Original Article

Endothelial Apoptosis in Pulmonary Hypertension Is Controlled by a microRNA/Programmed Cell Death 4/Caspase-3 Axis

Kevin White, Yvonne Dempsie, Paola Caruso, Emma Wallace, Robert A. McDonald, Hannah Stevens, Mark E. Hatley, Eva Van Rooij, Nicholas W. Morrell, Margaret R. MacLean, Andrew H. Baker

Abstract—Pulmonary endothelial cell apoptosis is a transient, yet defining pathogenic event integral to the onset of many pulmonary vascular diseases such as pulmonary hypertension (PH). However, there is a paucity of information concerning the molecular pathway(s) that control pulmonary arterial endothelial cell apoptosis. Here, we introduce a molecular axis that when functionally active seems to induce pulmonary arterial endothelial cell apoptosis in vitro and PH in vivo. In response to apoptotic stimuli, human pulmonary arterial endothelial cells exhibited robust induction of a programmed cell death 4 (PDCD4)/caspase-3/apoptotic pathway that was reversible by direct PDCD4 silencing. Indirectly, this pathway was also repressed by delivery of a microRNA-21 mimic. In vivo, genetic deletion of microRNA-21 in mice (miR-21−/− mice) resulted in functional activation of the PDCD4/caspase-3 axis in the pulmonary tissues, leading to the onset of progressive PH. Conversely, microRNA-21−overexpressing mice (CAG-microRNA-21 mice) exhibited reduced PDCD4 expression in pulmonary tissues and were partially resistant to PH in response to chronic hypoxia plus SU 5416 injury. Furthermore, direct PDCD4 knockout in mice (PDCD4−/− mice) potently blocked pulmonary caspase-3 activation and the development of chronic hypoxia plus SU 5416 PH, confirming its importance in disease onset. Broadly, these findings support the existence of a microRNA-21-responsive caspase-3 pathway in the pulmonary tissues that when active serves to promote endothelial apoptosis in vitro and PH in vivo. (Hypertension. 2014;64:00-00.) • Online Data Supplement

Key Words: apoptosis ■ endothelium ■ hypertension ■ pulmonary ■ microRNAs ■ PDCD4 protein, human

The pulmonary endothelium is essential for normal vascular homeostasis in the lung. Endothelial cell apoptosis in response to injury is a transient, yet defining pathogenic event that underlies many human vascular disorders such as pulmonary hypertension (PH). Cellularely, this is thought to initiate a selection-by-apoptosis event, whereby pathogenic death-resistant endothelial cells survive and establish in favor of healthy death-susceptible endothelial cells.1–3 In the context of human pathogenesis, this endothelial event is thought to precede the prototypic vasculopathy associated with PH and pulmonary arterial hypertension.4,5 Accordingly, discovery of the pathway(s) that govern this process would offer informative insight into the molecular pathology of the pulmonary hypertensive endothelium.

Programmed cell death 4 (PDCD4) is a proapoptotic factor and important regulatory molecule across many human diseases. In cancer, PDCD4 universally inhibits neoplastic transformation and growth6–8 and is considered a promising antineoplastic target for future therapies. In diabetes mellitus, PDCD4 is also thought to play an important role in disease pathogenesis through the induction of pancreatic β-cell apoptosis.9 Although ambiguous, PDCD4 is thought to at least partly drive apoptosis via activation of the caspase-3 pathway.10 Yet irrespective of context, the convergent outcome of these data seems to indicate that PDCD4 is a powerful inducer of apoptosis in vivo. Accordingly, given the importance of this cellular event in PH, we challenged the concept that a regulatory PDCD4/caspase-3 axis exists to control apoptosis in the endothelium in PH.

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Because of its pervasiveness, we also addressed whether the contribution of microRNA (miRNA) biology was relevant to this axis. As determined bioinformatically and validated experimentally, miRNA-21 (miR-21) targets encompass a broad range of biologically essential genes. Principal among these is PDCD4,8,11, a proapoptotic gene whose expression is closely and powerfully repressed by miR-21. MiRNAs are dynamic and pleiotropic post-transcriptional regulators that intricately govern cellular and tissue homeostasis.12,13 Recent and extensive literature has confirmed that miRNAs can also exist as pathoregulatory molecules central to the underlying pathogenesis of many diseases, such as cancer,14 respiratory,15 and cardiovascular disease.16,17 Appropriately, there is also an emergence of literature that connects miRNA biology and PH.18–25 Among these, miR-21 is suggested as a candidate miRNA inherent to this molecular connection. Broadly, miR-21 levels are induced in murine and human pulmonary hypertensive tissues,16,20 where it is assumed to protect against disease onset via beneficial repression of its direct target genes, including RhoB,20 a well-defined molecular mediator of PH.26 Nonetheless, beyond these elemental data, the comprehensive role of this miRNA in PH, including downstream interrogation of targets, phenotypic function within distinct pulmonary vascular cells (endothelial, smooth muscle, and fibroblast), and extensive in vivo modeling, remains to be challenged.

In this report, we present evidence of an existing regulatory PDCD4/caspase-3 pathway that controls endothelial apoptosis. In the healthy endothelium, this pathogenic axis is likely chronically repressed through archetypal silencing of PDCD4 by the PH-modifying molecule miR-21, whereas failure of this silencing mechanism leads to apoptosis in vitro and PH in vivo. Collectively, these data introduce an miRNA-responsive PDCD4/caspase-3 axis that controls endothelial apoptosis in experimental PH.

**Methods**

Detailed methods can be accessed via the online-only Data Supplement.

**Apoptosis Assay in Human Pulmonary Artery Endothelial Cells**

Caspase-3–dependent endothelial cell apoptosis occurs in response to growth factor–deficient, serum-deficient conditions (>60% apoptosis at 48 hours). Appropriately, this tractable model of apoptosis was used to investigate the molecular basis of pulmonary artery endothelial cell (PAEC) apoptosis. Cultured PAECs were seeded into plates and grown to >90% confluence before serum starvation. At 6- and 24-hour postapoptotic insult, PAEC number was quantitatively determined, as previously described.22 Synchronously, PAECs were seeded in 6-well plates for accompanying molecular analysis. At 24 hours, PAECs were collected for molecular analysis. For all small interference RNA and miR-mimic studies, transfection was accomplished using Lipofectamine (Life Technologies).

**Disease Modeling In Vivo**

Robust PH23 was established in age- and weight-matched adult CAG-miR-21 mice and PDPCD4−/− mice by dual serial injection (days 0 and 7) with the vascular endothelial growth factor Fk-1/KDR receptor inhibitor Semaxanib (SU 5416, 20 mg/kg subcutaneously; Sigma Aldrich) in combination with 14-day continuous exposure to hypoxia. On completion, right ventricular pressure and systemic arterial pressure were simultaneously measured.

**Reverse Transcription Polymerase Chain Reaction**

Tagman reverse transcription polymerase chain reaction analysis was performed in murine and human tissues, as previously described.14 For miRNA control, the housekeeping gene β-actin was used. For miRNA control, the small RNA U6 (murine) or RNU48 (human) was used.

**Western Blotting**

Protein expression analysis was derived by Western blot analysis as previously described.23,24 Briefly, protein was extracted from PAECs and lung tissues by sonication, ice solubilization, and cellular fractionation before quantification (Protein Assay Kit, BioRad) and electrophoresis separation. For protein immunodetection, anti-PDCD4 (1:1000, Abcam), anti–caspase-3 (pro- and cleaved–; 1:1000, Cell Signal), and anti-GAPDH (1:5000, Abcam) were used.

**Immunohistochemistry Analysis**

Immunohistochemistry was performed as described previously.27 Anti-α-smooth muscle actin (1:500, Sigma Aldrich), anti-von Willebrand (1:200, Dako), anti-PDCD4 (1:50, Abcam), anticleaved caspase-3 (1:200, Cell Signal), and IgG-control (required concentration, Vector) were used for detection.

**Statistics**

Before experimental analysis, power calculations were used to ensure appropriately powered experiments. For culture studies, 3 independent iterations were performed in duplicate (transcript and protein expression) or triplicate (blinded cell counting analysis). Statistical comparison analysis was performed with Prism 5.0 (GraphPad, CA) using nonparametric tests. For 2-group analysis, data were analyzed using the Mann–Whitney test. For multigroup comparison, data were analyzed using the Kruskal–Wallis test followed by Dunn multiple comparison test. A P value <0.05 (P < 0.05) was considered statistically significant. Data are expressed as mean±SEM.

**Results**

**PDPCD4 Induces Pulmonary Artery Endothelial Apoptosis Through Caspase-3 Activation**

Caspase-3–dependent50 PAEC apoptosis was achieved through serum deprivation. Cellular stress caused by serum deficiency induced significant and progressive PAEC apoptosis (Figure 1A). Molecularly, this was accompanied by induction of the proapoptotic factor PDPCD4 and cleaved activation of its downstream target caspase-3 (Figure 1B–1E), a key executioner zymogen essential for the induction of apoptosis in PAECs.2 Next, to determine whether PDPCD4 is an upstream regulator of caspase-3–dependent apoptosis, small interference RNA targeted against PDPCD4 was used to repress the downstream caspase-3/apoptosis axis. When compared with the appropriate control treatments, siPDPCD4 caused significant knockdown in PDPCD4 protein levels accompanied by a >90% decline in cleaved caspase-3 levels (Figure 1F–1I), the active variant of the pro–caspase-3 zymogen. Correspondingly, the progressive apoptosis noted at 24 hours in the appropriate control groups was abolished completely in PDPCD4-knockdown serum-deficient PAECs (Figure 1J). Thus, in the pulmonary endothelium, apoptosis in response to injurious stimuli seems to be part molecularly underpinned through PDPCD4 induction and consequent activation of the apoptotic zymogen caspase-3.

**MiRNA Silencing of the PDPCD4/Caspase-3 Axis Prevents Pulmonary Endothelial Cell Apoptosis**

Recent literature has shown that PDPCD4 is repressed by miRNA through archetypical miRNA–target interaction.8,11
Specifically, PDCD4 contains a conserved and validated 8mer miR-21 binding site positioned at its 3' untranslated region. Because miR-21 is abundantly expressed in the endothelium and also a well-established modifying gene in PH in vivo, we hypothesized that miR-21 acts to preserve endothelial apoptosis through inhibitory regulation of the PDCD4/caspase-3 axis. In serum-deficient PAECs, pharmacological transfection and delivery of 40-nmol/L miR-21 mimic repressed induction
of its target PDCD4, to consequently inhibit caspase-3 activation (Figure 2A–2E). Phenotypically, miR-21 mimic delivery reduced the rate of apoptosis in PAECs in response to serum-deficient stress, compared with the appropriate miR-control–treated cells (Figure 2F).

**Induction of the PDCD4/Caspase-3 Axis in the Pulmonary Tissues of miR-21−/− Mice In Vivo**

After confirmation of an existing miR-21/PDCD4/caspase-3/apoptosis axis in PAECs, we interrogated the potential existence of this regulatory axis in PH in vivo. Initially, status of this axis was experimentally tested by homozygous knockout of miR-21 in mice (miR-21−/− mice). In conformity with our cultured data, miR-21−/− murine lung tissue homogenates exhibited elevated PDCD4 levels accompanied by the enhanced detection of cleaved caspase-3 (Figure 3A–3D) when compared with wild-type (WT) littermate controls. Likewise, the chronic delivery of anti–miR-21 in mice also resulted in PDCD4 induction in the pulmonary tissues (Figure S1 in the online-only Data Supplement). Specific to the vasculature, elevated levels of PDCD4 and cleaved caspase-3 were also noted in the small pulmonary vessels in miR-21−/− lungs as visualized by in situ analysis (Figure 3E and 3F). Thus, these in vivo data coalesced with our cultured data (Figures 1 and 2) infer the existence of an miRNA-responsive PDCD4/caspase-3 axis in the pulmonary tissues that molecularly serves to regulate endothelial cell apoptosis.

**Induction of the PDCD4/Caspase-3 Axis in miR-21−/− Mice Causes the Progressive Onset of PH In Vivo**

In accordance with the hypothesis, we anticipated that the loss of miR-21 in vivo would lead to direct activation of the PDCD4/caspase-3 axis and as a consequence result in the onset of progressive PH. To analyze this, pulmonary hemodynamics and vasculopathy were assessed in the lungs of 8-week and 20-week miR-21−/− mice. Despite the absence of any change in systemic arterial pressure (Figure 4A and 4B), miR-21−/− mice displayed a progressive elevation in pulmonary pressures that manifested at 20 weeks of age (Figure 4C and 4D). These changes were spontaneous and reported under naïve normoxic conditions. Consistent with this and despite normal lung architecture in wild-type littermate mice, significant distal vascular thickening and remodeling were present in 20-week-old miR-21−/− mice (Figure 4E and 4F). Akin to this vascular pathology, the presence of rare but substantial (>90% vascular wall thickness) obliterated pulmonary vascular lesions were also noted in 20-week miR-21−/− murine lungs (Figure S2). Thereby, the loss of miR-21 in the pulmonary tissues results in activation of the PDCD4/caspase-3 axis and the onset of PH in vivo.

**Pulmonary MiR-21 Induction in CAG-miR-21 Mice Inhibits PDCD4 Induction and Prevents the Onset of PH In Vivo**

Given the observation that miR-21 deficiency leads to functional activation of the PDCD4/caspase-3 axis and onset of PH in vivo, we set out to determine whether restoration of miR-21 levels in vivo may abrogate the effects of miR-21 deficiency in PH. To analyze this, we transplanted a CAG (constitutively active) miR-21 transgene into the murine lungs (CAG-miR-21 mice).

**Figure 2.** MicroRNA silencing of the programmed cell death 4 (PDCD4)/caspase-3 axis prevents pulmonary artery endothelial cell apoptosis. A to E, Pharmacological delivery of miR-21 mimic in apoptotic (0.1% fetal bovine serum [FBS]) pulmonary artery endothelial cells (PAECs) significantly repressed expression of its direct target PDCD4 to consequently activate its downstream target caspase-3 (n/3, duplicate). F, Phenotypically, the delivery of miR-21 mimic significantly alleviated the rate of apoptosis in response to 0.1% FBS when compared with miR-control–treated cells (n/3, triplicate). *P<0.05 and ***P<0.001. Mean±SEM.
vivo, we anticipated that silencing of this axis through constitutive miR-21 induction in the pulmonary tissues would robustly protect against disease. To challenge this, CAG-miR-21 mice designed to constitutively overexpress miR-21 were exposed to 14-day chronic hypoxic plus SU 5416 pulmonary hypertensive injury (Figure 5A). Expression analyses in the lung tissues of these mice revealed miR-21 induction in the pulmonary tissues (Figure 5B), accompanied by significant repression of its target PDCD4 (Figure 5C). In vivo, systemic arterial pressure was unchanged (Figure 5D), whereas there was a significant alleviation in pulmonary pressures reported in CAG-miR-21 mice when compared with their wild-type controls (Figure 5E), indicative of their increased resistance to PH. Consistent with these hemodynamic changes, pulmonary vascular remodeling was similarly alleviated in these animals (Figure 5F and 5G). Together, our evidence suggests that miR-21 induction robustly protects against the onset of PH through in vivo silencing of the PDCD4/caspase-3/apoptosis axis.

**Direct Knockout of PDCD4 in Mice Alleviates the Onset of PH In Vivo**

Finally, we aimed to directly substantiate the putative importance of PDCD4 independent of miR-21. To test this, we challenged mice deficient of PDCD4 (PDCD4−/− mice) with 14-day chronic hypoxia and SU 5416 pulmonary injury. In reinforcement of our hypothesis, the direct loss of PDCD4 resulted in an emphatic reduction of caspase-3 activity in the pulmonary tissues in vivo (Figure 6A–6D). Hemodynamically, direct inhibition of this axis in PDCD4−/− mice had no effects on systemic arterial pressure (Figure 6E), yet partially alleviated against the pathological elevation in pulmonary pressures associated with chronic hypoxia plus SU 5416 insult (Figure 6F). Histopathologically, and consistent with the hemodynamic data, PDCD4−/− mice exhibited a significant alleviation in pulmonary vascular remodeling when compared with wild-type control mice (Figure 6G and 6H). Taken together, these data suggest that PDCD4 harbors a powerful and pathogenic role in the control of endothelial apoptosis in PH. In the normal endothelium, miR-21 acts to chronically and beneficially repress caspase-3 activation and maintain PAEC integrity (Figure 7A), whereas in response to injurious triggers or miR-21 loss, this results in direct activation of this pathway, to drive PAEC apoptosis and PH onset in vivo (Figure 7B).

**Discussion**

In this study, we present evidence that endothelial apoptosis in PH is molecularly executed through activation of an
miRNA-responsive PDCD4/caspase-3 pathway. Using a loss- and gain-of-function strategy in human cell models and in vivo, we report that inactivation of this pathway, either indirectly through miR-21 induction or directly through PDCD4 inhibition, robustly inhibits caspase-3 activation, preserves endothelial apoptosis, and increases resistance to the onset of PH. In vivo, functional activation of this pathway through miR-21 knockout results in derepression of its direct target PDCD4, endothelial activation of caspase-3, and the progressive onset of hemodynamic and vascular manifestations consistent with PH. When considered collectively, these data introduce a novel molecular axis that seems to control endothelial apoptosis in the pulmonary vascular system (Figure 7).

Extensive literature points toward a fundamental role for the endothelial cell in the genesis and evolution of PH.1,4,30,31 Definitively, pulmonary vessel obliteration and destruction caused by concentric migration of the proliferated endothelium (the plexiform lesion) remains the premier histopathologic hallmark32 of severe forms of PH (pulmonary arterial hypertension). Less well-defined are the cellular and molecular events that precede this pulmonary endotheliopathy. A conventional theory is that in response to genetic33 or exogenous3 triggers the pulmonary endothelial cell population undergo an initial and indefinite period of selection by apoptosis to result in the progenic emergence of an apoptotic-resistant proliferated cell population that synchronously drive the endothelial manifestations of PH. Our data support this hypothesis and introduce a molecular explanation to this, whereby execution of this event is at least in part driven through the induction of PDCD4 in the endothelium, a proapoptotic molecule that directly initiates downstream activation of the apoptotic enzyme caspase-3. By doing so, activation of this pathway causes prominent cell death within the endothelial population, with potential exception to those pathogenic cells that seem to thrive in response to highly apoptotic conditions. Our inference toward the centrality of caspase-3 in execution of this process is already substantiated by extensive evidence that shows abundant caspase-3 activation in the endothelium of remodeled pulmonary vessels in pulmonary hypertensive specimens.2,30 To this end, our evidence suggests that activity of this zymogen, through its direct upstream regulation by PDCD4,11 whereas its beneficial effects in pulmonary vascular remodeling and PH are widely established.19,20,24,34 Here,
we present evidence to offer a comprehensive and reasoned mechanistic explanation for the importance of miR-21 in the pulmonary hypertensive endothelium in vivo.

A longstanding challenge of PH research remains the lack of clinically accessible lung tissue specimens collected from patients with mild/early-stage PH. Contrarily, there is a near exclusive reliance on the analysis of end-stage/severe pulmonary hypertensive lung tissue specimens that are collected from patients at lung transplant or autopsy. Although informative, this failure to tractably analyze tissues throughout the progression of human disease halts our insight into the molecular events that underpin the early stages of disease pathogenesis. In respect of this, and although existing literature strongly shows that miR-21 expression levels are induced in the lungs and pulmonary vessels of patients diagnosed with end-stage PH,20,24 we cannot rule out the possibility that miR-21 is dynamically

Figure 5. Pulmonary miR-21 induction in CAG-miR-21 mice inhibits programmed cell death 4 (PDCD4) induction and prevents the onset of pulmonary hypertension (PH) in vivo. A, CAG-miR-21 mice and wild-type (WT) littermate mice were exposed to 14-day chronic hypoxia plus SU 5416 pulmonary injury. B and C, Molecularly, a ~2-fold induction of miR-21 (n/6 per group) accompanied by significant reduction in PDCD4 levels (n/5 per group) was reported in the lungs of CAG-miR-21 mice. D and E, Hemodynamically, systemic arterial pressure (n/6 per group) was unchanged in these mice, whereas a significant alleviation in pulmonary pressures (n/6–7 per group) was reported compared with their WT littermate controls, indicative of their resistance to PH after pulmonary hypertensive injury. F and G, Consistent with pulmonary hemodynamics, distal vascular thickness and remodeling were also significantly alleviated in CAG-miR-21 mice when compared with their WT littermate controls (n/4 per group). Scale bar, 30 μm. *P<0.05. Mean±SEM. αSMA indicates α-smooth muscle actin; MAP, mean systolic arterial pressure; and WT, wild type.

Figure 6. Direct knockout of programmed cell death 4 (PDCD4) in mice alleviates the onset of pulmonary hypertension in vivo. A to D, After 14-day chronic hypoxic plus SU 5416 pulmonary injury, PDCD4−/− mice exhibited an emphatic reduction in cleaved caspase-3 activity in the lungs when compared with wild-type (WT) mice (n/5 per group). E and F, Hemodynamically, direct inhibition of this axis in PDCD4−/− mice had no effects on systemic arterial pressure (n/5–6 per group), yet beneficially alleviated against the pathological elevation in pulmonary pressures associated with chronic hypoxia plus SU 5416 insult (n/5–7 per group). G and H, Histopathologically and consistent with the hemodynamic data, PDCD4−/− mice exhibited a significant alleviation in pulmonary vascular thickness and remodeling when compared with WT mice (n/4 per group). Scale bar, 20 μm. *P<0.05 and ***P<0.001. Mean±SEM. αSMA indicates α-smooth muscle actin; MAP, mean systolic arterial pressure; and ND, not detectable.

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Further supporting this thought, miR-21 is predicted to target at least an additional 6 genes known to be directly implicated in PH with ≥2, BMPRII and RhoB, already validated as directly targeted by this miRNA in the pulmonary vascular tissues in vivo.20 However, given that these genes converge to control endothelial function, this indicates that miR-21 modulatory effects are largely enriched to the context of the pulmonary endothelium and consistent with other evidence20; this supports the notion that miRNA–target interactions and modulatory effects are significantly concentrated when considered in a cellular, tissue, or disease-specific context. Notwithstanding the significant contribution of miRNA biology in regulation of the PDCD4/caspase-3 axis, we still acknowledge that additional miRNA-independent mediators may also exist to partially control function of this pathway.

Upstream, existing literature suggests that miR-21 expression levels are dynamically shifted by even subtle changes to the cellular microenvironment caused by pulmonary hypertensive triggers, such as hypoxia, inflammatory cytokines, and BMPRII signaling.20 Yet, less well-defined is regulation of this miRNA in the pulmonary vascular system by hormones. This could be a particularly relevant connection given that PH is a disorder with a more striking prevalence in women.37,38 Recent evidence has shown that miR-21 expression is downregulated by the hormone estrogen8 and given that this is also a pathogenic molecule in PH,27 it could be plausible that this axis represents another mechanism leading to disease formation. Although whether this is relevant in the context of male–female differences in PH remains to be fully explored and discovered.

Moreover, we provide unique in vivo evidence that PDCD4 expression can be pharmacologically manipulated in mice by chronic delivery of anti–miR-21 (Figure S1), a biosynthetically derived oligonucleotide that binds with complete complementarity to endogenous miR-21. These findings highlight the plausibility of this pathogenic axis as a molecular target that can be manipulated in vivo by pharmacologically derived miRNA reagents. In a therapeutic context, it could be envisaged that functional silencing of this pathway could be beneficially targeted by chronic delivery of synthetic miR-21 mimic, although limitations still remain concerning the lack of tissue specificity to the distal pulmonary vasculature and undesirable off-target effects31 of miRNA-based therapies. Of paramount importance, the biology of miR-21 in the context of global vascular remodeling must also be delineated. For example, despite its widely reported protective role in PH, miR-21 induction is assumed to play a central role in driving pathological neointimal formation and consequent vein graft failure.40 Aside from its contribution to key vascular remodeling processes in the setting of cardiovascular disease, miR-21 is additionally a well-illustrated oncomiR associated with pathology in various cancer types.5,11,41,42 Therefore, before miR-21–based therapies can undergo full clinical evaluation for their validity as a treatment for PH, both these technical and biological challenges must first be overcome.

In summary of these data, here, we have uncovered a regulatory axis that serves to control endothelial apoptosis and PH onset in vivo. This offers in part a molecular explanation as to why endothelial apoptosis occurs in response to pulmonary hypertensive injury, and in doing so also supports a
contributory role for miR-21 in PH. Given the principal role of the endothelium in pulmonary vasculopathy and PH, future anti-remodeling-based therapies designed to target this axis could prove an effective strategy in improving or restoring endothelial status in the pulmonary hypertensive vasculature.

Perspectives

PH is a debilitating and often fatal condition that is incurable at present. The reason for this failure to treat is largely restricted by our current understanding of the molecular processes that underpin its vascular and endothelial pathology. In this study, through a loss- and gain-of-function approach in human cell models and in vivo, we define the existence of a pathogenic miRNA-responsive PDCD4/caspase-3 axis that on activation drives apoptosis in vitro and PH in vivo. Because endothelial apoptosis and altered miRNA signaling are well reported in pulmonary hypertensive patients, we speculate that the pathogenic miR-21/PDCD4/caspase-3 pathway participates in the endothelial pathology associated with human disease pathogenesis.

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Disclosures

Dr. Van Rooij is a former employee and co-founder of MiRagen Therapeutics. The authors report no conflicts.

References


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**Novelty and Significance**

**What Is New?**
- Programmed cell death 4 (PDCD4) knockdown in human pulmonary endothelial cells, either directly or via microRNAs, alleviates cellular apoptosis. In vivo loss of function of PDCD4, as observed in PDCD4−/− mice and miRNA-21 overexpressing mice, protected against the development of pulmonary hypertension, whereas PDCD4 gain of function in miRNA-21 knockout mice caused the progressive onset of disease.

**What Is Relevant?**
- Endothelial apoptosis and dysfunction is a dominant vascular hallmark in patients with pulmonary hypertension, yet little information exists concerning its active role in the initial stages of disease progression. Recent published evidence suggests that microRNA signaling is altered in the pulmonary hypertensive vasculature. This study addressed the potential link between microRNA and the endothelium in pulmonary hypertension and through a comprehensive in vivo and in vitro approach demonstrates an important role for the miRNA-21/PDCD4/caspase-3 axis in disease progression.

**Summary**
Transition of the pulmonary endothelium from healthy to diseased is an early and defining pathogenic event in pulmonary hypertension. This study used a comprehensive approach to help identify the potential molecular pathways that regulate this process. Through this strategy, the proapoptotic factor PDCD4 was discovered to contribute to endothelial apoptosis in vitro and pulmonary hypertension in vivo. Knockdown of PDCD4, either directly or indirectly via microRNA biology, was beneficial against the development of disease in mice, whereas induction of PDCD4 in miR-21 knockout mice caused the progressive onset of disease. Supported by these data, we speculate that the microRNA-responsive proapoptotic factor PDCD4 is an important inducer of apoptosis in the pulmonary endothelium, and in doing so offers a mechanistic explanation surrounding endothelial apoptosis in pulmonary hypertension.
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A microRNA-responsive PDCD4/caspase-3 axis controls endothelial apoptosis in pulmonary hypertension


SUPPLEMENTAL MATERIAL
Expanded Methods

Ethical Approval
All animal procedures conform with the United Kingdom Animal Procedures Act (1986), and “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Animal use approval was institutionally granted by the University Committee Board. Experimental procedures using human pulmonary cells conform to the principles outlined in the Declaration of Helsinki.

Human pulmonary artery endothelial cells
Human pulmonary artery endothelial cells (PAECs) were grown from surgically discarded lung tissues collected from donors that underwent pulmonary resection for reasons not related to PH (PromoCell). Cultured PAECs were grown in Endothelial Cell Growth Medium 2 (EGM-2; PromoCell) and utilized for experimental analysis from passage 2–8 inclusive.

Apoptosis assay in PAECs
Caspase-3 dependent endothelial cell apoptosis occurs in response to growth factor-deficient, serum-deficient conditions (>60% apoptosis noted at day 2). Appropriately, this tractable model of apoptosis was utilized to investigate the molecular basis of PAEC apoptosis. Cultured PAECs were seeded into plates (triplicate for each experimental condition) and grown to ~90% confluency. To induce apoptosis, EGM-2 media was replaced with Endothelial Cell Basal Medium 2 (EBM-2; PromoCell) supplemented with 0.1% [v/v] fetal bovine serum (FBS). EBM-2 supplemented with 2% [v/v] FBS served as the serum-enriched control treatment group. At 0hr and 24hr post-apoptotic insult PAEC number was quantitatively determined, as previously described 1. Synchronously, PAECs were seeded in 6-well plates (duplicate for each experimental condition) for accompanying molecular analysis. At 24hr, PAECs were rinsed with ice-cold PBS (Sigma Aldrich) and collected for transcript (Qiazol lysis reagent, Qiagen) and protein (1% [v/v] lauryl maltoside, Abcam) expression analysis.

siRNA and miRNA transfection studies in PAECs
Synthetically derived small interference RNA (siRNA) targeting human PDCD4 (20nmol/L siPDCD4, SCBT) were used for targeted PDCD4 knockdown in human PAECs. siRNA with a nontargeting sequence was used as negative control (20nmol/L, SCBT). siRNAs were transfected with Lipofectamine (Life Technologies) 48 hours before serum conditioning, according to manufacturer instructions. Naïve (untreated) PAECs served as control for siRNA transfection. Synthetically derived mature-sequence miR-21 mimic molecules (Ambion) were transfected in human PAECs at a pre-optimized concentration (40nmol/L) that both overexpressed miR-21 levels and repressed target expression. Random sequence miR-mimic was used as a negative scramble control (40nmol/L, Ambion). MiR-21 and miR-control mimic molecules were transfected with Lipofectamine (Life Technologies) 48 hours before serum conditioning, according to manufacturer instructions.

Disease modeling in vivo
MiR-21 null mice (miR-21-/- mice; C57BL/6 background) and miR-21 over-expressing mice (CAG-miR-21 mice; B6C3F1 background) were generated and provided by Eric N. Olson, PhD (University of Texas Southwestern Medical Center, Texas, USA). In naïve unchallenged conditions, both transgenic miR-21 strains are viable, healthy and fertile with no other presenting anatomical phenotype. Specifically, CAG-miR-21 transgenic mice do not develop lung tumors or heart failure. Mice deficient of PDCD4 (PDCD4-/- mice; C57BL/6 background) were generated and provided by David B. van Dijk, PhD (University of Texas Southwestern Medical Center, Texas, USA). In naïve unchallenged conditions, both transgenic PDCD4-/- strains are viable, healthy and fertile with no other presenting anatomical phenotype.
background) were generated as previously described, and purchased from Jackson Laboratories (Maine, USA). Littermate controls were utilized for all miR-21/- and CAG-miR-21 studies, while genetically matched inbred C57BL/6 mice (Jackson Laboratories, Maine, USA) were utilized as controls for PDCD4/- studies. All mice utilized for investigation were female and age-matched.

Robust PH was established in age- and weight-matched adult CAG-miR-21 mice and PDCD4/- mice by dual serial injection (day 0 and 7) with the vascular endothelial growth factor (VEGF) Flk-1/KDR receptor inhibitor Semaxanib (SU 5416, 20mg/kg/subcutaneous, Sigma Aldrich) in combination with 14 day continuous exposure to hypoxia in a pressure- and temperature-humidity controlled airflow chamber maintained at an atmospheric pressure equivalent to 10% oxygen. Upon completion of the 14 day chronic hypoxic and SU 5416 injury, heart and arterial catheterization was performed prior to euthanasia and tissue harvest.

**AntimiR-21 delivery to mice in vivo**
Locked nucleic acid antimiR-21 biosynthetic oligonucleotides (targeted against nucleotides 2-17 of miR21) and random sequence control biosynthetic oligonucleotides (antimiR-Control) were synthesized and provided by miRagen Therapeutics (Colorado, USA). AntimiR-21 and antimiR-Control were administered subcutaneously (25mg/kg) at day 0 and 7 in C57BL/6 mice (n/6 per group, 8-10 weeks), while a saline injection group (vehicle) was also included as antimiR control. At day 14, mice were euthanized and lungs harvested for PDCD4 expression analysis.

**Right heart and arterial catheterization**
Right heart catheterization was adopted as a tractable surrogate for pulmonary pressure, and procedurally described elsewhere. Briefly, under general anaesthetic conditions (1-2% isoflurane supplemented with medical oxygen) a heparinized saline-filled 22-gauge pressure calibrated catheter (AD Instruments) was directly introduced into the right ventricle to obtain right ventricular pressure, from which right ventricular systolic pressure (RVSP) was derived. Simultaneously, mean systemic arterial pressure (MAP) was measured in each mouse through micro-catheterization of the right carotid artery.

**Tissue Harvest**
Immediately following hemodynamic evaluation by catheterization, the heart and lungs were flushed (<25mmHg pressure) with ice-cold PBS (Sigma Aldrich) to remove erythrocytes. For molecular analysis, right lung tissues were flash-frozen. For *in situ* analysis, the left lung was tracheal inflated with 10% neutral-buffered formalin (Sigma Aldrich) at a pressure of ~20cm H$_2$O and immersed in 10% neutral-buffered formalin for ~16 hours at 25°C under gentle agitation.

**RT-PCR**
TaQMan RT-PCR analysis was performed in tissues, as previously described. Briefly, total RNA extraction (RNeasy mini kit, Qiagen) and reverse transcription was applied to synthesize cDNA (High Fidelity cDNA Amplification Kit, Applied Biosystems) or mature miRNA molecules (MicroRNA Assay Kit, Applied Biosystems). cDNA amplification was achieved via fluorescently labeled Taqman probes and readout using an Applied Biosystems ViiA7 PCR machine. RNA fold-change was calculated using the formula (–ΔΔCt). For mRNA control, the housekeeping gene β-actin was used. For miRNA control, the small RNA U6 (murine) or RNU48 (human) was used.
Western Blotting
Protein expression analysis was derived by Western blot analysis, as previously described. Briefly, protein was extracted from PAECs and lung tissues by sonication, ice-solubilization, and cellular fractionation before quantification (Protein Assay Kit, BioRad) and electrophoresis separation. For protein immunodetection blotting, anti-PDCD4 (1:1000, Abcam), anti-caspase-3 (pro- and cleaved-; 1:1000, Cell Signal) and anti-GAPDH (1:5000, Abcam) was used. Blots were electronically formatted using a Perfection V600 Photo Scanner (Epson) and quantitatively analysed using Adobe Photoshop. For protein loading control, the housekeeping protein GAPDH was used.

Immunohistochemistry analysis
Immunohistochemistry was performed as described previously. Briefly, high temperature citrate buffer antigen-retrieval was performed followed by 10% [v/v] normal goat serum block. Anti-alpha smooth muscle actin (1:500, Sigma Aldrich), anti-Von Willebrand (1:200, Dako), anti-PDCD4 (1:50, Abcam), anti-cleaved caspase-3 (1:200, Cell Signal) and IgG-control (required concentration, Vector) was used with the alkaline phosphatase stain system. Protein presence was confirmed by color formation, which appeared pink/dark-pink. To better define architecture, lung tissues were counterstained with Harris modified hematoxylin (Sigma Aldrich). Photomicrographs were obtained using a Zeiss Axio Imager M1 microscope.

Analysis was performed by photomicrograph inversion, and highlight of two representative opposing areas within the pulmonary vascular wall to generate a mean intensity value between 0 - 255 arbitrary units, prior to conversion to percentage (%) expression units. Five small pulmonary vessels [defined by an external diameter less than 100µm and non-association with airway] were analysed to generate a single mean value per mouse.

Pulmonary vascular pathology analysis
Pulmonary vascular pathology was used to quantitatively score [by a blinded Investigator] pulmonary hypertensive-associated vasculopathy in vivo. In alpha-smooth muscle actin stained lung tissues, a ratio of vascular wall thickness: vascular diameter was determined and used as a surrogate value to define the extent of pulmonary vascular thickness and remodelling. Using this approach, five small pulmonary arteries were quantitatively analyzed to generate a mean vasculopathy score per animal.

Scoring of pulmonary vascular lesions in miR-21-/−-mice
The presence of obliterated pulmonary vascular lesions was reported in miR-21-/− murine lungs. To define these, WT (n/4) and miR-21-/− lungs (n/8 per group) were stained for alpha-smooth muscle actin and Von Willebrand. Quantitatively, small pulmonary vessels that fulfilled the criteria of an “obliterated pulmonary vascular lesion” (ratio value >0.9, vessel wall thickness: vessel diameter) were expressed as a percentage against the collective small pulmonary vasculature population.

Statistics
Prior to experimental analysis, power calculations were utilized to ensure appropriately powered experiments. For culture studies, three independent iterations were performed in duplicate (transcript and protein expression) or triplicate (blinded cell counting analysis). Statistical comparison analysis was performed with Prism 5.0 (GraphPad, California, USA)
by use of non-parametric tests. For two group analysis, data was analysed using the Mann-Whitney test. For multi-group comparison, data was analysed using the Kruskal-Wallis test followed by Dunn’s multiple comparison test. A P-value less than 0.05 (P<0.05) was considered statistically significant. Data are expressed as mean ± SEM.
References


Figure S1. *In vivo* modulation of lung PDCD4 expression in WT mice by chronic pharmacological administration of anti-miR-21. The chronic delivery of anti-miR-21 in WT mice represses PDCD4 levels in the lung. (n/6 per group). P<0.05. Mean ± SEM.
Figure S2. Progressive formation of obliterated pulmonary vascular lesions in miR-21-/- mice. Scale bar 30µm. (n/4-8 per group). Mean ± SEM.