Loss of Adenomatous Poliposis Coli-\(\alpha\)3 Integrin Interaction Promotes Endothelial Apoptosis in Mice and Humans

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**Rationale:** Pulmonary hypertension (PH) is characterized by progressive elevation in pulmonary pressure and loss of small pulmonary arteries. As bone morphogenetic proteins promote pulmonary angiogenesis by recruiting the Wnt/\(\beta\)-catenin pathway, we proposed that \(\beta\)-catenin activation could reduce loss and induce regeneration of small pulmonary arteries (PAs) and attenuate PH.

**Objective:** This study aims to establish the role of \(\beta\)-catenin in protecting the pulmonary endothelium and stimulating compensatory angiogenesis after injury.

**Methods and Results:** To assess the impact of \(\beta\)-catenin activation on chronic hypoxia-induced PH, we used the adenomatous polyposis coli (\(Apc^{Min/+}\)) mouse, where reduced APC causes constitutive \(\beta\)-catenin elevation. Surprisingly, hypoxic \(Apc^{Min/+}\) mice displayed greater PH and small PA loss compared with control C57Bl6J littermates. PA endothelial cells isolated from \(Apc^{Min/+}\) demonstrated reduced survival and angiogenic responses along with a profound reduction in adhesion to laminin. The mechanism involved failure of APC to interact with the cytoplasmic domain of the \(\alpha\)3 integrin, to stabilize focal adhesions and activate integrin-linked kinase-1 and phosho Akt. We found that PA endothelial cells from lungs of patients with idiopathic PH have reduced APC expression, decreased adhesion to laminin, and impaired vascular tube formation. These defects were corrected in the cultured cells by transfection of APC.

**Conclusions:** We show that APC is integral to PA endothelial cells adhesion and survival and is reduced in PA endothelial cells from PH patient lungs. The data suggest that decreased APC may be a cause of increased risk or severity of PH in genetically susceptible individuals. (Circ Res. 2012;111:1551-1564.)

Key Words: adenomatous poliposis coli ■ angiogenesis ■ integrin signaling ■ pulmonary hypertension ■ Wnt signaling

- Devastating condition in which progressive elevation in pulmonary pressure and resistance to flow are associated with the loss and obliterator narrowing of small distal pulmonary arteries (PAs).  
- The disease affects mostly women of reproductive age and, in the absence of treatment, results in worsening chronic right heart failure and death.  
- Because current pulmonary arterial hypertension (PAH) therapies are largely vasodilators, many patients ultimately need to be considered for lung transplantation.

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The discovery of the link between mutations in the bone morphogenetic protein (BMP) receptor 2 (BMPR2)\(^{14}\) or reduced expression of this receptor\(^{4}\) and the development of IPAH led to efforts directed at understanding the role of BMP signaling in pulmonary blood vessel biology. Our group recently reported\(^{6}\) that activation of BMP signaling promotes pulmonary angiogenesis by simultaneously recruiting the Wnt/\(\beta\)-catenin (\(\beta\)C) and the Wnt/planar cell polarity signaling pathways, to induce pulmonary arterial endothelial cell (PAEC) proliferation and motility, respectively. Cellular levels of \(\beta\)-catenin are regulated by a cytoplasmic protein complex composed of Axin, adenomatous poliposis coli (APC), and glycogen synthase kinase (GSK) 3\(\beta\). In human PAECs, we found that BMP-mediated phosphorylation of extracellular signal-regulated kinase (ERK1/2) inactivates GSK3\(\beta\), and disassembles the Axin/APC/GSK3\(\beta\) complex resulting in \(\beta\)C accumulation and

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translocation to the nucleus, to regulate genes important in endothelial survival and growth. \(^6\) Human PAECs deficient in βC failed to form functional vessels in a murine model of angiogenesis. \(^7\) Based on our findings, we reasoned that constitutive activation of βC in a patient with dysfunctional BMPR2 signaling could protect the pulmonary endothelium against injury by promoting PAEC survival and might induce regeneration of lost vessels by stimulating PAEC growth.

To test this hypothesis, we used the adenomatous polyposis coli (Apc\(^{Min/\pm}\)) mouse in which constitutive elevation of βC in all tissues results from truncation and functional loss of an APC allele. Mice develop colonic polyps in a manner similar to that seen in patients that suffer from familial adenomatous polyposis (FAP), an autosomal-dominant disease also associated with loss of function mutations in APC. \(^8\) We exposed Apc\(^{Min/\pm}\) mice to chronic hypoxia, a stimulus that is known to produce pulmonary hypertension (PH) and loss of small distal PAs. \(^9\) In this study we show that, contrary to our expectations, patients express less APC, and transfection of APC reverses impaired tube formation of these cells. Taken together, our findings reveal a novel role for APC in mediating PAEC adhesion and survival, suggesting that reduced expression or activity of APC could increase the risk of developing PH in individuals that may be susceptible because of the specific environmental exposures and abnormalities in other genes, such as BMPR2.

Methods

An expanded Methods section is available in the Online Data Supplement.

Hemodynamic and Morphometric Studies in Mice

The Animal Care Committee at Stanford University approved all the experimental protocols used in this study. Animals used in the experiments were obtained by crossing a male C57 Apc\(^{Min/\pm}\) with a female C57 mouse. For hypoxia studies, mice were placed in a hypoxia chamber where they were exposed to 10% inspired O\(_2\) with access to food and water ad libitum for 3 weeks. Echocardiographic measurements of cardiac function and right ventricular systolic pressure (RVSP), left ventricular end diastolic pressure, and heart rate were measured under isoflurane anesthesia (1.5%–2.5% in 2 L O\(_2\)/min) in unventilated mice using a closed-chest technique as previously described. \(^10\)

Cell Culture

Primary human mvPAECs and EC growth medium were obtained from Sciencell (Sciencell, Carlsbad, CA). Cells were grown in EC growth medium and used between passages 4–8. Cells were starved in EC starvation medium (0.2% fetal bovine serum and gentamycin/amphotericin) for 24 hours before the experiment. For hypoxia studies, cells were placed in a hypoxia chamber (Biospherix, New York, NY) that provided 1% O\(_2\) concentration for 24 hours.

Mouse mvPAECs were isolated from lung tissue as previously described. \(^7\) To ensure the purity of the culture, we repurified these cultures with CD31 antibody-coated beads after the first passage.

Integrin Blockade assay

Cells were incubated with integrin blocking antibodies (α1, α2, α3, α4, α5, αV, α6, β1, and β4, Millipore, Billerica, MA) for 1 hour at 4°C and then seeded in FN, CIV, or LN-coated 96-well plates. The average number of adherent cells was calculated by counting the total number of cells in 6 random fields per well (magnification ×200).

Plasmids and Transfection Methods

A pCMV-Neo-Bam plasmid containing the Homo sapiens wild-type APC sequence was a kind gift from Dr Bert Vogelstein (Johns Hopkins University). Plasmids containing inserts with the integrin α3 and α4 sequences in a pBluescript II KS (Sac to Kpn poly linker orientation) phagemid were obtained from American Type Culture Collection. The short interfering RNA (siRNA) duplexes (Dharmacon, Lafayette, CO) specific for βC (Dharmacon on-target plus; accession number NM_001012329, NM_020248), and APC (Ambion-validated siRNA, Ambion, Grand Island, NY), were transfected into human mvPAECs using a Nucleofector II (Program T-032) using the basic endothelial Nucleofection Kit (Lonza, Basel, Switzerland).

Generation of the ΔITGA3 and Integrin Chimeric Mutants

For generation of the integrin chimeras, the cytoplasmic tail of α3 (3039G-3136A) or α4 (3129C-3225C) was excised from their native sequence and swapped followed by subcloning into a pcDNA3.1 vector. For the generation of the integrin alpha (ITGA)3 mutant (Δα3), we substituted the serines found in the QPXXXE motif with alanines as illustrated below:

AGCCCCAGCCGTCAGACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
Statistical Analysis
The number of samples or animals studied per experiment is indicated in the figure legends. Values from multiple experiments are expressed as mean±SEM. Statistical significance was determined using unpaired t test or 1-way ANOVA followed by Dunnett or Bonferroni multiple comparison tests unless stated otherwise. A value of \( P<0.05 \) was considered significant.

Results

**ApcMin/+ Mice Demonstrate Greater RVSP, Right Ventricular Hypertrophy, and Reduction in Small Distal PAs After Chronic Hypoxia and Recovery in Room Air**

To determine whether increased levels of total \( \beta \text{C} \) in the pulmonary circulation protect against small distal PA loss and development of PH, we placed 15-week-old male and female Apc\textsuperscript{Min/+} and C57 wild-type littermate control mice in a hypoxia chamber (fractional inspired oxygen: 10%) for 3 weeks, and compared their structural and functional response immediately after exposure, and after 3 weeks of recovery in room air, as in our previous studies.\(^{10}\) Compared with C57, the Apc\textsuperscript{Min/+} mice of both genders developed more severe PH as judged by significantly higher RVSP after 3 weeks of hypoxia not attributable to a change in left ventricular end diastolic pressure. Moreover, the elevation in RVSP persisted after 3 weeks of recovery in room air (Figure 1A). Consistent with these findings, Apc\textsuperscript{Min/+} mice, when compared with C57, had greater right ventricular hypertrophy as judged by Fulton index (the ratio of the right ventricle to left ventricle and septum), both after chronic hypoxia and after the room air-recovery period (Figure 1B). Of note, differences in Fulton index were solely because of the increase in right ventricle mass as no significant differences in left ventricle + septum mass were seen between C57 and Apc\textsuperscript{Min/+} mice in any of the experimental conditions. Similar values for RVSP and right ventricular hypertrophy were observed in C57 and Apc\textsuperscript{Min/+} mice maintained over the same period of time exclusively in room air (Figure 1A–B).

Chronic hypoxia-induced PH is associated with muscularization of normally nonmuscular small distal alveolar duct and wall arteries, and Apc\textsuperscript{Min/+} and C57 mice demonstrated a similar increase in the muscularization of these vessels after chronic hypoxia and recovery (Figure 1C). However, we observed a significant reduction in the number of these distal vessels in the Apc\textsuperscript{Min/+} versus C57 mice in chronic hypoxia that, in contrast to the control group, failed to normalize at the end of the recovery period (Figure 1D).

Echocardiographic analyses in the C57 and Apc\textsuperscript{Min/+} mice after chronic hypoxia and recovery in room air revealed similar values for left ventricular function as judged by fractional shortening, cardiac output, ejection fraction, and heart rate (Online Figure IA–D). Systemic blood pressure (Online Figure IE), left ventricular end diastolic pressure (Online Figure IF), and pulmonary artery acceleration times (Online Figure IG) were also similar in both genotypes under all conditions of study. Similar values for left ventricular end diastolic pressure and cardiac output suggested that the elevated RVSP could represent an increase in pulmonary vascular resistance. However, the hematocrit levels were lower in the Apc\textsuperscript{Min/+} versus C57 mice in room air (30%±5% versus 40%±10%) and hypoxia (54±10 versus 65±8%). We then set out to investigate how, despite the heightened \( \beta \text{C} \) expression in Apc\textsuperscript{Min/+} mice, there could be increased hypoxia-mediated loss of small distal PAs and impaired recovery in room air.

**Apc\textsuperscript{Min/+} mvPAECs Demonstrate Reduced Survival After Serum Withdrawal but a Preserved Proliferative Response Under Normoxia and Hypoxia**

We harvested mvPAECs using CD31 antibody–coated beads (see Methods) to determine whether the impaired survival of these cells when exposed to hypoxia could be a cause of loss of vessels in the Apc\textsuperscript{Min/+} mice. Compared with C57, Apc\textsuperscript{Min/+} mvPAECs demonstrated a >50% reduction in APC protein, accompanied by an increase in total \( \beta \text{C} \) (Figure 2A). Apc\textsuperscript{Min/+} and C57 cells were then either incubated in room air (fractional inspired oxygen, 21%) or in a hypoxia chamber (fractional inspired oxygen, 1%) for 24 hours, as described in the Methods section, and both genotypes were exposed to decreasing serum concentrations ranging from 10% (full growth medium) to 0% (serum free). Impaired cell survival or apoptosis, judged by active caspase 3/7, was significantly greater in Apc\textsuperscript{Min/+} versus C57 mvPAECs and correlated with decreasing serum supplementation in normoxia (Figure 2B, left) that was further aggravated by hypoxia (Figure 2B, right).

Next, we compared the proliferative response of the Apc\textsuperscript{Min/+} versus C57 mvPAEC in response to an angiogenic stimulus. We counted the cells 24 hours after stimulation with vascular endothelial growth factor at concentrations ranging from 0 to 50 ng/mL.\(^{11}\) We observed a lower initial number of adherent Apc\textsuperscript{Min/+} versus control mvPAECs cells, but the rate of proliferation of the Apc\textsuperscript{Min/+} cells that did adhere was comparable to that of C57 mvPAECs in both normoxia (Figure 2C, left) and hypoxia (Figure 2C, right).

Although most studies show that elevated \( \beta \text{C} \) is a survival factor in mammalian cells, there are reports that excessive \( \beta \text{C} \) can also be linked to apoptosis.\(^{12}\) We, therefore, investigated whether the impaired survival of the Apc\textsuperscript{Min/+} mvPAEC could be linked to elevated \( \beta \text{C} \) levels. Apc\textsuperscript{Min/+} cells were transfected with either scrambled or \( \beta \text{C} \)-specific siRNA, with the goal of reducing endogenous \( \beta \text{C} \) levels to those seen in C57 cells (see Figure 2A). After confirming that \( \beta \text{C} \) levels were reduced to the target range (Online Figure IIA), we starved the cells and measured active caspase 3/7 as an indication of apoptosis (see Methods). We found no difference in apoptosis in Apc\textsuperscript{Min/+} mvPAEC treated with \( \beta \text{C} \) siRNA versus scrambled siRNA after 24 hours (Online Figure IIB), suggesting that elevated \( \beta \text{C} \) levels are not responsible for the reduced survival of Apc\textsuperscript{Min/+} mvPAEC.

**APC Deficiency Is Associated With Reduced Tube Formation in Matrigel**

To further understand the consequences of reduced APC on angiogenesis, we seeded Apc\textsuperscript{Min/+} and C57 mvPAECs in wells coated with matrigel, a biological matrix similar in composition to the endothelial basement membrane.\(^{13}\) We found that, after 30 minutes, significantly fewer Apc\textsuperscript{Min/+} versus C57 cells attached to matrigel (Figure 3A), and Apc\textsuperscript{Min/+}
ApcMin/+ mvPAECs formed less complex tube networks when assessed 6 hours after seeding (Figure 3B). In association with reduced adhesion of Apc Min/+ mvPAECs to matrigel, we observed impaired formation of focal adhesions in these cells, as assessed by vinculin and actin staining (Figure 3F–H versus 3C–E), as well as reduced clustering of microtubules at the cell periphery (Online Figure III).

**Adhesion Defect of APC-Deficient mvPAECs Is Prominent on LN**

It has been proposed that APC is required for cell adhesion to the extracellular matrix by promoting the activation of integrin complexes and their association with the underlying actin cytoskeleton.14,15 To determine whether the adhesion defect of Apc Min/+ mvPAECs is selective to a specific component of the extracellular matrix, we used human mvPAECs transfected with either scrambled or APC-targeting siRNA and measured APC expression using an antibody in which the specificity for APC was validated using mass spectrometry (see Methods). A >50% reduction in APC protein was documented 48 hours after transfection, associated with a greater than 2-fold increase in βCat (Figure 4A). Similar to Apc Min/+, APC siRNA-treated mvPAECs demonstrated a preserved growth response to vascular endothelial growth factor in

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**Figure 1.** Adenomatous polyposis coli (ApcMin+) mice in chronic hypoxia demonstrate increased right ventricular systolic pressure (RVSP) and RV hypertrophy and small vessel loss compared with wild-type C57Bl6J (C57) littermate controls. Measurements of (A) RVSP, (B) right ventricle (RV) weight relative to that of left ventricle (LV) and septum (RV/LV+S), (C) muscularization of peripheral arteries at alveolar wall and duct level, and (D) number of peripheral alveolar duct and wall arteries per 100 alveoli in mice exposed to room air (normoxia), 3 weeks of 10% O2 (hypoxia) and 3 weeks of recovery in room air (recovery) as described in the Methods section. Representative images of muscularized pulmonary arteries (C) and vessel number (D) are shown above the corresponding measurements. Bars represent mean±SEM from experiments involving 10 animals per group. **P<0.01, ***P<0.001 vs normoxia; #P<0.01, ##P<0.001 vs C57, 1-way ANOVA with Bonferroni posttest. Scale bar=25 μm (C) and 100 μm (D).
Reduced serum conditions (Online Figure IVA) but reduced survival when incubated in hypoxia (Online Figure IVB). Reduced expression of APC by siRNA resulted in a mild (10%–20%) decrease in adhesion to FN (Figure 4B) and a somewhat more impaired adhesion (30%–40%) to CIV (Figure 4C) at all tested concentrations of substrate. Adhesion of human APC siRNA-transfected mvPAECs to LN was, however, severely reduced (>80%) relative to adhesion to an uncoated substrate and failed to improve despite an increase in the amount of LN coating (Figure 4D). The reduced adhesion to all 3 substrates was unrelated to elevated levels of βC, as shown in experiments using βC-targeting siRNA to reduce βC levels in ApcMin/+ mvPAECs (Figure 4E).

**LN Interaction With α3β1 Integrin Triggers an Interaction With Cytoplasmic APC in mvPAECs**

To determine which integrin receptor complex regulates adhesion of human mvPAECs to LN, we incubated cells with blocking antibodies against specific α and β integrins before seeding (see Methods). Although adhesion to LN was reduced with high concentrations of α1, α4, α5, and αv blocking antibodies, and with lower concentrations of α6 and β4 blocking antibodies, the most profound effect was noted with the blockade of α3 and β1 integrins (Online Figure VA).

Binding to extracellular matrix proteins can result in conformational changes that facilitate the formation of active signaling complexes on α- and β-integrin cytoplasmic tails that can impact cell adhesion and survival. To establish whether LN promotes recruitment of APC to the cytoplasmic tail of the β3 integrin to enable signaling, we first immunoprecipitated human mvPAEC lysates after 1 hour of exposure to LN using antibodies specific to this integrin, followed by western immunoblot for APC. We also assessed APC interaction with the α1-integrin in cells adherent to CIV, or with α5 integrin in cells plated on FN. We found that although APC can associate with all of these α-integrins, there was a striking increase in

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**Figure 2. Adenomatous polyposis coli (Apcmin/+ ) microvascular pulmonary artery endothelial cells (mvPAECs) demonstrate reduced survival and growth.**

A. Representative Western immunoblots for β-catenin (βC) and APC in lysates from C57Bl6J (C57) littermate and Apcmin/+ mvPAECs. Densitometric values are shown relative to α-tubulin. ***P<0.0001, unpaired t test. B. Apoptosis was measured by the Caspase 3/7 assay in C57 and Apcmin/+ mvPAECs exposed to a range of serum concentrations (0%–10%) under either normoxia (left) or hypoxia (right). After 24 hours, lysates were analyzed for luciferase activity (LU) as described in the Methods section. Camptothecin was used as a positive control. C. Proliferation was assayed by cell count assays in C57 and Apcmin/+ mvPAECs exposed to a range of vascular endothelial growth factor (VEGF) concentrations (0–50 ng/mL) under either normoxia (left) or hypoxia (right). Cell numbers were measured 72 hours after the addition of VEGF as described in the Methods section. Bars represent mean±SEM from N=3 experiments. *P<0.01, **P<0.001, ***P<0.0001 vs C57 at 10% fetal bovine serum (FBS; apoptosis assay) or baseline (VEGF proliferation assay); #P<0.01, ##P<0.001, ###P<0.0001 vs C57, 1-way ANOVA with Bonferroni posttest. FiO2 indicates fractional inspired oxygen.
the formation of a $\alpha_3$-APC complex after seeding of the mv-PAECs on LN, likely because of clustering of $\alpha_3$ integrins on LN (Online Figure VB).

Taken together, our studies suggest that binding to LN induces recruitment of APC to the $\alpha_3$-(likely $\beta_1$) integrin complex. We next sought to examine the nature of this interaction and why recruitment of APC to the $\alpha_3$ integrin is required for adhesion to LN.

**LN-Dependent APC Binding to $\alpha_3$ Integrin Facilitates Focal Adhesion Complex Formation by Selectively Activating ILK-1**

Binding of the integrin receptor complex to the extracellular matrix triggers a sequence of intracellular events that not only stabilizes cell–matrix interactions but also controls cell survival. Central to these biological events is the recruitment of ILK-1, a 52-kDa protein composed of 3 structurally distinct domains: 3 ankyrin repeats near the N terminus, a short linker sequence, and a kinase domain at the C terminus. Activation of cytoplasmic adaptor proteins, such as paxillin and vinculin, via direct or indirect binding to ILK-1 is necessary for the formation of mature integrin–actin focal adhesion complexes. Thus, a reduction in ILK-1 expression or activity can change cell shape and decrease the strength and number of focal adhesions. Indeed both proteins colocalize at the cell periphery at the site of focal adhesions (Online Figure VI), suggesting that they may cooperatively interact in the mechanism responsible for cell adhesion to the extracellular environment.

To determine whether APC is required for the recruitment of ILK-1 to integrin receptors on binding to extracellular substrates, we performed coimmunoprecipitation studies. Using...
α-integrin antibodies, we precipitated protein complexes in cell lysates from human mvPAECs treated with either scrambled or APC siRNA after binding to CIV, FN, and LN, and then carried out Western immunoblotting for ILK-1. We found that the amount of ILK-1 precipitated using the α3 integrin antibody was independent of the extracellular matrix substrate (LN, CIV, or FN) to which the cells adhered (Figure 5A) and was preserved despite reducing levels of APC (Figure 5A). Thus, recruitment of ILK-1 by the α3 integrin is independent of its interaction with APC, even on LN.

We next determined whether APC deficiency could influence ILK-1 activation. We incubated ILK-1 precipitated from lysates of human mvPAEC seeded on FN, CIV, and LN with GSK3β, a known substrate of ILK-1 (see Methods). We found that GSK3β phosphorylation by ILK-1 was higher in cells cultured on LN compared with CIV and FN. Moreover, ILK-1 activity was reduced in cells treated with APC-targeting siRNA when cultured on LN, but not in cells cultured on FN or CIV (Figure 5B). Thus, the increase in APC associated with the α3 integrin in mvPAECs seeded on LN is a requirement for the activation of ILK-1.

ILK-1 activation also increases cell survival via the protein kinase B/Akt signaling pathway.20 Given the poor survival response seen in APC-deficient mvPAECs (see Figure 2), we investigated whether loss of APC by APC-targeting siRNA reduced activation of protein kinase B/Akt in mvPAECs cultured on LN versus cells cultured on CIV or FN. Although there was considerable variability in the levels of pAkt (ie, protein kinase B active) in mvPAECs on the different substrates, reducing APC decreased pAkt only in LN-bound cells (Figure 5C).

Taken together, these studies in human mvPAECs cultured on LN show that the interaction between APC and the α3 integrin is required for the activation of ILK-1 and that ILK-1 activity promotes both adhesion of mvPAEC on LN and survival via protein kinase B/Akt (Figure 8).

Adhesion to LN Is Dependent on the QPSXXE Motif of α3 Integrin

The next series of experiments was carried out to determine whether the specific interaction of APC with the α3 integrin that is required for ILK-1 activity is also required for cell
adhesion to LN. The cytoplasmic portion of the α3 integrin is composed of 52 amino acids and can undergo a series of posttranslational modifications. One such modification is the phosphorylation of serine 1042 within the QPSXXE motif that strengthens adhesion to LN via paxillin and focal adhesion kinase activity.21

Given the specificity of our findings, we hypothesized that the association of APC with the α3 cytoplasmic tail would promote cell adhesion independent of the extracellular substrate. To this end, we engineered an integrin chimera in which the cytoplasmic tail of the α3 integrin was switched with that of the FN-specific integrin, α4 (α3Δ4)22–24 and another in which the α4 cytoplasmic tail was switched with that of the α3 integrin (α4Δ3) (schema in Figure 6A). We transfected these constructs individually into mvPAECs cotransfected with either scrambled or APC siRNA. We found that cells transfected with the α4 chimera (α4Δ3) demonstrated loss of adhesion to FN after treatment with APC siRNA (Figure 6B) whereas the α3 chimera (α3Δ4) demonstrated preserved LN adhesion despite transfection with APC siRNA (Figure 6C). As expected, under conditions of reduced APC, we also observed reduced LN binding in both vector and α4Δ3-transfected human mvPAECs (Figure 6B).

We also investigated whether disruption of the QPSXXE sequence in the α3 integrin cytoplasmic tail, which contains the serine residue critical to LN binding (S1042), could reproduce the adhesion defect seen with APC deficiency. To this end, we mutated the QPSXXE motif in the α3 integrin cDNA sequence by substituting alanines for serines within the motif (Figure 6A). Consistent with our expectation, we found that cells transfected with the mutant α3 integrin construct (Δα3) demonstrated reduced adhesion to LN, compared with cells transfected with an empty vector (Figure 6D).

Taken together, our results demonstrate that when human mvPAECs bind LN, a functional QPSXXE motif in the cytoplasmic tail of the α3 integrin recruits APC to activate ILK-1, stabilize focal adhesions, and activate pAkt (Figure 8). This property is not unique to mvPAECs, as we also documented reduced adhesion to LN compared with FN and CIV in the SW480 colon cancer cell line that expresses a truncated version of APC missing the cytoplasmic tail (Online Figure VII).

Reduced APC Expression in mvPAECs From IPAH Patients Is Related to Decreased Adhesion and Tube Formation

Previous studies showed that PAECs isolated from IPAH patients demonstrate reduced survival and form smaller vascular tube networks compared with those of healthy donors,25 but the mechanism involved was poorly understood. To determine whether the reduced angiogenic potential of IPAH mvPAECs could be related to decreased expression of APC, we isolated cells from the lungs of 5 healthy subjects (unused donor lungs) and lungs explanted from 5 IPAH patients undergoing lung transplant (see Online Table I for patient characteristics). These cells were obtained from tissues procured through the Pulmonary Hypertension Breakthrough Initiative (see
Methods). We confirmed reduced tube formation in matrigel in mvPAECs from IPAH patients compared with those from healthy donors (Figure 7A). IPAH mvPAECs exhibited decreased adhesion to LN-coated surfaces (Figure 7B) compared with healthy donor mvPAECs, associated with lower levels of APC as demonstrated by Western immunoblot (Figure 7C) and immunohistochemistry (Figure 7B). As with APC siRNA-treated mvPAECs (Figure 7B), we found evidence of reduced ILK-1 activation in IPAH mvPAECs incubated with LN (Figure 7D). To determine whether the APC deficiency is directly responsible for the adhesion defect seen in IPAH mvPAECs, we transfected these cells with an APC expression construct. When transfected in healthy donor cells, wild-type APC protects against apoptosis secondary to hypoxia supporting its role as a prosurvival factor (Online Figure VIII). APC-transfected IPAH mvPAECs recovered the ability to adhere to LN (Online Figure IX) and, when seeded in matrigel, were able to form vascular networks of similar size and density as those produced by healthy donor mvPAECs (Figure 7D).

Discussion

Based on our findings, we propose that in mvPAECs, APC promotes cell survival in response to formation of α3 integrin-rich focal adhesions on binding to LN (Figure 8).
Interaction of QPSXXE motif of the α3 integrin cytoplasmic tail with APC is a prerequisite for the activation of ILK-1, formation of focal adhesions, and phosphorylation of pAkt to mediate cell survival. To the best of our knowledge, this is the first demonstration that APC can promote mvPAEC survival independent of its role in regulating Wnt signaling. Moreover, a reduction in APC appears to contribute to the decreased angiogenic potential of mvPAECs in IPAH patients. Although the current findings appear to contradict our previous work on the proangiogenic effects of βC in BMP-stimulated PAECs, we have discovered a novel function of APC that also promotes cell survival.

Since its original characterization, studies of the Apc Min/+ mouse established a firm link between increased Wnt/βC activation and development of colonic polyps. However, few studies have investigated whether there is a vascular phenotype in these animals, or whether APC might play a role in tumor pathogenesis independent of Wnt signaling. A study...
revealed that APC mRNA and protein levels fall in response to hypoxia as a result of repression of the APC promoter by hypoxia inducible factor-1α, a phenomenon that we were able to reproduce in human mvPAECs incubated in hypoxia (fractional inspired oxygen: 1%) for 24 hours (Online Figure X). It is surprising that the hypoxia-mediated endothelial apoptosis and loss of vessels and the exaggerated PH in the \( \text{Apc}^{\text{Min/+}} \) versus C57 mice were not accompanied by greater muscularization of peripheral arteries as is seen in other murine models of PH. This may, however, be related to a confounding effect of loss of APC in PA smooth muscle cells. Future studies creating a transgenic mouse with EC- or smooth muscle cell-specific deletion of APC would allow us to better understand the nature of the smooth muscle cell response to APC deletion. Finally, it is also intriguing that mice develop PH only when exposed to hypoxia even though they can develop malignant tumors at baseline.

A prominent finding in our studies in APC-deficient mvPAECs was the relative specificity of the adhesion defect to LN and LN-enriched substrates, such as matrigel. Because the basement membrane of blood vessels is rich in LN, inability to properly attach to this substrate would substantially impair the physiological angiogenic response to a vascular injury. In HeLaS3 cells, microtubule-associated APC is recruited to focal adhesions by disheveled after the binding of Wnt5a to the Wnt receptor frizzled 2. In this setting, it was thought that APC is brought in close proximity to the integrin receptors by its association with disheveled and frizzled 2 and that this fosters cytoskeletal changes that influence cell adhesion, polarity, and directed migration. Our studies demonstrate not only that there is a physical interaction between APC and the \( \alpha_3 \) integrin but that this interaction is required for ILK-1 activity and stabilization of focal adhesions. Moreover, this interaction can only occur if the QPSXXE motif in the \( \alpha_3 \) cytoplasmic tail is preserved.

At the center of the interaction between LN, \( \alpha_3 \) integrin and APC is the activation of ILK-1, a protein that facilitates integrin–actin interactions and activates numerous signaling pathways in response to integrin binding to the extracellular matrix. Our studies show that the \( \alpha_3 \) integrin can recruit ILK-1 independent of APC but that the APC interaction with \( \alpha_3 \) integrins clustering on LN is required to activate ILK-1. Other intermediary proteins like APC can promote a functional interaction between integrin receptors and ILK-1 and are likely associated with mvPAEC binding to other substrates. An example is the calponin homology ILK-binding protein (or \( \alpha_-\text{parvin/actopaxin} \)), an integrin adaptor protein that binds \( \beta_1 \) integrin and recruits ILK-1 by interacting with its C terminal. This event also ensures the activation of ILK-1 and consequent or concomitant downstream signaling via protein kinase B (pAkt).

In contrast to the strong APC-dependent activation of ILK-1 seen in LN-bound mvPAECs, the extent of ILK-1 activation seen in cells cultured on FN or CIV was minimal and did not correlate with pAkt activation. One possible explanation for this observation is that FN and CIV-specific integrin receptors preferentially used focal adhesion kinase, to ensure maturation of focal adhesion complexes and to trigger activation of pAkt. Indeed, studies performed in mesenchymal stem cells, intestinal epithelial cells, and human umbilical vein ECs have demonstrated that both CIV and FN promote focal...
adhesion formation and pAkt through activation of focal adhesion kinase.\textsuperscript{38,39} It was interesting that transfection of a constitutively active Akt expression construct failed to reverse or improve survival of APC-deficient cells (Online Figure XI). Based upon previous studies, it is probable that recruitment of APC to the LN-bound α\textsubscript{3}β\textsubscript{1} integrin complex initiates a signaling cascade involving additional survival pathways responsible for protecting mvPAECs during stress and injury. Alternatively, recruitment of constitutively active Akt to the integrin cytoplasmic scaffold is necessary to properly target signaling to downstream effectors of survival.\textsuperscript{40}

Reduced APC levels in SW480 colon cancer cells\textsuperscript{41} could contribute to heightened metastatic behavior by allowing the cells to detach from the LN-based extracellular matrix and assume an invasive phenotype. In a study investigating 22 colorectal cancer cell lines with different levels of APC expression, a strong correlation was found between CpG methylation in 2 regions of the APC promoter and the degree of reduced APC expression.\textsuperscript{42} Moreover, treatment of colon cancer cells with 5-aza-2′-deoxycytidine, a known DNA methyltransferase inhibitor, reduced CpG methylation and concomitantly increased levels of APC. Other agents with demethylating properties, such as selenite, also increase levels of APC in prostate cancer cells and reduce tumor growth and metastasis.\textsuperscript{43} It is possible that these strategies to increase APC could be used to reverse the angiogenic defect in IPAH mvPAECs, as we showed, by transfecting the cells with an APC construct.

Although the mechanism leading to a reduction in APC in IPAH mvPAECs is unknown, several possibilities could be explored. Using a microRNA (miR) microarray to analyze colorectal cancer cells exposed to the novel antineoplastic agent CM-1, Li et al discovered that CM-1–dependent suppression of miR-135a/b was associated with an increase in APC expression and anticancer activity.\textsuperscript{44} Also, Nagel et al\textsuperscript{45} found that untreated colorectal cancer cells exhibit higher level of miR-135a/b that was inversely related to APC expression. Other miRs, such as miR-27, suppress APC expression in osteoblasts and this regulates their differentiation.\textsuperscript{46} Thus, it is possible that in IPAH cells there is an increase in miR-135a/b or in miR-27. Caruso et al\textsuperscript{47} described a different miRNA expression profile in IPAH compared with control lungs but miR-135a/b and miR-27 were not mentioned in their study.

Given the potential clinical relevance of our findings, the fact that there is no reported link between FAP and PAH is surprising. Recent reports describe increased vascularity in the oral mucosa of patients with FAP, which contrasts with our findings in the lungs of Apc\textsuperscript{min/+} mice.\textsuperscript{48} A possible explanation for this discrepancy may be related to an inherent difference in the subendothelial matrix of systemic versus pulmonary microvessels. Alternatively environmental hypoxia may also cause systemic microvessel dropout. That is, specific environmental and genetic modifiers may be necessary to unmask FAP or PAH. It is conceivable that patients who are carriers of the BMPR2 mutation may be at a higher risk of developing IPAH when they have loss of function of APC. It is also possible that some patients with FAP have mild or moderate PH, but their symptoms are masked by the underlying pathology of their colonic condition, or that some patients with BMPR2 mutations and PAH have mild colonic disease.

Given the rarity of both conditions (ie, the lack of penetrance of the APC mutation in causing colon disease\textsuperscript{49,50} and the BMPR2 mutation in causing PAH), it is likely that screening FAP patients with APC mutations for PAH may be necessary to further characterize the presence of PAH in FAP, as well as the contribution of reduced APC to IPAH, particularly in patients with BMPR2 dysfunction.

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**Disclosures**

None.

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What Is Known?

- Idiopathic pulmonary arterial hypertension (IPAH) is associated with progressive loss of small vessels and impaired regeneration following genetic and/or environmental injury.
- Activation of Wnt/β-catenin (βC) signaling in pulmonary artery endothelial cells (PAECs) promotes angiogenesis by targeting expression of βC specific genes involved in proliferation and survival.
- Loss of adenomatous poliposis coli (APC) leads to increased proliferation and survival of cancer cells by increasing both the amount and transcriptional activity of βC.

What New Information Does This Article Contribute?

- Despite increasing βC levels in PAECs, partial loss of APC leads to significant small vessel loss and more severe PAH in transgenic (ApcMin/+ ) mice exposed to chronic hypoxia when compared to controls.
- Loss of APC results in reduced α3 integrin-dependent laminin adhesion and increased endothelial cell apoptosis.
- APC deficiency is seen in PAECs isolated from IPAH patients and correlates with reduced adhesion and impaired angiogenesis. Restoring APC in IPAH results in normalization of PAEC phenotype.

Novelty and Significance

Pulmonary arterial hypertension (IPAH) is characterized by progressive elevation in pulmonary pressures and loss of small pulmonary arteries. As BMPs promote pulmonary angiogenesis by recruiting the Wnt/βC pathway, we proposed that βC activation could reduce loss and/or induce regeneration of small PAs and attenuate PAH. To assess the impact of βC activation on chronic hypoxia-induced PAH, we used the adenomatous poliposis coli (ApcMin) mouse, where reduced APC causes constitutive βC elevation. Surprisingly, hypoxic ApcMin/+ mice displayed greater elevation in pulmonary pressures and small pulmonary artery loss compared to control littermates. PAECs isolated from ApcMin/+ demonstrated reduced survival and angiogenic responses along with profound decrease in adhesion to the laminin component of the basement membrane. We discovered that APC is required to interact with the laminin α3 integrin cytoplasmic domain to stabilize focal adhesions and trigger pAkt by activating integrin-linked kinase (ILK-1). This signaling mechanism is clinically relevant as PAECs from IPAH patients also have reduced APC expression, impaired laminin adhesion and vascular tube formation, that were corrected by restoration of APC expression. Thus, we propose that APC is integral to endothelial cell adhesion and survival and a reduction in APC could increase the risk of IPAH in genetically susceptible individuals.
Loss of Adenomatous Poliposis Coli-α3 Integrin Interaction Promotes Endothelial Apoptosis in Mice and Humans

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ONLINE DATA SUPPLEMENT: METHODS
Hemodynamic and Morphometric Studies on Mice

All the experimental protocols used in this study were approved by the Animal Care Committee at Stanford University and adhered to the published guidelines of the National Institutes of Health and the American Physiological Society.

C57Bl6J and C57 Apc<sup>Min/+</sup> mice were purchased from the Jackson Laboratory (Jackson Laboratories, Bar Harbor, ME). All animals used in our experiments were obtained by crossing male C57 Apc<sup>Min/+</sup> mice and a female C57Bl6J for at least five generations. Tail based genotyping of the progeny was used to identify littermate controls and Apc<sup>Min/+</sup> mice. For hypoxia studies, mice were placed in a hypoxia chamber where they were exposed to 10% inspired O<sub>2</sub> with access to food and water ad libitum for 3 weeks. To establish the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen, and the gas was re-circulated continuously. The chamber environment was monitored using an oxygen analyzer (Servomex, Sugar Land, TX). Carbon dioxide was removed by soda lime granules and chamber temperature was maintained at 22–24°C. The chamber was inspected daily to document temperature, O<sub>2</sub> + CO<sub>2</sub> atmospheric concentration and animal welfare, and was opened every third day for 1 hour to clean the cages and replenish food and water supplies. RVSP, RV and heart rate were measured under isoflurane anesthesia (1.5–2.5% in 2 L O<sub>2</sub>/min) in unventilated mice using a closed chest technique as previously described in detail. A 1.4-F Millar catheter (Millar Instruments, Houston, TX) was inserted into the jugular vein and directing to the right ventricle. Systemic blood pressure was determined in conscious animals by a noninvasive computerized tail-cuff method. Pulmonary artery acceleration time (PAAT) was determined from Doppler echocardiograms acquired with a GE Vivid 7 ultrasound system (GE Healthcare, Milwaukee, WI) equipped with 13-MHz transducer. The Doppler sample volume was centrally positioned within the main PA, just distal from the pulmonary valve with the beam oriented parallel to the flow as described. The sweep speed for flow recordings was 200 mm/s. PAAT was defined as the interval between the onset of systolic pulmonary arterial flow and peak flow velocity. LVEDP measurements were performed under isoflurane anesthesia (1.5% in 1L/min oxygen flow). A 1.4-F Millar catheter was inserted into the right carotid artery and advanced into the left ventricle. Intraventricular pressure signals from Millar transducer were analyzed using a PowerLab/4sp data acquisition system (AD Instruments Ltd, Castle Hill, Australia) connected to a ThinkPad computer. Systolic and diastolic left ventricular pressure were measured directly from the pressure waveforms. Left ventricular shortening fraction and cardiac output were evaluated by echocardiography (Acuson Sequoia 256, Garvenville, NY). The mice were euthanized by exsanguination. The heart and lungs were removed en bloc, and right ventricular hypertrophy was evaluated by the Fulton index, i.e., weight of right ventricle/left ventricle plus septum (RV/LV+S). The pulmonary circulation was flushed with 3ml of PBS at 37°C, and the lungs were prepared for morphometric analyses by barium gelatin injection of the pulmonary arterial vasculature and formalin inflation-fixation of the lung. Morphometric analyses were performed on paraaffin-embedded lung sections stained using elastic van Giessen or Movat pentachrome stains. The total number of peripheral arteries was calculated as a ratio of the number of arteries per 100 alveoli in each of 5–6 different microscopic fields (200x magnification) per section from each lung. Musclearization was assessed in 15 higher magnification fields/mouse (400x magnification) by calculating the proportion of fully and partially muscularized peripheral (alveolar duct and wall) PAs to total peripheral PAs. All morphometric analyses were performed by one observer, blinded as to genotype and condition, i.e., room air, hypoxia, and recovery.
Isolation of Mouse and Human Microvascular Pulmonary Endothelial Cells

Mouse mvPAEC were isolated by digesting whole lung tissue with collagenase IA (0.5mg/ml) for 45 minutes at 37°C. The cell suspension was filtered through 70µm cell strainers, and then centrifuged at 250G for five minutes. The cell pellet was then washed three times with PBS and the cell suspension was incubated with sheep anti-rat IgG magnetic beads (Invitrogen, Carlsbad, CA) coated with rat anti-mouse CD-31 antibody (BD Biosciences, San Diego, CA) to select out mvPAEC for culture. Characterization of the culture after isolation was performed by labeling with Dil-conjugated Ac-LDL (Dil-Ac-LDL) and CD31 staining and confirmed as 95% pure ECs.

Human mvPAEC were isolated from fresh lungs of control (unused donor) and IPAH patients obtained through the PHBI Network (PHBI). The tissues were procured at the Transplant Procurement Centers at Stanford University, Cleveland Clinic and Allegheny General Hospital and de-identified patient data were obtained via the Data Coordinating Center at the University of Michigan. Lung tissue was digested with collagenase IA (1.0mg/ml) (Sigma-Aldrich, St Louis, MO) for 1h and followed the mouse mvPAEC protocol. Anti-human CD31-coated beads were used for EC purification (Cat: 111.55D, Invitrogen). To ensure the purity of the culture we re-purified these cultures with CD31 beads after first passage. Staining using Dil-conjugated Ac-LDL (Dil-Ac-LDL, Invitrogen) and CD31 show over 95% purity for ECs. The expression analyses were done at passage 2. All cells were used between passages 3 and 6.

Cell Culture

Primary human mvPAECs and EC growth medium were obtained from Sciencell (Sciencell, Carlsbad, CA). Cells were grown in EC growth medium (2% FBS, 1 µg/ml hydrocortisone, 10ng/ml human epidermal growth factor, 3ng/ml basic fibroblast growth factor, 10µg/ml heparin, and gentamycin/amphotericin); subcultured at a 1:4 ratio in 100mm dishes (Corning, Lowell, MA) and used between passages 4-8. Cells were starved in EC starvation medium (0.2% FBS and gentamycin/amphotericin) for 24 hours prior to the experiment. SW480 cells were obtained from ATCC (ATCC, Manassas, VA) and maintained in Leibovitz F12 media (ATCC). For hypoxia studies, cells were placed in an incubator containing a hypoxia chamber (Biospherix, New York, NY) that provided 1% O₂ concentration for 24 hours.

For affinity purification (see “Affinity Purification” below), 293T cells were used and maintained in DMEM high glucose/10% FCS plus Penicillin/Streptamycin.

Adhesion Assay

A 96-well plate was coated with fibronectin (purified from human plasma and purchased from Sigma-Aldrich, St. Louis MO), collagen IV (purified from human fibroblasts and purchased from Sigma-Aldrich, St. Louis, MO) and laminin (derived from Engelbreth-Holm-Swarm murine sarcoma basement membrane and purchased from Sigma-Aldrich, St. Louis, MO) overnight at 4°C using concentrations of 1.0, 5.0 and 10.0 µg/ml for each ECM component per well, respectively. Human mvPAECs between passages three and five were seeded (20,000 cells/well) and incubated at 37°C for 30 minutes. At the end of the experiment, the cells were washed in PBS and fixed in 4% PFA at room temperature for 10 minutes. Subsequently, cells were stained with crystal violet for 10 minutes.
followed by a water wash for 10 minutes. The average number of cells was calculated by
counting the total number of cells in six random fields per well (200x magnification). All
assays were done in triplicate, and three separate cell harvests were assessed.

**Tube Formation Assay**

Human and murine mvPAECs (5×10³ per well) were cultured in a 96-well plate (Corning,
Lowell, MA) coated with 50µl Matrigel Basement Membrane Matrix or Growth Factor
Reduced Matrigel (Trevigen, Gaithersburg, MD). Tube length was quantified after 6
hours by measuring the cumulative tube length in three random microscopic fields using
a Leica computer-assisted microscope with the program KS300 3.0 (Zeiss).

**Integrin Blockade Assay**

A 96-well plate was coated with LN overnight at 4°C using a concentration of 5.0µg/ml.
Prior to seeding in the cell culture wells, mvPAECs between passages three and five
were incubated with different integrin blocking antibodies (α1, α2, α3, α4, α5, αV, α6, β1
and β4, Millipore, Billerica, MA) at the following concentrations: 0.1, 0.5, 1.0 and 5 µg/ml,
for one hour at 4°C. Following integrin blockade, the cells were seeded in the coated 96
well plates and adhesion studies performed as described above.

**Proliferation and Survival assays**

To measure proliferation, cells were seeded at 25,000 cells per well on 24-well plates in
EC growth medium and allowed to adhere overnight. The next day, cells were washed
3x with PBS and incubated in EC starvation medium for 24 hours. Cells were then trypsinized and counted in a hemocytometer as previously described⁴. All assays were
done in triplicate, and three separate cell harvests were assessed. For Caspase 3/7
assays, cells were seeded in a 96-well plate (4 wells per condition and 5,000 cells per
well) and allowed to attach overnight, then incubated for 24 hours in serum-free media to
induce apoptosis. Then cells were incubated for 1 hour in 100µl of Caspase 3/7
Luciferase Reagent Mix (Promega, Madison, WI) and total luminescence measured in a
Turner 20/20 luminometer (Turner Biosystems, Sunnyvale, CA).

**RNA Interference**

The siRNA duplexes (Dharmacon, Lafayette, CO) specific for β-catenin (Dharmacon on-
target plus; accession number NM_001012329, NM_020248), and APC (Ambion
Validated siRNA, Ambion, Grand Island, NY), were transfected into human mvPAECs
using nucleofection as described previously ⁴. Knockdown efficiency was evaluated 48
hours later by measuring protein levels in lysates using a western immunoblot as
described below.

**Plasmids and Transfection Methods**

A pCMV-Neo-Bam plasmid containing the H. sapiens WT APC sequence was a kind gift
from Dr. Bert Vogelstein (Johns Hopkins University). A constitutively active (CA) Akt
eexpression construct (pcDNA3 T7 Akt1 K179M T308A S473A) was a kind gift from Dr.
William Sellers (Novartis). Plasmids containing inserts with the integrin α3 and α4
sequences in a pBluescript II KS phagemid were obtained from ATCC. Cells were
transfected in a Nucleofector II (Program T-032, Lonza) using the basic endothelial
Nucleofection Kit (Lonza, Basel, Switzerland).

**Western Immunoblotting**

Cells were washed three times with ice-cold PBS and lysates prepared by adding boiling lysis buffer (10mM Tris HCl, 1% SDS, 0.2mM PMSF) containing 1X protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO), scraping into a 1.5ml microcentrifuge tube and boiling for 10 minutes prior to centrifugation. Protein concentration was determined by the Lowry assay (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded onto each lane of a 4-12% Bis-Tris gel and subjected to electrophoresis under reducing conditions. After blotting, PVDF membranes were blocked for one hour in blocking buffer (nonfat milk powder 5% in TBS/Tween 0.1%) and incubated with primary antibodies overnight at 4°C. Binding of secondary HRP-antibodies was visualized by ECL or ECL plus (Amersham Biosciences, Piscataway, NJ). A loading control was evaluated by re-probing the membrane with a mouse monoclonal antibody to α-tubulin (Sigma-Aldrich). The following antibodies were used to probe the membranes: β-catenin (BD Biosciences), APC and ILK-1 (Santa Cruz Biotechnology, Santa Cruz, CA), α1, α3 and α5 integrins (Millipore), phospho and total Akt (Cell Signaling Technologies, Boston, MA).

**Affinity purification (AP) and mass spectrometry to validate specificity of APC antibody**

For AP, 293T cells were seeded to 20% subconfluence in 15 cm plates. When cells were attached, cells were transfected with 20 µg pCMV-APC or pCMV empty vector using calcium phosphate. 48 h after transfection, cells were detached and washed with PBS. Cells were lysed in 1 ml cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, 2 mM sodium orthovanadate, 2 mM PMSF and HALT protease inhibitor (Pierce)). Cells were dounced 20x on ice, and spun at 10,000 rpm for 30 min. The supernatant was pre-cleared with 20 µl Dynabeads Protein G (Invitrogen) for 1 hr. The pre-cleared lysate was incubated with 4 µl of APC antibody (Santa Cruz) overnight. The next day the antibody was chased with 40 µl of Dynabeads for 3 hr. The beads were washed 4 times with lysis buffer but containing 0.05% Nonidet P40. Proteins were eluted with 50 µl of IgG Elution Buffer (Pierce) for 10 min using gentle vortex. The IP solution elutes were neutralized with 5 µl of 1M Tris pH 9.0. Half of the elutes were analyzed by 4% SDS-PAGE (Invitrogen) followed by silver staining (Pierce). Protein bands were excised and sent to the Stanford University Mass Spectrometry core facility (Stanford, CA) for analysis. Excised gels were digested using Promega MS grade trypsin overnight as previously reported (1), with the addition of the acid labile surfactant protease max (Promega). Prior to digestion the gel slices were cut into approximately 1mm x 1mm cubes, reduced with 5 mM DTT and alkylated with acrylamide. Peptides were extracted and dried down using a speed-vac prior to reconstitution and analysis. The remaining IP solution elutes were sent to the SUMS facility. Elutes were digested using the previously reported FASP (2) protocol using 30 µM filters from Pall. Nano reversed phase HPLC was done using an Eksigent 2D nanoLC (Eksigent, Dublin, CA) with buffer A consisting of 0.1 % formic acid in water and buffer B 0.1 % formic acid in acetonitrile. A fused silica column self packed with duragel C18 (Peeke, Redwood City, CA) matrix was used with a linear gradient from 2 % B to 40 % B at a flow rate of 600 nL/minute. The nanoHPLC was interfaced with a Bruker/Michrom Advance Captive spray source for nanoESI into the mass spectrometer. The mass spectrometer was a
LTQ Orbitrap Velos (Thermo Fischer Scientific) which was set in data dependent acquisition mode to perform MS/MS on the top twelve most intense multiply charged cations. The .RAW data were searched using Sequest on a Sorcerer platform against the Uniprot database. Data was validated and visualized using Scaffold software.

Confocal Microscopy

Cells were plated in four-chamber polystyrene glass slides (15,000 cells per chamber). For stimulation studies, cells were starved for 48 hours and then stimulated with BMP-2 as specified. Next, cells were fixed for ten minutes in 4% paraformaldehyde followed by three washes with PBS. Cells were permeabilized for 30 minutes on room temperature with 0.1% triton X-100 and 1% BSA in ice-cold PBS prior to overnight incubation with primary antibody. The next day, samples were washed in PBS three times and incubated with Alexa 488 or 555 tagged secondary antibody ( Molecular Probes, Carlsbad, CA) for one hour at room temperature. For actin labeling studies, slides were treated with Alexa 488 labeled phalloidin for 20 minutes to stain for actin followed by addition of Gold Antifade solution containing DAPI (Molecular Probes) and stored at 4°C until analysis.

Confocal analysis was performed using a Leica SP2 AOBS confocal laser scanning microscope using HCX PL APO 63X oil objective (N.A. 1.32-0.60) to locate areas of interest on the slides. Image acquisition was performed using the Leica Confocal, v 2.5, build 1347 software. Images were processed and saved in TIFF format using Adobe Photoshop Creative Suite 2 (Adobe Systems, San Jose, CA).

ILK-1 Kinase Assay

Cells were starved for 48 hours prior to the experiment. Cells were washed in PBS and lysed with ice-cold RIPA buffer (500ml per T75 flask). Equivalent amounts (250 µg) of lysates were incubated overnight at 4°C with 5 µl of goat polyclonal anti-ILK1 antibody (Santa Cruz Biotechnology). The next day, the immune complexes were precipitated with protein G sepharose 4 fast flow beads (Amersham) and washed three times with RIPA lysis buffer and three times with kinase buffer (50mM HEPES [pH=7], 2mM MgCl2, 2mM MnCl2, 20mM Na3VO4, protease inhibitors). The kinase assay was performed using 2µg of GSK3β fusion protein (Cell Signaling Technologies, Boston, MA) as a substrate. This fusion protein is a low molecular weight (~28kDa) peptide that contains the two serine residues (Ser21/9) which undergo phosphorylation in the presence of ILK-1. GSK3β fusion protein is then incubated with 200µM ATP in the reaction buffer (50mM HEPES [pH=7], 2mM MgCl2, 2mM MnCl2, 20mM Na3VO4, 20mM NaF) for 30 minutes at 30°C. Next, Laemmli buffer was added and samples were boiled for 10 minutes prior to loading onto a 4-12% Bis-Tris SDS-PAGE gel. Phosphorylation of the substrate was detected by western immunoblot with anti-GSK3β serine 21/9 antibodies (Cell Signaling Technologies).

Co-Immunoprecipitation (Co-IP)

Analysis of protein-protein interaction was carried out using a previously published protocol\[5]. Briefly, after stimulation, cells were washed in ice-cold PBS followed by cell lysis with 300µl of Co-IP buffer (50mM HEPES, pH=7.8, 300mM NaCl, 1% NP-40, 1.2mM EDTA pH=8.0, 5mM MgCl2 with protease inhibitors). To the lysate, a total of 3-5
ml of the IP antibody was added followed by overnight incubation at 4°C. The next day, the cell lysate was incubated with 20ml of equilibrated protein G beads for 2-3 hours at 4°C. The bead mixture was centrifuged and washed with Co-IP washing buffer (same as Co-IP buffer but with 0.1% NP-40). The beads were then resuspended in Laemmli Buffer and boiled for five minutes, followed by western immunoblot analysis of the beads. Loading was confirmed by western immunoblot for α-tubulin in a sample of the whole cell lysate obtained prior to addition of antibodies.

**Generation of the ΔITGA3 and Integrin Chimeric Mutants**

For generation of the integrin chimeras, the cytoplasmic tail of α3 (3039G-3136A) and α4 (3129C-3225C) were excised from their native sequence and swapped followed by subcloning into a pcDNA 3.1 vector. For the generation of the ITGA3 mutant (Δα3), we substituted the serines found in the QPSXXE motif with alanines as illustrated below:

```
AGCCCAGCCGTCAGAGACAGA (Native Sequence)
AGaCCAGCCGcAGAGcGA (Mutant Sequence)
```

Site-directed mutagenesis and Chimera generation were performed at Mutagenex Labs (Mutagenex, Somerset, NJ).

**Statistical analysis**

The number of samples or animals studied per experiment is indicated in the Figure Legends. Values from multiple experiments are expressed as mean±SEM. Statistical significance was determined using unpaired t-test or one-way ANOVA followed by Dunnett’s or Bonferroni’s multiple comparison tests unless stated otherwise. A value of P<0.05 was considered significant.
REFERENCES:


Supplement Figure I. \( \text{Apc}^{\text{Min/+}} \) mice demonstrate no differences in echocardiographic parameters compared to C57 controls.

(A) Left ventricular fractional shortening, (B) ejection fraction, (C) cardiac output and (D) heart rate of C57 littermate and \( \text{Apc}^{\text{Min/+}} \) mice in normoxia and chronic hypoxia. (E) Systolic blood pressure was measured using a tail cuff method. (F) Left ventricular end diastolic pressure (LVEDP) and (G) pulmonary artery acceleration times (PAAT) measurements were carried out as described in the Methods.
Supplement Figure II. βC knockdown does not promote survival in APC deficient human mvPAECs.

(A) siRNA mediated βC knockdown and (B) caspase 3/7 activation assay in scrambled and βC siRNA treated C57 littermate and ApcMin/+ mvPAECs was performed as described in the Methods. Bars represent mean ±SEM from N=3 experiments. **P<0.001, ***P<0.0001 vs. C57, one way ANOVA with Bonferroni’s post-test.
Supplement Figure III. APC<sup>Min/+</sup> mvPAECs demonstrate reduced clustering of microtubule networks.

Representative IF images of C57 (left panel) and APC<sup>Min/+</sup> (right panel) mvPAECs probed for α-tubulin (red). Scale bar=50μm.
Supplement Figure IV. APC siRNA treated human mvPAECs demonstrate preserved proliferation response and reduced survival in hypoxia.

(A) Cell count and (B) caspase 3/7 luciferase assay were performed as described in the Methods. Bars represent mean ±SEM from N=3 experiments. ***P<0.0001 vs. scrambled siRNA control at baseline and #P<0.01, ##P<0.001 vs. corresponding scrambled siRNA mvPAECs, one way ANOVA with Bonferroni post-test.
Supplement Figure V. Human mvPAECs bind to laminin via α3β1 integrins resulting in the formation of an α3/APC protein complex.

(A) Adhesion to laminin (LN) in human mvPAECs pretreated with increasing concentrations of α and β integrin blocking antibodies was evaluated 30 minutes after seeding the cells. Cells incubated with nonspecific IgG served as controls. (B) Immunoprecipitation of whole cell lysates of mvPAECs recovered following adhesion to collagen IV (CIV), LN and fibronectin (FN) using α1, α3 and α5 integrin antibodies and immunoblotting using APC antibody. Bars represent mean ±SEM from N=3 experiments. *P<0.01, **P<0.001, ***P<0.0001 versus IgG, one way ANOVA with Bonferroni’s post-test.
Supplement Figure VI. APC and ILK-1 colocalize in the periphery of laminin adhered mvPAECs.

Representative confocal images of C57 mvPAECs probed for APC (green) and ILK-1 (red). Merged images are shown in the right panels. Nuclei were stained blue with DAPI. Scale bar=50µm.
Supplement Figure VII. SW480 cells demonstrate reduced adhesion to laminin.

Adhesion assay of SW480 cells seeded in collagen IV (CIV), laminin (LN) and fibronectin (FN). Bars represent mean ±SEM from N=3 experiments. *P<0.01, ***P<0.0001, one way ANOVA with Bonferroni's post-test versus noncoated (NC).
Supplement Figure VIII. WT APC transfected human mvPAECs demonstrate increased survival in hypoxia.

Caspase 3/7 luciferase assay were performed as described in the Methods. Bars represent mean ±SEM from N=3 experiments. ***P<0.0001 vs. normoxia control at baseline and #P<0.01, ##P<0.001 vs. corresponding normoxia mvPAECs, one way ANOVA with Bonferroni post-test.
Supplement Figure IX. IPAH mvPAECs recover adhesion to laminin after transfection with WT APC expression construct.

Adhesion assay of IPAH mvPAECs cells seeded in collagen IV (CIV), laminin (LN) and fibronectin (FN). Bars represent mean ±SEM from N=3 experiments. ***P<0.0001 versus empty vector, one way ANOVA with Bonferroni's post-test.
Supplement Figure X. APC expression is reduced in human mvPAECs incubated in hypoxia.

Western immunoblot for APC in whole cell lysates of human mvPAECs exposed to normoxia (FiO$_2$: 20%) and hypoxia (FiO$_2$: 1%) for 24 hours. Densitometry values are shown relative to α-tubulin in whole cell lysates. ***P<0.0001, unpaired t-test.
**Supplement Figure XI.** Transfection of a constitutively active (CA) Akt construct fails to protect APC siRNA treated mvPAECs incubated under serum free conditions.

Survival was measured using the Caspase 3/7 luciferase assay and results were compared against scrambled or APC siRNA treated mvPAECs transfected with an empty vector. ###P<0.0001 vs. corresponding scrambled siRNA and *P<0.01 vs. scrambled siRNA+empty vector, one way ANOVA with Bonferroni’s post-test.
### Unused Donor Control

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<tr>
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### IPAH Patients

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PAP, pulmonary artery pressures (mmHg), s: systolic, d: diastolic, m: mean.
PVR, pulmonary vascular resistance (dyne.sec.cm⁻².m²). 6MW: distance (m) walked in 6 minutes.

**Supplement Table I.** Clinical characteristics of the patients who served as the source for unused donor and IPAH mvPAECs.