels at the Golgi (correlated against TGN46) compared with the non-wounded control (Figure 4C). In contrast, both scrambled and α -catenin siRNA treatment enabled cells to release cPLA₂ α , with Golgi levels relative to TGN46 reduced by 50 and 42%, respectively (Figure 4C). These results suggest that the presence of δ -catenin is the determining factor controlling release of cPLA₂ α upon VE-cadherin disengagement.

To further define the mechanism underlying communication between VE-cadherin-catenin complexes and cPLA₂ α , a functional association between α -catenin and cPLA₂ α was examined. Coimmunoprecipitation experiments demonstrated a functional link in which both VE-cadherin and α -catenin could be detected in immunoprecipitated cPLA₂ α protein complexes (Figure 5A). This suggests that a physical association exists between VE-cadherin complexes and cPLA₂ α , although the reciprocal α -catenin immunoprecipitates did not reveal associated cPLA₂ α , suggesting a weak, transient, or indirect interaction between the components (Figure 5A). Next we examined whether the effects of α -catenin depletion on cPLA₂ α localization were due to a reduction in plasma membrane levels of VE-cadherin complexes (as evidenced by microscopic analysis; Figure 3D) and subsequent release of cPLA₂ α . To test this idea, we isolated biotinylated cell surface proteins and analyzed them for VE-cadherin, VEGFR2, β_1 -integrin, and transferrin receptor (TfR) levels (Figure 5B). VE-cadherin knockdown significantly reduced the biotinylated cell surface pool of VE-cadherin without significantly altering surface TfR or β_1 -integrin levels, although a small (~25%) reduction in VEGFR2 was observed (Figure 5, B and C). VE-cadherin depletion also decreased the levels of

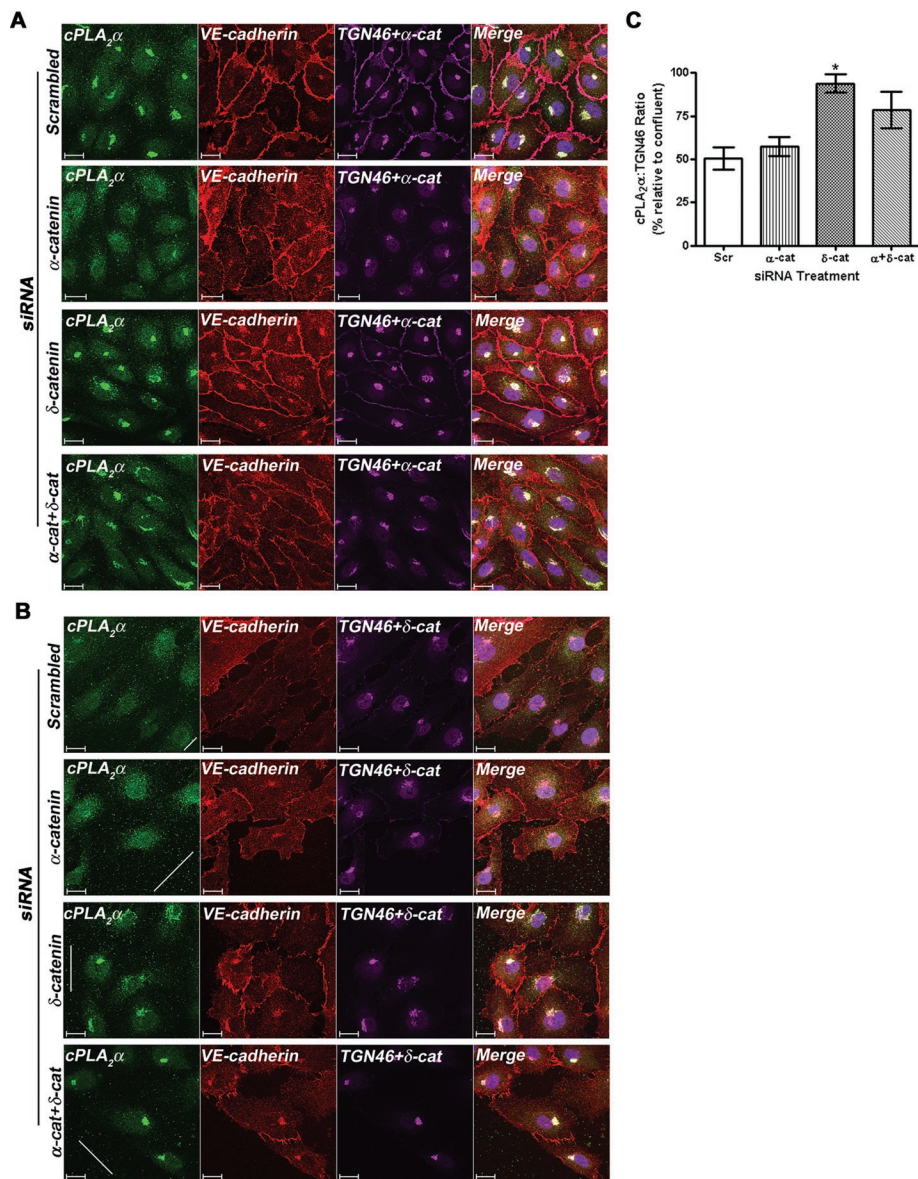


FIGURE 4: Loss of α -catenin is unable to induce $cPLA_2\alpha$ redistribution from the Golgi in the absence of δ -catenin. (A) HUVECs treated with either or both α -catenin- and δ -catenin-targeted siRNA or a scrambled control were fixed and processed with the indicated antibodies. (B) Cells treated as in A were scratch wounded, recovered for 18 h, fixed, and processed as described. Scale bar, 20 μ m. (C) Ratio of $cPLA_2\alpha$:TGN46 localization at the Golgi apparatus was determined from B and expressed relative to ratios from confluent scrambled siRNA-treated cells. * $p < 0.05$ compared with scrambled siRNA-treated control.

plasma membrane-associated α -, β -, γ -, and δ -catenins; however, this may reflect the reduction in total catenin levels (Figure 5D). These effects were observed using either pooled siRNAs or a specific prevalidated siRNA duplex targeted against VE-cadherin (unpublished data).

In contrast, α -, β -, or γ -catenin knockdown did not significantly affect VE-cadherin, VEGFR2, β_1 -integrin, or TfR surface levels or total protein (Figure 5, B–D), although γ -catenin knockdown tended to raise plasma membrane VE-cadherin and associated catenin levels. However, δ -catenin depletion decreased plasma membrane VE-cadherin levels by $\sim 60\%$ (Figure 5, B and C); consistent with the reduced VE-cadherin noted at cell–cell junctions (Figure 3). Depletion of δ -catenin also reduced α -, β -, and γ -catenin levels associated with biotinylated plasma membrane proteins without perturbing VEGFR2,

β_1 -integrin, or TfR levels (Figure 5, B and C). These results suggest δ -catenin controls VE-cadherin–catenin complex stability and turnover while potentially negatively modulating $cPLA_2\alpha$ redistribution to the Golgi. In contrast, α -catenin regulates $cPLA_2\alpha$ localization without influencing VE-cadherin surface expression.

Partitioning defective protein 3 regulates annexin A1-dependent $cPLA_2\alpha$ Golgi localization

Are there other proteins that regulate the cellular distribution of $cPLA_2\alpha$? The partitioning defective protein 3 (PAR3) is a large scaffolding protein consisting of several isoforms involved in diverse regulatory roles (Gao et al., 2002; Rivers et al., 2008). Given that partitioning-defective proteins are implicated in cadherin complex stability and function, as well as in angiogenesis (Iden et al., 2006; Zhao et al., 2010; Zovein et al., 2010; Herbert and Stainier, 2011), could PAR3 be involved in the controlling $cPLA_2\alpha$ Golgi localization? To test this idea, we used immunofluorescence microscopy to assess cellular protein distribution. Here PAR3 exhibited largely cytoplasmic accumulation, with enrichment at the cell periphery, showing an overlapping codistribution with CD31 (Figure 6A) and VE-cadherin (Supplemental Figure S6A); the latter was unaffected by β -catenin depletion (Supplemental Figure S6A), with wound-border cells from control cells displaying reduced PAR3 staining at the cell periphery (Supplemental Figure S6B). Next PAR3 immunoprecipitates contained VE-cadherin, which was absent in complexes from an isotype-matched control (Figure 6B). This suggests that PAR3 and VE-cadherin form a functional complex, as previously demonstrated (Lampugnani et al., 2010; Tyler et al., 2010). Immunoblotting of endothelial cell lysates showed the presence of all three PAR3 isoforms comprising 180-, 160-, and 110-kDa polypeptides (Figure 6C). An siRNA duplex specific for the PAR3 mRNA was able to effectively reduce the cellular levels of the 180- and 160-kDa species without significantly altering levels of annexin A1 (AnxA1), a known regulator of $cPLA_2\alpha$ Golgi localization (Figure 6, C and D; Herbert et al., 2007). However, PAR3 knockdown also caused $\sim 25\%$ reduced VE-cadherin levels, although this failed to reach significance (Figure 6E). Furthermore, VEGF-A-stimulated VEGFR2 activation was not altered, although there was a modest decrease in phospho-AKT levels (Supplemental Figure S6, C–G). Of importance, PAR3 depletion caused a dramatic loss in the Golgi $cPLA_2\alpha$ staining (Figure 6F), resulting in a 40% reduction in the $cPLA_2\alpha$:TGN46 ratio, similar to VE-cadherin depletion (Figure 6G). In contrast, the localization of control proteins such as TGN46, VE-cadherin, or catenins was not affected by PAR3 depletion (Figure 6F; unpublished data).

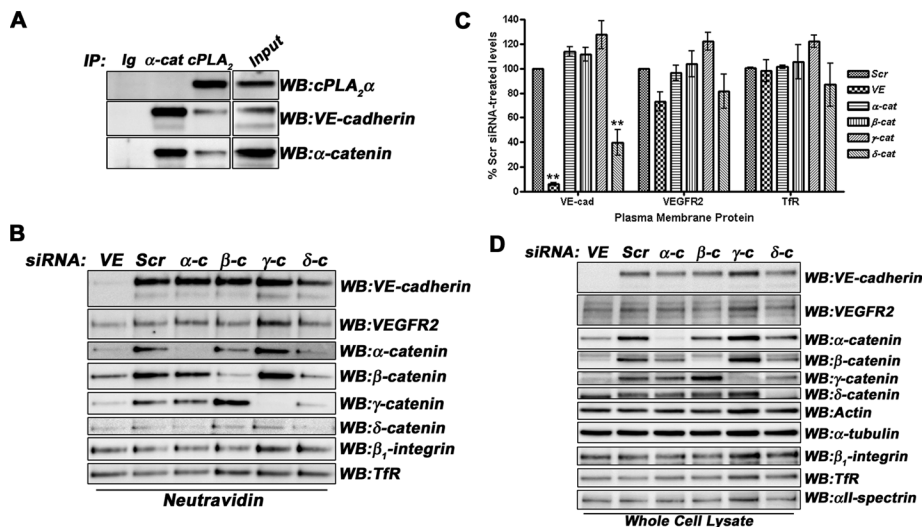


FIGURE 5: Depletion of α -catenin does not affect VE-cadherin plasma membrane levels. (A) Confluent HUVECs lysates were immunoprecipitated with protein G Sepharose and antibodies against either α -catenin, cPLA₂ α , or a rabbit isotype control Ig. Bound complexes were separated by SDS-PAGE and immunoblotted with indicated antibodies. (B) Biotinylated surface proteins from HUVECs treated with siRNA against indicated catenins, VE-cadherin, or a scrambled sequence were isolated with NeutrAvidin agarose and analyzed by immunoblotting with antibodies against indicated proteins. (C) Immunoblots from B were quantified and collated from three independent experiments. ** $p < 0.01$. (D) Whole-cell lysates from A were analyzed by immunoblotting with indicated antibodies.

The effects of PAR3 knockdown were similar to that previously observed following AnxA1 depletion, where Golgi apparatus cPLA₂ α staining was much reduced (Herbert *et al.*, 2007; Supplemental Figure S7A). One question was whether VE-cadherin, PAR3, and AnxA1 are also functionally linked to regulate cPLA₂ α Golgi localization. To test this, we depleted AnxA1 levels using siRNA and analyzed VE-cadherin distribution by immunofluorescence microscopy. AnxA1 knockdown did not affect VE-cadherin or PAR3 localization at cell–cell contacts despite reducing cPLA₂ α Golgi localization (Supplemental Figures S7A and S8 and Figure 6G). AnxA1 depletion similarly did not affect short-term ligand-stimulated signaling through VEGFR2 (Supplemental Figure S7B). In addition, neither AnxA1 nor PAR3 distribution was affected by the loss of β -catenin (Supplemental Figure S7C), and AnxA1 depletion did not alter cPLA₂ α localization at the wound border (Supplemental Figure S7D). Depletion of either PAR3 or AnxA1 did not significantly alter plasma membrane VE-cadherin, TfR, or β ₁-integrin levels (Supplemental Figures S8 and S9A). However, there was a ~25% reduction in cell surface biotinylated VEGFR2 upon AnxA1 depletion, similar to that observed upon VE-cadherin depletion (Supplemental Figure S9B). There was no significant change in total levels of other membrane or cytoplasmic proteins examined, including catenins, upon either PAR3 or AnxA1 knockdown (Supplemental Figure S9C). Furthermore, PAR3 depletion did not affect AnxA1 levels or vice versa (Supplemental Figures S8 and S9C), thus discounting mutual down-regulation as a mechanism regulating cPLA₂ α localization.

Does PAR3 exist in a complex with either AnxA1 or VE-cadherin to provide a mechanism linking VE-cadherin to cPLA₂ α ? To answer this, we analyzed AnxA1 immunoprecipitates and found that both the 180-kDa (predominantly) and 160-kDa PAR3 isoforms were present together with VE-cadherin (Supplemental Figure S9D). Of importance, β -catenin immunoprecipitates contained not only VE-cadherin, but also AnxA1, PAR3, and cPLA₂ α (Supplemental Figure S9E). However, δ -catenin was not detectable in any immunoprecipitates

examined (unpublished data). Furthermore, immunoprecipitates of atypical PKC contained all proposed components of the VE-cadherin–cPLA₂ α pathway, including PAR3, AnxA1, and cPLA₂ α (Supplemental Figure S9F), strengthening arguments for formation of a functional signaling complex. Taken together, these findings suggest that both AnxA1 and PAR3 are key regulatory elements controlling the VE-cadherin–instigated localization of cPLA₂ α to the Golgi apparatus in confluent endothelial cells.

Given that a principal binding partner for VE-cadherin–catenin complexes is F-actin, the actin cytoskeleton may also be involved in regulating cPLA₂ α distribution. To address this, we added the actin polymerization inhibitor cytochalasin D (CytD) to confluent endothelial cells and examined cPLA₂ α distribution over a 30-min period. CytD (1 μ M) rapidly disrupted VE-cadherin-rich contacts and reduced cPLA₂ α Golgi localization after 30 min (Figure 7A). This was accompanied by a dramatic alteration in actin distribution and cell morphology. To discount effects of the latter on cPLA₂ α localization, we applied a sustained (4 h) low dose (200 nM) of CytD to the cells and reex-

amined the cPLA₂ α distribution (Figure 7B). CytD treatment again altered cPLA₂ α distribution, resulting in a 50% reduction in cells exhibiting Golgi-localized cPLA₂ α (Figure 7C). Cell surface levels of VE-cadherin and TfR, but not VEGFR2, were down-regulated by CytD treatment (30 min, 400 nM; Figure 7, D and E), consistent with the microscopy data (Figure 7, A and B). Of importance, pretreatment of confluent endothelial cells with CytD increased the association of VE-cadherin, β -catenin, and AnxA1 with PAR3 immunoprecipitates but decoupled cPLA₂ α from this complex (Figure 7F). This suggests that the F-actin cytoskeleton may physically link cell membrane–localized VE-cadherin–catenin–PAR3 complexes (with or without AnxA1) with cPLA₂ α , to tether the latter to the Golgi apparatus.

Depletion of either α -catenin or PAR3 stimulates cPLA₂ α -dependent angiogenesis

VE-cadherin not only regulates cell–cell adhesion, but also modulates endothelial proliferation and differentiation during angiogenesis (Vestweber, 2008; Abraham *et al.*, 2009). Given that VE-cadherin depletion liberates active cPLA₂ α from the Golgi apparatus and active cPLA₂ α is associated with increased endothelial proliferation and angiogenesis (Herbert *et al.*, 2005, 2009), we sought to examine the influence of VE-cadherin depletion on these key endothelial properties. The Matrigel-based assay promotes the formation of interlinked “tubule-like” endothelial networks independent of cell proliferation and requires formation and maintenance of strong cell–cell contacts. HUVECs, when grown under such conditions and subjected to microscopy analysis, showed formation of VE-cadherin contacts; in addition, these endothelial tubules exhibited strong cPLA₂ α Golgi staining (Figure 8A). Such staining likely indicates the proliferation-inhibited state of these cells.

Loss of VE-cadherin contacts has been reported to promote the formation of angiogenic tubules (Abraham *et al.*, 2009). Given that increased cPLA₂ α activity can enhance the angiogenic response to

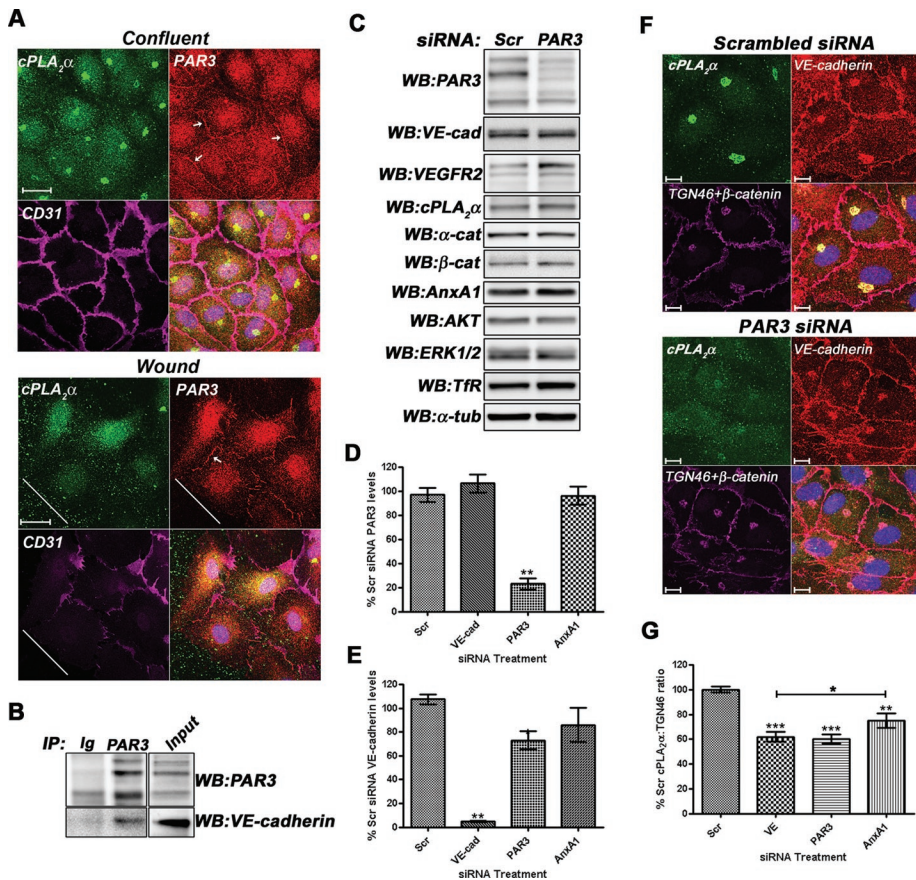


FIGURE 6: PAR3 participates in the control of cPLA₂α localization upon cellular confluence. (A) Confluent (left) or borders of recovered scratch-wounded (right; scratch indicated by line) HUVECs were fixed and stained for cPLA₂α (green), PAR3 (red), and CD31 (purple). Scale bar, 20 μm. Arrows indicate accumulation of PAR3 at cell–cell junctions. (B) PAR3 was immunoprecipitated from confluent HUVECs and bound proteins analyzed for VE-cadherin and PAR3. (C) Protein lysates from HUVECs treated with scrambled or PAR3 siRNA were analyzed by immunoblotting with indicated antibodies. Images are representative of four independent experiments. (D) PAR3 and (E) VE-cadherin levels from C were determined and expressed as a percentage of scrambled siRNA-treated controls. **p* < 0.01. (F) HUVECs treated with either scrambled or PAR3 siRNA were processed for confocal immunofluorescence microscopy with antibodies against cPLA₂α, VE-cadherin, and TGN46. Scale bar, 10 μm (G) Ratio of cPLA₂α to TGN46 staining was determined from >200 siRNA-treated cells (as indicated) across five independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.005.

VEGF-A (Herbert *et al.*, 2009), can disruption of the VE-cadherin complex influence this phenomenon? To test this, we used an endothelial–fibroblast coculture assay and analyzed HUVECs for tubule length and branching after 9 d in medium containing excess VEGF-A (Figure 8B). Similar to the Matrigel tubulogenesis assay, cPLA₂α also displays a Golgi apparatus–like distribution in endothelial tubules formed in coculture (Figure 8B and Supplemental Figure S10A), indicative of low enzymatic activity. The “tips” of these angiogenic tubules exhibit a similar morphology to vessels undergoing *in vivo* vessel sprouting (i.e., display numerous membrane protrusions at the leading edge; Supplemental Figure S10B). In contrast to a recent report (Abraham *et al.*, 2009) but in agreement with earlier studies (Martin *et al.*, 2001; Strlic *et al.*, 2009), VE-cadherin knockdown reduced endothelial tubulogenesis (Figure 8, C–E). This was further decreased by inhibition of cPLA₂α (Figure 8C). In contrast, knockdown of either α-catenin or PAR3, which releases active cPLA₂α, promoted a significant increase in endothelial tubules and branches compared with controls (Figure 8, C–E). Of importance, this increase in endothelial branching was blocked upon cPLA₂α in-

hibition with pyrrolidine (Figure 8, C–E) or less effectively with siRNA against cPLA₂α (Supplemental Figure S10C). In addition, targeted knockdown of either β- or δ-catenin had no significant effect on tubule formation in the coculture assay (Supplemental Figure S10, C and D). Codepletion of δ-catenin with α-catenin also significantly reduced the latter’s enhancement of tubule length (Supplemental Figure S10D). Finally, depletion of α-catenin, PAR3, or VE-cadherin all resulted in a trend toward increased cPLA₂α-dependent PGE₂ release following ionophore stimulation (Figure 8F). When taken together, these findings reinforce the functional link between the status of VE-cadherin complexes and cPLA₂α activity in regulating endothelial function.

DISCUSSION

Specific endothelial proteins can form a circuit or pathway leading to the production of prostaglandins and leukotrienes that regulate varied phenomena, including blood clotting, vessel tone, and angiogenesis (Haeggstrom *et al.*, 2010). In this study, we present a mechanism by which endothelial cell confluence, cell cycle progression, and vascular physiology are regulated by a pathway involving communication from a cell adhesion complex to the intracellular enzyme cPLA₂α. The intact endothelium displays a unique mode of cPLA₂α control by which sequestration to the Golgi apparatus reduces enzyme activity (Herbert *et al.*, 2005, 2007). This may limit the access of cPLA₂α to the perinuclear membrane and its preferred phospholipid substrates. Here we show that the localization and inactivation of the cPLA₂α enzyme to the Golgi apparatus depends on the formation of VE-cadherin–based junctional complexes.

Down-regulation of VE-cadherin levels is sufficient to release cPLA₂α from the Golgi apparatus of a confluent endothelial monolayer. Of importance, this soluble released cPLA₂α is now able to respond to elevated cytosolic Ca²⁺ and liberate AA from membrane-bound phospholipids. VE-cadherin depletion by siRNA may mimic the physiological response that occurs during cell migration, proliferation, and sprouting, in which uncoupling and turnover of VE-cadherin cell–cell contacts leads to cPLA₂α release and enzyme activation. The liberated cPLA₂α then cleaves membrane phospholipids to generate AA and its metabolites (such as prostaglandin E₂ and thromboxane A₂) to regulate remodeling of the endothelium and broader blood vessel function (Antoniotti *et al.*, 2003; Bogatcheva *et al.*, 2005; Fiorio Pla *et al.*, 2008; Linkous *et al.*, 2010). Indeed, the growing importance of cPLA₂α as a controller of angiogenesis, and in particular tumor vascularization, was recently highlighted (Nie and Honn, 2002; Fiorio Pla *et al.*, 2008). Identifying the molecular components regulating cPLA₂α activity will enable exploitation of this pathway as a novel therapeutic target (Linkous *et al.*, 2009).

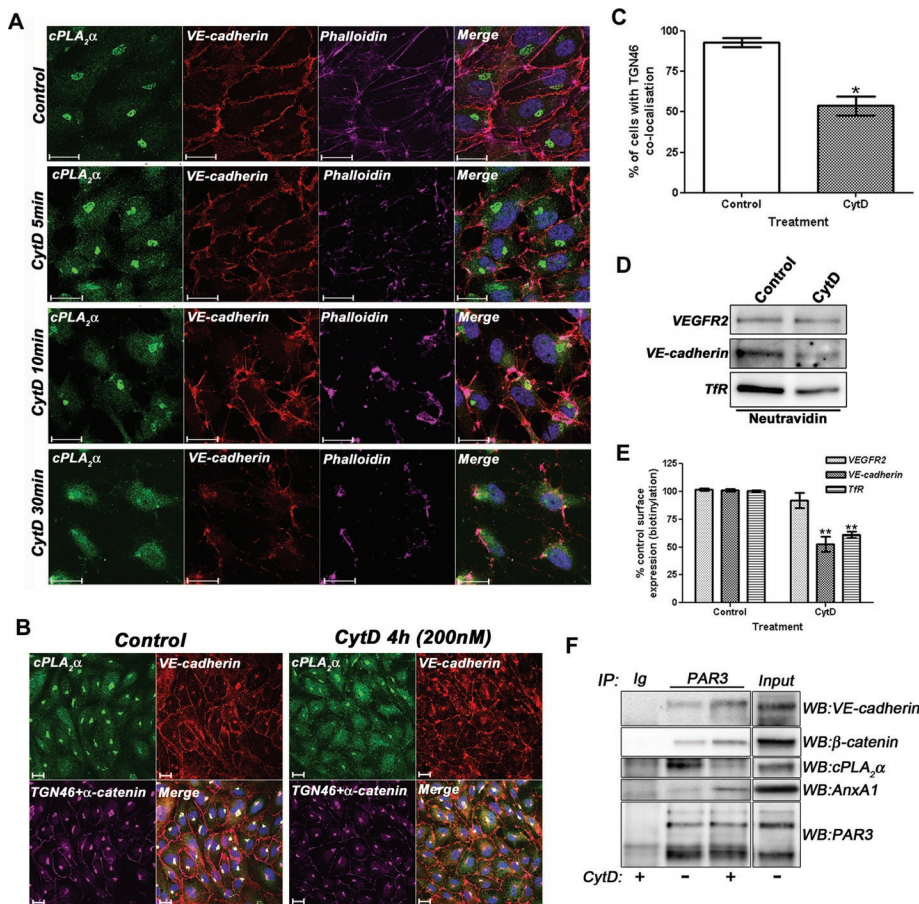


FIGURE 7: F-Actin is required for tethering cPLA₂α to the Golgi apparatus in confluent endothelial cells. (A) Confluent HUVECs were treated with CytD (1 μM) for the indicated times prior to fixation and processing with antibodies against cPLA₂α and VE-cadherin, together with Alexa Fluor 633–phalloidin. Scale bar, 20 μm. (B) Confluent HUVECs were treated for 4 h with CytD (200 nM) prior to fixation and localization analysis. Scale bar, 20 μm. (C) The percentage of cells displaying Golgi-localized cPLA₂α was determined from B and expressed relative to dimethyl sulfoxide (DMSO)–treated control. *p < 0.01; three independent experiments analyzing 200 cells. (D) Confluent HUVECs were pretreated with CytD (400 nM, 30 min) prior to biotinylation, lysis, and isolation of labeled proteins with NeutrAvidin agarose. Bound proteins were analyzed by immunoblotting. (E) Plasma membrane levels of VE-cadherin, VEGFR2, and TFR were quantified and expressed relative to control DMSO-treated cells. **p < 0.01; three independent experiments. (F) Confluent HUVECs were treated with CytD (400 nM, 30 min) or DMSO prior to lysis, preclearing, and immunoprecipitation with anti-PAR3 antibodies. Bound proteins were analyzed for VE-cadherin, β-catenin, AnxA1, PAR3, and cPLA₂α.

There is interdependence between VE-cadherin and its classic binding partner β-catenin in regulating their stability and turnover within endothelial cells (Lampugnani et al., 1995), suggesting that depletion of such factors may induce cPLA₂α release from the Golgi apparatus. In agreement, there was a dramatic loss of β-catenin from both the cytoplasm and cell periphery upon VE-cadherin depletion. Numerous studies demonstrated an increase in cytoplasmic and nuclear β-catenin following tyrosine phosphorylation by various kinases, including Src, Fer, and EGF receptor (Kim and Lee, 2001; Cadigan and Liu, 2006). This translocation coincides with reduced cadherin adhesiveness, down-regulation of cadherin complexes, and increased endothelial permeability (Lilien and Balsamo, 2005). In contrast, nonphosphorylated β-catenin is known to bind tightly to VE-cadherin complexes and increase anchorage to the actin cytoskeleton (Dejana et al., 2008). Surprisingly, depletion of β-catenin failed to disrupt cPLA₂α Golgi localization in confluent endothelial cells, despite the presence of both proteins in PAR3,

AnxA1, and β-catenin immunoprecipitates. This appears to discount a critical role for β-catenin in transmitting the Golgi apparatus localization signal from VE-cadherin to cPLA₂α.

However, in contrast to β-catenin, α-catenin was required for efficient cPLA₂α Golgi localization upon establishment of cell confluence; cells lacking α-catenin displayed reduced Golgi cPLA₂α distribution. How does α-catenin regulate cPLA₂α localization? It was proposed that α-catenin is recruited to β-catenin-containing VE-cadherin adherens junctions, where it not only assists to stabilize the junction, but also competes with the Arp2/3 complex for binding to polymerizing actin (Pokutta et al., 2008). This competition prevents further actin filament growth and stabilizes the protruding plasma membrane (Drees et al., 2005). It is conceivable that formation of cell–cell contacts initiates the interaction of α-catenin with the cortical actin cytoskeleton. This in turn may link with the Golgi apparatus and tether cPLA₂α to the organelle. Thus, when α-catenin is depleted, a scaffold for actin recruitment is lost and cPLA₂α is subsequently liberated from the Golgi. An absence of α-catenin could also promote excess actin polymerization and disruption of normal cytoskeletal linkages, thereby leading to loss of cPLA₂α sequestration. This is a concept under investigation.

In contrast, γ-catenin (plakoglobin) and δ-catenin (p120-catenin) are not essential for cPLA₂α Golgi localization, although δ-catenin is uniquely needed for cell adhesion complex stability. Its loss caused a reduction in levels of VE-cadherin and associated α-, β-, and γ-catenin. Down-regulation of VE-cadherin itself does not appear responsible for this phenotype. Reduced VE-cadherin would be expected to recapitulate the VE-cadherin siRNA distribution, but the opposite effect is observed. In epithelial cells, δ-catenin is known to control rates of E-cadherin internalization, with depletion weakening cell–cell contacts (Sato et al., 2011). A similar role was demonstrated in endothelial cells, where δ-catenin depletion reduced both surface VE-cadherin and transendothelial resistance (Chiasson et al., 2009; Herron et al., 2011). Analogously, δ-catenin may stabilize VE-cadherin complexes at the plasma membrane, enabling formation of an α-catenin-mediated signal, which culminates in cPLA₂α translocation to the Golgi apparatus. This is supported by the double-knockout experiments in which the absence of δ-catenin prevents α-catenin depletion from inducing cPLA₂α release. Given that δ-catenin depletion failed to liberate cPLA₂α from the Golgi apparatus despite disruption of VE-cadherin adhesions (Hatanaka et al., 2011; Herron et al., 2011), this suggests an involvement of this catenin in the negative regulation of cPLA₂α Golgi localization. This concept was supported by the lack of cPLA₂α Golgi release following monolayer wounding when δ-catenin was absent. In addition, without δ-catenin, depletion of α-catenin failed to

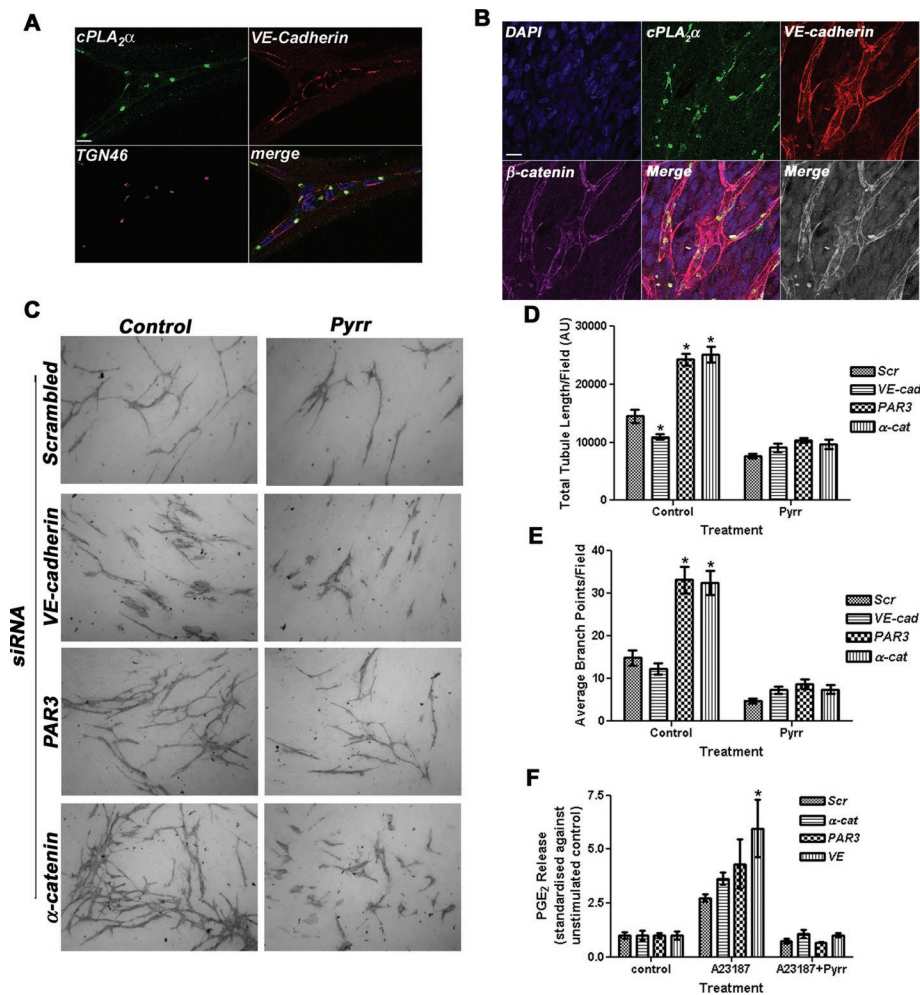


FIGURE 8: Inhibition of $cPLA_2\alpha$ reduces HUVEC angiogenesis induced by PAR3 and α -catenin depletion. (A) HUVECs were grown overnight on Matrigel-coated coverslips prior to fixation and processing for $cPLA_2\alpha$ (green), VE-cadherin (red), and TNG46 (purple) expression. Scale bar, 20 μ m. (B) HUVECs were grown for 9 d upon a confluent monolayer of primary human foreskin fibroblasts prior to fixation and processing for confocal immunofluorescence microscopy analysis with the indicated antibodies. Scale bars, 20 μ m. (C) HUVECs treated with indicated siRNA were grown for 9 d as in B, except that VEGF-A (25 ng/ml) and pyrrolidine (1.5 μ M) were added after 4 d in culture. Cells were fixed and stained with anti-CD31 antibody and visualized by DAB staining. (D) Total tubule length per field and (E) average branch points per field were calculated and compiled from across three independent experiments performed in triplicate. * $p < 0.05$. (F) HUVECs treated with indicated siRNA were treated with A23187 in the presence or absence of pyrrolidine (1.5 μ M). Released PGE2 was measured by enzyme-linked immunosorbent assay and expressed as a value of control unstimulated levels.

liberate $cPLA_2\alpha$. Thus α -catenin must control the localization of $cPLA_2\alpha$ by a mechanism downstream of VE-cadherin homophilic engagement with δ -catenin, providing a higher level of control, perhaps independent of a direct complex formation with $cPLA_2\alpha$.

We propose that δ -catenin normally stabilizes and strengthens VE-cadherin plasma membrane complexes. However, upon depletion of δ -catenin, VE-cadherin–catenin– $cPLA_2\alpha$ complexes are shuttled to an intracellular compartment, where interactions are maintained. Here complexes are unable to be disrupted and release Golgi $cPLA_2\alpha$ upon generation of an appropriate signal (i.e., wounding or growth factor exposure). An additional role may be to limit formation of this signaling complex and ensure that it is rapidly disrupted when active $cPLA_2\alpha$ is required. Alternatively, the loss of δ -catenin may expose additional binding sites for the recruitment of PAR3 (as suggested by Sato et al., 2011) and the

continued localization of $cPLA_2\alpha$ to the Golgi. Given that VE-cadherin contains separate binding motifs for δ -catenin and α/β -catenin complexes, it is conceivable that both are bound simultaneously, along with additional adapters, including PAR3, to coordinate $cPLA_2\alpha$ localization. However, elucidation of the exact mechanisms requires further investigation.

The partitioning-defective PAR3 regulator collaborates with the calcium-sensitive protein annexin A1 to regulate Golgi $cPLA_2\alpha$ localization. It has been shown that PAR3 is needed for VE-cadherin stability, endothelial cell polarity, and lumen formation; the last requires interactions with the actinomyosin machinery via Rac1, cdc42, and RhoE GTPases (Iden et al., 2006; Koh et al., 2008; Tyler et al., 2010; Zovein et al., 2010). In *Drosophila melanogaster*, clusters of the PAR3 orthologue (Bazooka) were shown to reposition cadherin–catenin complexes, enabling the formation of functional adherens junctions (McGill et al., 2009). We found that loss of PAR3 dramatically reduces $cPLA_2\alpha$ Golgi localization in confluent endothelial cells without significantly altering VE-cadherin surface levels or β -catenin/VE-cadherin localization to cell–cell junctions. Biochemical analysis demonstrated the formation of a large protein complex that incorporates elements of the VE-cadherin complex with PAR3 and AnxA1 in endothelial cells. AnxA1 appears to participate in controlling $cPLA_2\alpha$ localization by acting at both the Golgi apparatus and the plasma membrane.

Coimmunoprecipitation experiments suggest that levels of AnxA1 and β -catenin bound with VE-cadherin–PAR3 complexes are diametrically opposed, that is, if high levels of AnxA1 are bound, low levels of β -catenin are present and vice versa. This may enable the functional output of the complex to be precisely controlled. Alternatively, AnxA1 may promiscuously translocate between the two compartments. Of importance, PAR3–VE-cadherin complexes are internalized following F-actin disruption. This specifically liberates $cPLA_2\alpha$ from the complex, leaving AnxA1 associated with PAR3. The “free” $cPLA_2\alpha$ is then able to dissociate from the Golgi apparatus. This suggests that recruitment of the actin cytoskeleton to the VE-cadherin complex, potentially through binding to α -catenin, is required for tether $cPLA_2\alpha$ to the Golgi apparatus following VE-cadherin engagement. Thus AnxA1 and PAR3, together with F-actin, are critically important in controlling the localization of $cPLA_2\alpha$ to the Golgi apparatus in confluent endothelial cells. These results can be extrapolated to in vivo blood vessel formation. Here leading “tip” cells migrate away from the main vessel wall while maintaining attachments to the base “stalk” cells partially via VE-cadherin engagements. By reorganizing their cytoskeleton following PAR3-mediated polarization, tip cells can respond to chemical cues and begin to form new vessels (Herbert et al., 2007). It is possible that

enhanced activation of cPLA₂α in tip cells may accompany this process due to disengagement of VE-cadherin–PAR3 complexes and liberation of cPLA₂α from the Golgi. This is an exciting and intriguing possibility that warrants further investigation.

It is known that VE-cadherin is important for endothelial function and angiogenesis (Harris and Nelson, 2010). We established that VE-cadherin was needed for endothelial tubule formation and angiogenesis *in vitro*. However, VE-cadherin inhibition was recently reported to promote branching from preformed tubules via enhanced activation of Rho kinase (Abraham *et al.*, 2009). One explanation for these differing conclusions may be the need for VE-cadherin in the initial endothelial differentiation stage for tubule formation as opposed to promoting branches from preformed tubules. This suggests that VE-cadherin is required for efficient endothelial cell proliferation, and its absence overrides the ability of cPLA₂α to influence proliferation. In addition, VE-cadherin may not influence cPLA₂α activity in subconfluent endothelial cells, which lack cell–cell adhesions and already contain a substantial cPLA₂α cytosolic pool.

Further discrepancies arise in the role of PAR3 in tubule formation. Previously, PAR3 was found to promote endothelial lumen formation in a three-dimensional collagen assay by controlling cell polarity (Koh *et al.*, 2008; Herbert and Stainier, 2011). However, by using a coculture assay, we demonstrated that depletion of PAR3 or α-catenin (but not δ-catenin or β-catenin) increases angiogenic tubule formation, consistent with increased liberation of active cPLA₂α. This enhanced angiogenesis was prevented by cPLA₂α ablation using either chemical inhibitors or specific siRNA. This suggests increased cPLA₂α activity induced by PAR3/α-catenin down-regulation may promote endothelial tubule formation. Our novel findings may reflect differences in assay design or PAR3 isoforms targeted during inhibition. The coculture assay relies on a mixture of proliferation, migration, and differentiation. We propose that the enhanced proliferation that accompanies cPLA₂α cytoplasmic redistribution (Herbert *et al.*, 2007) is responsible for enhanced tubule generation. However, it is unclear whether there is disrupted lumen formation under these circumstances.

Another important aspect of our study is the apparent distinction between growth factor (e.g., VEGF-A)–stimulated signaling pathways and the cPLA₂α localization phenomenon. VEGFR2 activation and downstream intracellular signaling does not contribute to the signal for cPLA₂α localization to the Golgi apparatus. In addition, the depletion of VE-cadherin does not appear to significantly affect short-term (0–1 h) signaling through this key receptor–ligand complex, which regulates endothelial cell migration, proliferation, and tubulogenesis. These conclusions argue for a hitherto unknown and novel mechanism to control long-range signaling between different compartments, for example, plasma membrane and Golgi apparatus. This mechanism enables a soluble enzyme (cPLA₂α) to be mobilized between membrane-bound (inactive) and soluble (active) states. This determines its ability to hydrolyze phospholipids and provide key metabolites (AA) that are substrates for essential bioactive compounds that regulate vascular and animal function (prostaglandins and leukotrienes).

In summary, VE-cadherin attachments are important for regulating many aspects of endothelial function, including cell migration, proliferation, and tumor neovascularization. The formation of VE-cadherin homophilic complexes are required for transmitting an inhibitory signal independent of β- or γ-catenin to cPLA₂α, thus resulting in its sequestration to the Golgi apparatus and subsequent inhibition of enzyme activity (presumably by preventing access to sites of substrate enrichment). This signal depends on the presence

of α-catenin, PAR3, and AnxA1 and is modulated by the activity of δ-catenin on VE-cadherin complex formation. The attachment of α- and β-catenin to the cytoplasmic tail of VE-cadherin following cell–cell interactions provides the platform for recruiting PAR3 and AnxA1 to the complex. AnxA1 may play a dual role in binding to both cPLA₂α at the Golgi and VE-cadherin complexes in the plasma membrane. Formation of the VE-cadherin–α-catenin–PAR3 complex allows F-actin to bind, possibly through α-catenin, which subsequently stabilizes or drives cPLA₂α to the Golgi. Disruption of this complex liberates cPLA₂α, thereby increasing AA and PGE₂ production and enhancing angiogenic tubule formation. Thus activation of this pathways upon formation of cell–cell contacts provides an effective means of controlling the activity of cPLA₂α and, through this, regulating AA release, eicosanoid production, phospholipid turnover, cell proliferation, and ultimately angiogenesis. These findings demonstrate the importance of VE-cadherin contacts in regulating angiogenesis and that cPLA₂α is a potential therapeutic target for antiangiogenic strategies in disease such as cancer and macular degeneration.

MATERIALS AND METHODS

Cell culture and materials

Human umbilical vein endothelial cells were isolated from human umbilical cords as previously described (Jaffe, 1984; Howell *et al.*, 2004). Cells were cultured in endothelial cell basal medium supplemented with Endothelial Cell Growth Factor Kit 2 (Promocell, Heidelberg, Germany). All cells were grown on 0.1% (wt/vol) gelatin-coated cultureware and were not used in excess of four passages. HeLa cells were cultured as previously described and transfected using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, Amsterdam, Netherlands). The following antibodies were purchased: anti-cPLA₂α (C20; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-VE-cadherin BV9 (Santa Cruz Biotechnology); goat anti-VEGFR2 extracellular domain (R&D Systems, Minneapolis, MN); rabbit anti-VEGFR2, anti-c-AKT, anti-phospho-Ser473-c-AKT, anti-p44/42 ERK1/2, anti-phospho-Thr202/Tyr204-p44/42 ERK, and all catenin antibodies (Cell Signaling Technology, Beverly, MA); and rabbit anti-PAR3 (Upstate Biotechnology, Milton Keynes, United Kingdom). Horseradish peroxidase (HRP)–conjugated secondary antibodies were from ThermoFisher Scientific (Leicester, United Kingdom), and Alexa Fluor–conjugated secondary antibodies were from Invitrogen. Pyrrolidine was a kind gift from M. H. Gelb (University of Washington, Seattle, WA). All other reagents were obtained from Sigma-Aldrich (Poole, United Kingdom) or Invitrogen unless otherwise stated.

Biochemistry

Lysate preparation and Western analysis were performed as described previously (Herbert *et al.*, 2005). Immunoprecipitations were performed overnight at 4°C using protein G Sepharose (Upstate Biotechnology), 2 μg of antibody, and 500–1000 μg of total protein in either 1% (vol/vol) NP40 lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid, 1% [vol/vol] NP40, pH 7.4) or 0.5% CHAPS lysis buffer (0.5% [wt/vol] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], 10 mM Tris/HCl, 150 mM NaCl, 0.5 mM CaCl₂, and 0.5 mM MgCl₂, pH 7.5). Samples (20–50 μg of protein or total bead volume) were resolved by SDS–PAGE on 7–14% gradient gels. Protein was transferred onto nitrocellulose membranes, which were blocked in 5% (wt/vol) nonfat milk and then incubated overnight with primary antibody at room temperature or 4°C. After incubation with HRP-conjugated anti-goat immunoglobulin G (IgG; 1:3000) for

1 h, immunoreactive bands were visualized using a West Pico enhanced chemiluminescence (ECL) detection kit (ThermoFisher Scientific). Images were captured on a FujiFilm LAS-3000 system (FujiFilm, Tokyo, Japan). Band intensities were determined using Advanced Image Data Analyzer 2.11 software.

RNA interference

HUVECs were transfected with 6 pmol/13-mm² dish of nontargeting annealed control siRNA (Scrambled D-001210-01; Dharmacon, Lafayette, CO), annexin A1 (sc-29198, Santa Cruz Biotechnology, or 146987, Ambion, Austin, TX), cPLA₂ α (Ambion; Herbert *et al.*, 2009), α -catenin (sc-29190, Santa Cruz Biotechnology; or L-010505, Dharmacon), β -catenin siRNA (s436; Ambion), γ -catenin (sc-29324, Santa Cruz Biotechnology; or L-011708, Dharmacon), δ -catenin (sc-43021, Santa Cruz Biotechnology; or L-012572, Dharmacon), PAR3 (s32128, s32127, s32126; Ambion) or VE-cadherin siRNA (sc-36814, Santa Cruz Biotechnology; or s2780, Ambion) for 5–6 h using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Media were replaced and cells used for analysis between 48 and 72 h later.

Immunofluorescence

Immunofluorescence was performed as previously described (Herbert *et al.*, 2005). Briefly, cells grown on coverslips were fixed in 10% (vol/vol) formalin in neutral buffered saline (HT50-1-128; Sigma-Aldrich) for 5 min at 37°C. After permeabilization with 0.1% (vol/vol) Triton X-100 for 5 min, cells were refixed (5 min) and washed with phosphate-buffered saline, and nonspecific binding sites were blocked with 5% (vol/vol) donkey/5% (vol/vol) horse serum. Cells were incubated overnight with primary antibody, followed by the appropriate secondary antibodies. Coverslips were mounted on microscope slides in Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL). For functional-blocking antibody experiments, HUVECs were incubated for 18 h with either anti-cadherin BV9 antibody (5 μ g/ml) or isotype-matched control IgG1 antibody prior to fixation and analysis as described. The ratio of cPLA₂ α to the Golgi-resident protein TGN46 (cPLA₂ α :TGN46) was determined using either ImageJ (National Institutes of Health, Bethesda, MD) or LSM5 (Carl Zeiss, Jena, Germany) software to determine the relative intensity of protein staining at the Golgi apparatus. Three random fields of between 10 and 30 cells were routinely analyzed per coverslip performed in triplicate. Data were compiled from at least four independent experiments.

Endothelial cell differentiation, tubulogenesis, and migration assays

To assess HUVEC differentiation, 10⁵ cells were seeded in 24-well dishes coated with 100 μ l of Matrigel (BD Biosciences, San Diego, CA) for 16 h prior to imaging. Tubule length was quantified using ImageJ software. Coculture assays were performed by culturing siRNA-treated HUVECs onto a confluent monolayer of primary human foreskin fibroblasts for 7–10 d in the presence or absence of VEGF-A. Tubules were fixed and either stained for immunofluorescence microscopy as outlined or processed by diaminobenzidine (DAB) staining (Sigma-Aldrich). Tubule length and branching were determined by ImageJ analysis. For migration assays, HUVECs (5 \times 10⁴ cells) were seeded in serum-free media to the top chamber of 24-well modified Boyden chambers (Transwell filters, 8- μ m pore size; Costar, Cambridge, MA). Cells were allowed to migrate toward VEGF-A- or serum-containing media for 16 h. Migrated cells were fixed, stained with Harris hematoxylin stain, and then viewed using a phase contrast light microscope and photographed using a 6-megapixel Nikon D40 digital camera (Nikon, Melville, NY).

Random fields from each digital image were counted and the number of migrated cells expressed as a percentage of control.

Arachidonic acid release assay and PGE2 enzyme-linked immunosorbent assay

This assay was performed as described previously (Herbert *et al.*, 2005). Briefly, HUVECs were cultured to the required density in either six-well or 60-mm² dishes and labeled for 24 h with 0.3 μ Ci/ml [³H]AA in growth media. Subconfluent, confluent, or wounded siRNA-treated cells were washed with phosphate-buffered saline and incubated overnight. The media was collected and cells stimulated with 5 μ M A23187 in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–Tyrode buffer supplemented with 0.3% (wt/vol) fatty acid-free bovine serum albumin for 15 min. Cleared media were assayed for radioactivity by liquid scintillation along with cell lysates prepared in 0.5% (vol/vol) Triton X-100. Arachidonic acid release was calculated relative to total cellular radioactivity and expressed as a percentage of control levels. PGE2 release was determined from siRNA-treated HUVECs in 48-well plates according to manufacturer's instructions (Invitrogen) and expressed as a fold change of control unstimulated levels.

Microscopy and quantification

Phase contrast images were acquired using an Olympus CK2 inverted microscope linked to an Olympus OM-1 camera (Olympus, Center Valley, PA). Confocal immunofluorescence images were collected using a Zeiss LSM510 Meta Axiovert 200M confocal microscope. Fluorescence intensity was determined using LSM510 Meta software or ImageJ. Multiple comparisons were performed using one-way analysis of variance and Tukey's posttest analysis with Prism software (GraphPad, La Jolla, CA).

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