MicroRNA-124 Controls the Proliferative, Migratory, and Inflammatory Phenotype of Pulmonary Vascular Fibroblasts


Rationale: Pulmonary hypertensive remodeling is characterized by excessive proliferation, migration, and proinflammatory activation of adventitial fibroblasts. In culture, fibroblasts maintain a similar activated phenotype. The mechanisms responsible for generation/maintenance of this phenotype remain unknown.

Objective: We hypothesized that aberrant expression of microRNA-124 (miR-124) regulates this activated fibroblast phenotype and sought to determine the signaling pathways through which miR-124 exerts effects.

Methods and Results: We detected significant decreases in miR-124 expression in fibroblasts isolated from calves and humans with severe pulmonary hypertension. Overexpression of miR-124 by mimic transfection significantly attenuated proliferation, migration, and monocyte chemotactic protein-1 expression of hypertensive fibroblasts, whereas anti–miR-124 treatment of control fibroblasts resulted in their increased proliferation, migration, and monocyte chemotactic protein-1 expression. Furthermore, the alternative splicing factor, polypyrimidine tract–binding protein 1, was shown to be a direct target of miR-124 and to be upregulated both in vivo and in vitro in bovine and human pulmonary hypertensive fibroblasts. The effects of miR-124 on fibroblast proliferation were mediated via direct binding to the 3′ untranslated region of polypyrimidine tract–binding protein 1 and subsequent regulation of Notch1/phosphatase and tensin homolog/FoxO3/p21cip1 and p27kip1 signaling. We showed that miR-124 directly regulates monocyte chemotactic protein-1 expression in pulmonary hypertension/idiopathic pulmonary arterial hypertension fibroblasts. Furthermore, we demonstrated that miR-124 expression is suppressed by histone deacetylases and that treatment of hypertensive fibroblasts with histone deacetylase inhibitors increased miR-124 expression and decreased proliferation and monocyte chemotactic protein-1 production.

Conclusions: Stable decreases in miR-124 expression contribute to an epigenetically reprogrammed, highly proliferative, migratory, and inflammatory phenotype of hypertensive pulmonary adventitial fibroblasts. Thus, therapies directed at restoring miR-124 function, including histone deacetylase inhibitors, should be investigated. (Circ Res. 2014;114:67-78.)

Key Words: anoxia ■ hypertension, pulmonary ■ migration ■ neoplasms ■ proliferation

Chronic pulmonary hypertension (PH), in all its forms, is characterized by robust fibroproliferative changes in pulmonary arteries.1,2 Importantly, one of the most consistent findings in experimental models of PH and in models of vascular injury and hypertension in the systemic circulation is early and dramatic remodeling of the perivascular layer, namely adventitia. The vascular adventitia acts as a biological processing center for the retrieval, integration, storage, and release of key regulators of vessel wall function. During the remodeling process, the principal cell type of the vascular adventitia, fibroblast, undergoes profound phenotypic changes characterized by heightened proliferative, migratory, fibrotic, and inflammatory activity.3-10 These observations raise the possibility of the emergence and expansion of adventitial fibroblasts that have lost their ability to limit stimulus-induced proliferation and cytokine production.11 Experimental evidence suggests that...
miRNAs are classes of small noncoding RNAs that can regulate gene expression by binding to the 3′ untranslated region (3′ UTR) of target mRNA to cause degradation or translational repression.\textsuperscript{15} miRNAs play important roles in the regulation of gene expression, developmental processes, cell differentiation, cell proliferation and migration, apoptosis, and stress responses.\textsuperscript{16,17} Aberrant miRNA expression is directly related to initiation and progression of pathophysiological processes, including diabetes mellitus, cancer, and cardiac hypertrophy.\textsuperscript{18–20} Furthermore, miRNAs have been implicated in atherogenesis, control of vascular tone, and hypertension, as well as neointimal formation.\textsuperscript{18,21–23} Recent studies also implicate a role for miRNAs in the complex pathology and molecular dysregulation that characterizes PH.\textsuperscript{23–28} Several miRNA target genes, including BMPR2, RhoB, SHP2, NFAT, and Mst1, have been predicted to be highly enriched in PH-associated pathways, suggesting extensive miRNA-regulated control of this disease.\textsuperscript{23–25,28–30} However, all studies on miRNAs in PH disease process to date have exclusively examined changes in endothelial and smooth muscle cells, whereas the role of miRNAs in controlling the phenotype of adventitial fibroblast remains undefined.

Our previous findings of dramatic differences in the proliferative and inflammatory characteristics of adventitial fibroblasts from the PH vessel are caused by epigenetic modifications such as might occur in response to altered histone acetylation, DNA methylation, and changes in microRNA (miRNA or miR) expression profiles.\textsuperscript{10,12–14}

miR-124 expression in the acquisition of a highly proliferative, invasive phenotype.\textsuperscript{33} Downregulation of miR-124 has also been documented in carcinogenesis in other cell systems\textsuperscript{32,33} and in rheumatoid arthritis where altered expression of miRNAs in general and miR-124 specifically have been shown to regulate cell proliferation and inflammation in synovial fibroblasts.\textsuperscript{34,35} Studies in cancer and neuronal cells have begun to establish the downstream targets of miR-124, which may associate with proliferation or other aspects of activated cell phenotype, such as Sox9, Jag1, polypyrimeridine tracts–binding protein 1 (PTBP1), small C-terminal domain phosphatase 1, ephrin-B1, NfatC3, and monocyte chemotactic protein-1 (MCP-1).\textsuperscript{27,36,37} PTBP1 is especially interesting because of its effects on Notch signaling and other aspects of proliferation in cancer cell types.\textsuperscript{38}

We thus sought to determine whether the activated (highly proliferative, promigratory, and proinflammatory) phenotype of pulmonary adventitial fibroblasts was controlled by suppression of miR-124 and subsequent increases in PTBP1. We determined the effects of restoration of miR-124 expression on the activated fibroblast phenotype using cells and tissues from both large mammalian experimental animal (calf) and humans to better understand mechanisms controlling adventitial fibroblast function in human PH disease process.

### Methods

Details are provided in the Online Data Supplement.

#### Animals

Neonatal calves, Wistar-Kyoto rats, and C57BL/6 mice with hypoxia-induced PH were used in the study.

#### Patient Characteristics

Human lung tissues from subjects with idiopathic pulmonary arterial hypertension (IPAH) or donors were obtained during lung transplantation. Clinical information on patients is summarized in Online Table I.

#### Cell Culture

Bovine pulmonary artery adventitial fibroblasts were isolated from control and hypoxic hypertensive calves. Human pulmonary artery fibroblasts were derived from patients with idiopathic PH or from patients undergoing lobectomy or pneumonectomy.

Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cell migration was evaluated by scrape assay.

#### Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted and reverse-transcribed to cDNA as described in online-only Data Supplement. The intron-spanning primers were designed using sequence information from the National Center for Biotechnology Information database (Online Table II). Results are presented as fold changes in gene expression calculated using the 2ΔΔCt cycle threshold method.

#### Cell Transfection

Transfection was performed with 50 nmol/L of miR-124 mimic, scramble, anti-miR, short interfering RNAs (scramble), or short interfering RNA targeting PTBP1, PTBP2, and phosphatase and tensin homolog (PTEN) (Online Table II). Total RNA/miRNA was extracted 48 hours after transfection using mirVana miRNA isolation kit.

#### Luciferase Reporter Assay

Wild-type or mutant PTBP1 reporter constructs were cotransfected with miR-124 (50 nmol/L) into cells using DharmaFECT Transfection Reagents (Dharmacon). Firefly and Renilla luciferase activities were measured using a Dual-Luciferase Assay (Promega, January 3, 2014)
Wang et al

miR-124 Controls Activated Fibroblast Phenotype

69

Madison, WI) 24 hours after transfection. Firefly luciferase values were normalized to Renilla.

Statistics
Values are expressed as fold-change mean±SEM. Student t test and 1-way ANOV A were used for statistical analysis. Differences with P<0.05 were considered statistically significant.

Results

miR-124 Is Decreased in Fibroblasts Exhibiting a Highly Proliferative and Promigratory Phenotype

Pulmonary adventitial fibroblasts derived from calves with severe PH (PH-Fibs) and from humans with IPAH (IPAH-Fibs) demonstrated markedly enhanced proliferative (in the absence of serum-derived growth factors) and migratory capabilities compared with fibroblasts derived from control calves (CO-Fibs) or human donors (HCO-Fibs; Figure 1A–1C). These highly proliferative and promigratory PH-Fibs/IPAH-Fibs exhibited significantly decreased expression of miR-124 compared with corresponding control fibroblasts (Figure 1D). The observed marked decrease in miR-124 expression in PH/IPAH-Fibs was not because of proliferation-stimulating culture conditions because decreased miR-124 levels were also observed in lung tissue of PH calves and in pulmonary arteries isolated by laser-assisted microdissection from human patients with IPAH (Figure 1E). Interestingly, no decrease in miR-124 expression was detected in chronically hypoxic male mouse or rat lung tissue or in sugen+hypoxia male mouse lungs, suggesting species-specific changes in miR-124 expression in PH (Online Figure I).

Highly Proliferative and Migratory Fibroblast Phenotype Is Controlled by miR-124

To investigate the function of miR-124 in these highly activated fibroblasts, we transiently transfected mature miR-124 mimic, anti–miR-124, and scrambled mimic (as control) into both bovine and human PH- and CO-Fibs. Transfection efficiency was >95% as monitored by Cy5 oligonucleotides. Mature miR-124 expression in transfected cells was confirmed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR; bovine, Figure 2A; human, Figure 2E). Transfection with miR-124 mimic did not result in cell death (Online Figure II).

Overexpression of miR-124 in PH/IPAH-Fibs caused a significant time-dependent decrease in proliferation rate compared with PH-Fibs treated with the scrambled miRNA (bovine, Figure 2C; human, Figure 2G). Flow cytometric analysis demonstrated that overexpression of miR-124 contributed to a G1/S growth arrest in both PH- and CO-Fibs (Online Figure II). Transfection of PH/IPAH-Fibs with anti–miR-124 did not significantly affect their growth (Figure 2C and 2G). However, transfection of slowly growing CO-/HCO-Fibs with

Figure 1. Decreased microRNA-124 (miR-124) expression in fibroblasts isolated from pulmonary hypertensive calves (PH-Fibs) and fibroblasts isolated from human patients with idiopathic pulmonary arterial hypertension (IPAH-Fibs) correlates with their highly proliferative and migratory phenotype. Proliferation rates (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] assay, 5 days; A and B) and migration capabilities (scrape assay, 24 hours; C) of PH-Fibs and IPAH-Fibs are markedly higher than those of fibroblasts isolated from control calves (CO-Fibs) and fibroblasts isolated from normal human donors (controls) (HCO-Fibs). D, Real-time polymerase chain reaction (PCR) analysis shows that miR-124 expression is significantly decreased in PH-/IPAH-Fibs compared with CO-/HCO-Fibs. All experiments (A–D) were performed with different bovine PH-Fibs and CO-Fibs populations (n=5, each) and with different human IPAH-Fibs and HCO-Fibs populations (n=5, each). Results are expressed as fold-change mean±SEM of PH-/IPAH-Fibs vs CO-/HCO-Fibs. E, Real-time PCR analysis demonstrates that miR-124 expression is significantly decreased in bovine PH lung tissue (n=5) and in human laser-assisted microdissection (LAM)–isolated IPAH pulmonary arteries (n=17, Online Table I) compared with corresponding control tissues (bovine lungs, n=5; human LAM-isolated arteries, n=7). Tissues used were from a cohort of patients previously described and reported by Savai et al39. *P<0.05.
anti–miR-124 significantly increased their proliferative capability, whereas transfection with miR-124 mimic slightly, yet significantly, decreased their proliferation (bovine, Figure 2B; human, Figure 2F).

Both the elevated promigratory capabilities of PH-/IPAH-Fibs and the moderate migratory capabilities of CO-/HCO-Fibs decreased on treatment with the miR-124 mimic (Figure 2D and 2H). Notably, the migratory potential of PH-/IPAH-Fibs was decreased to the levels of untreated control cells. Anti–miR-124 treatment markedly increased the migratory potential of CO-/HCO-Fibs (Figure 2D and 2H).

miR-124 Regulates Expression of Cell Cycle–Related Genes

We next sought to determine the mechanisms involved in miR-124 regulation of augmented PH-Fibs proliferation. We found that mRNA expression levels of Notch1, PTEN, FOXO3, p21Cip1, and p27Kip1 were reduced in PH-/IPAH-Fibs compared with control cells (Figure 3A and 3B). Transfection with miR-124 mimic markedly increased mRNA expression levels of these genes in both hypertensive (PH-/IPAH) and control (CO-/HCO) fibroblasts. Furthermore, anti–miR-124 treatment caused a significant decline in mRNA expression of Notch1, PTEN, FOXO3, p21Cip1, and p27Kip1 in both cell types (Figure 3A and 3B).

PTBP1 Is a Direct Downstream Target of miR-124 in Adventitial Fibroblasts

miRNAs regulate gene expression by binding to target sites of mRNAs and causing their degradation or translational repression. As such, we sought to determine the targets—upstream of the cell cycle regulator genes PTEN, p21Cip1, and p27Kip1—that are increased in cells with low miR-124 expression. We first confirmed that miR-124 regulates FOXO3 and p21Cip1 expression in PH-Fibs via a PTEN-dependent pathway because short interfering (si)–PTEN-treated cells failed to upregulate FOXO3 and p21Cip1 in response to miR-124 overexpression (Online Figure III). This suggested that direct miR-124 targets are located upstream of the PTEN pathway. Because Notch1 is a key regulator of PTEN and, as shown in Figure 3A and 3B, Notch1 is upregulated on overexpression of miR-124, we predicted that a direct target of miR-124 controls the Notch1 pathway in activated fibroblasts.

RT-PCR and Western blot analyses showed that PTBP1 expression levels were consistently increased in PH-/IPAH-Fibs compared with CO-/HCO-Fibs (Figure 4A, 4B, 4D, and 4E). Immunostaining analysis demonstrated strong PTBP1 expression in the pulmonary artery adventitia (where fibroblasts reside) of severely hypertensive (bovine and human) but not control tissues (Figure 4C and 4G). No increase in...
PTBP1 mRNA expression was noted in chronically hypoxic male mouse lungs (Online Figure I). Quantitative RT-PCR analysis confirmed increased mRNA expression of PTBP1 in human IPAH pulmonary arteries compared with control arteries (obtained by laser-assisted microdissection; Figure 4F). Transfection of both Fib populations with the miR-124 mimic decreased PTBP1 expression (Figure 4A, 4B, and 4D), whereas transfection with anti-miR-124 attenuated expression of each of these genes. Experiments were performed with different bovine PH-Fibs and fibroblasts isolated from control calves (CO-Fibs) populations (n=10, each) and with different human IPAH-Fibs and fibroblasts isolated from normal human donors (controls) (HCO-Fibs) populations (n=5, each). Data are presented as fold-change means±SEM of PH- or IPAH-Fibs vs CO- or HCO-Fibs. *P<0.05 vs CO- or HCO-Fibs; #P<0.05 vs PH- or IPAH-Fibs.

To establish that miR-124 targets PTBP1 directly, a dual-luciferase reporter assay was performed. The potential binding sites of miR-124 in the 3′ UTR of the bovine cPTBP1 mRNA were determined by TargetScan. To test the specific regulation of PTBP1 by miR-124, we cloned the PTBP1 3′ UTR and mutant sequence into a pmirGLO Dual-Luciferase miRNA target Expression Vector (Figure 5A). Luciferase activity of the wild-type pLuc-PTBP1 3′ UTR construct was significantly inhibited after transfection of miR-124 mimic into both CO- and PH-Fibs (Figure 5B and 5C, respectively), whereas anti–miR-124 treatment resulted in increased PTBP1 expression (Figure 5A, 5B, and 5D).

miR-124 Regulates Fibroblast Proliferation Through PTBP1

To determine whether PTBP1 mediates the antiproliferative effects of miR-124, we generated a PTBP1 expression vector without the 3′ UTR, pcDNA3.1-CMV-PTBP1, which encoded the full-length PTBP1 protein and that cotransfection of miR-124 and pcDNA3.1-CMV-PTBP1 abrogated miR-124-induced PTBP1 down-regulation, whereas miR-124 expression remained elevated (Online Figure V). When PH-Fibs were cotransfected with pcDNA3.1-CMV-PTBP1 vector and miR-124 mimic, the antiproliferative effects of miR-124 were abrogated (Figure 6A).

To further investigate the signaling pathways involved in regulation of cell cycle–related genes by miR-124, we transiently cotransfected PH-Fibs with miR-124 and pcDNA3.1-CMV-PTBP1. Real-time PCR analysis demonstrated that induction of Notch1, PTEN, FOXO3, p21Cip1, and p27Kip1 expression by miR-124 was markedly attenuated (Figure 6B–6F).

miR-124 Expression Is Controlled by Histone Deacetylases

To elucidate the stimulus responsible for decreased expression of miR-124 in PH/IPAH-Fibs, we first tested hypoxia. Even prolonged (≤1 week) exposure of CO-Fibs to 3% hypoxia failed to
decrease miR-124 expression (data not shown). Because hypermethylation of miR-124 was shown to be responsible for its suppression in cancer cells and to be mediated through increases in EVI1 expression, we next evaluated EVI1 expression in PH-Fibs. No significant increases in EVI1 expression were detected (Online Figure VI). Treatment of PH-Fibs with 5-aza-deoxycytidine (2, 5, and 10 µmol/L) for 5 and 7 days also did not increase miR-124 (Online Figure VI). Because histone deacetylase (HDAC) activity is required to maintain the activated PH-Fib phenotype and miRNA expression can be modulated by acetylation, we next tested whether small molecule HDAC inhibitors can rescue miR-124 expression in PH-Fibs. As shown in Figure 7A(a) and 7A(b), treatment of PH-Fibs with suberoylanilide hydroxamic acid (10 µmol/L), apicidin (3 µmol/L), or OSU42 (2.5 µmol/L) led to a significant increase in miR-124 expression and concomitantly decreased PTBP1 expression.

miR-124 Controls HIF-2α Expression

Previous studies have documented that the hyperproliferative and prom migratory phenotype of adventitial PH-Fibs is directly related to hypoxia-inducible factor (HIF)-2α expression and function. We, therefore, evaluated whether miR-124 controlled expression of either HIF1α or HIF2α in fibroblasts. Under basal conditions, PH-/IPAH-Fibs constitutively overexpressed HIF2α compared with CO-/HCO-Fibs (Figure 7B(a) and 7B(c)). No differences in HIF1α expression were observed between these cell types (Figure 7B(b) and 7B(d)). Administration of miR-124 mimic significantly decreased mRNA expression levels of HIF2α in PH-/IPAH-Fibs and, although to a lesser extent, in CO-/HCO-Fibs (Figure 7B(a) and 7B(c)). Conversely, anti–miR-124 treatment significantly increased HIF2α expression in PH-/IPAH-Fibs and only moderately in CO-/HCO-Fibs (Figure 7B(a) and 7B(c)).

miR-124 Directly Controls MCP-1 Expression in PH-Fibs

Bovine PH-Fibs (consistent with our previous observations), as well as human IPAH-Fibs, demonstrated significantly increased expression levels of MCP-1 mRNA (Figure 8A) and protein (Online Figure VII). Transfection with miR-124 mimic markedly decreased MCP-1 expression in both bovine (Figure 8B) and human (Figure 8C) fibroblasts and in both mRNA (Figure 8) and protein (Online Figure VII). Treatment with anti–miR-124 increased MCP-1 mRNA expression in CO-/HCO and PH-/IPAH fibroblasts (Figure 8B and 8C) and increased MCP-1 protein expression in CO-/HCO-Fibs (Online Figure VII).
To evaluate whether MCP-1 is a direct target of miR-124, we made a 3' UTR MCP-1 promoter luciferase construct and observed that MCP-1 luciferase activity was significantly reduced in cells transfected with miR-124 mimic (Figure 8D). With use of manipulated reporter, transfection of miR-124 mimic no longer suppressed reporter activity (Figure 8F).

Because miR-124 gene expression is controlled by HDACs (Figure 7A(a)), we next tested whether HDAC inhibitors affect MCP-1 expression. Treatment of PH/IPAH-Fibs with suberoylanilide hydroxamic acid (10 µmol/L) and apicidin (3 µmol/L) for 48 hours (for RT-PCR) and 72 hours (for Western blot) resulted in markedly decreased MCP-1 gene (Figure 8E) and protein (Figure 8F) expressions.

**Discussion**

Adventitial fibroblasts have been described to exhibit the earliest, most dramatic, and most sustained proliferative, fibrotic, and inflammatory responses to vascular stress and thus to play a key role in PH process. Failure of activated fibroblasts to deactivate contributes to chronic inflammatory and fibrotic diseases. The mechanisms contributing to sustained activation of vascular adventitial fibroblasts in PH are yet to be defined. Numerous studies have shown that miRNAs are pivotal in the gene regulation process, playing important roles in controlling development, tissue-specific cell differentiation, cell proliferation and migration, apoptosis, and stress responses. However, it was previously not known whether changes in miRNA expression directly control the activated phenotype of pulmonary fibroblasts in PH.

The present study demonstrates an important role for miR-124 and its direct downstream target, the alternative splicing factor PTBP1, in the generation of epigenetically reprogrammed, activated adventitial fibroblast, characterized by heightened proliferative, migratory, and inflammatory properties from calves and humans with severe PH. Our studies are consistent with observations in cancer cells and rheumatoid arthritis synovial fibroblasts, diseases to which PH is thought to have similarities, where involvement of miR-124 in the regulation of cell proliferation, migration, and inflammation (MCP-1 production) has been documented.

miR-124 is a frequently epigenetically silenced tumor-suppressive miRNA in cancer cells, which contributes to their proliferative and invasive capabilities. Our study demonstrates that the proximal direct target of miR-124, the RNA-binding protein PTBP1, is a critical regulator of effects of miR-124 on fibroblast proliferation and migration, consistent with studies in some cancer cells and recently in vascular smooth muscle cells. PTBP1 is a nucleic acid–binding protein, also known for its role in multiple aspects of mRNA life cycle and function, including regulation of RNA processing and internal ribosome entry site–dependent translation, localization, stability, and translation. PTBP1 may also bind to DNA and act as a transcription factor. PTBP1 is strongly implicated in cancer initiation and progression, and removal of PTBP1 from tumor cells results in decreased cell proliferation and invasion.

Our study demonstrated that miR-124–mediated downregulation of cell proliferation was because of its effects on PTBP1 and that PTBP1 was a direct downstream target of miR-124 that can exert exquisite regulation of many downstream molecules important in cell proliferation, such as cell cycle–related genes Notch1, PTEN, FOXO3, p21Cip1, and p27Kip1, as well as potential cell migration, where p27Kip1 has been implicated.

Our results also support the notion that miR-124, through direct regulation of PTBP1, controls Notch1 expression and subsequent downstream proliferative signaling pathways (AKT-2, p21Cip1, cyclin-dependent kinase 4 [CDK4]), likely
through PTEN-related mechanisms. Reports in other cell systems demonstrated that Notch1 can function as an oncogene by regulating the PTEN/phosphatidylinositide 3-kinases/AKT pathway.59,60 A potential role for Notch1 in regulating proliferation has been demonstrated in some, but not all, studies in cancer cells.61,62 It should be noted, however, that the role of Notch signaling in cancer and in control of proliferation in other pathological conditions is highly dependent on the spatial and temporal context of Notch1 activation, as well as the status of other signaling pathways operating in cells.63 Thus, highly proliferative PH-/IPAH-Fibs express a distinct phenotype that is dependent on decreased Notch1 signaling and downregulation of PTEN expression, similar to a phenotype of at least some cancer cells64 and vascular smooth muscle cell phenotype achieved with PTEN knockdown in vivo.65

The mechanisms contributing to stable decreases in miR-124 in PH/IPAH-Fibs are not fully understood. The ability of an initial stimulus, such as hypoxia, to engage an miRNA circuit could potentially result in stable changes in pulmonary fibroblasts, which persist even after the hypoxic stimulus is removed. However, in contrast to a recent report where acute hypoxia lead to an immediate reduction in miR-124,35 we did not detect an ability of even prolonged exposure to hypoxia to drive a stable change in the control fibroblast phenotype toward the activated PH-Fibs phenotype or a reduction in miR-124 expression in these cells. Thus, the acquisition of stable, functional phenotypic changes likely requires the involvement of epigenetic processes such as might occur in response to altered histone acetylation, DNA methylation, and changes in miRNA expression profiles.12

Histone-dependent packaging of genomic DNA into chromatin is a central mechanism for gene regulation. Expression of inflammatory genes, DNA repair genes, and proliferation genes is controlled by the degree of acetylation of histone and nonhistone proteins produced by histone acetyltransferase and HDACs.66–68 Several reports have documented such changes in HDAC activity in fibroblasts in rheumatoid arthritis and juvenile idiopathic arthritis, with specific increase in HDAC-1 activity.68,69 Our study demonstrates that HDAC inhibitors can restore miR-124 levels toward normal and reverse characteristics of the activated phenotype. These findings thus begin to explain the molecular basis for the constitutively activated fibroblast phenotype, which is critical in many aspects of the pulmonary vascular disease process. In the present study, apicidin, which selectively inhibits class I HDACs, led to significant miR-124 and concomitant decreases in PTBP1 expression. This is consistent with our previous reports that PH-Fibs exhibited significantly elevated catalytic activity of class I HDACs (HDAC-1, -2, and -3) and that selective inhibition of class I HDACs is sufficient to suppress pulmonary vascular remodeling and hypoxic PH.13,14,70 Similarly, observations in cancer cells showed that HDAC inhibitors have important antitumor effects through modulation of miRNAs.42 Reports in cancer have also suggested that epigenetic modulation of mRNA expression by HDAC inhibitors could be an important adjunctive treatment approach in cancer chemotherapy.71

Figure 6. Polypyrimidine tract–binding protein 1 (PTBP1) abrogates antiproliferative effects of microRNA-124 (miRNA-124) mimic. A, miR-124 mimic (50 nmol/L) when cotransfected with pcDNA3.1-CMV empty vector, exhibited antiproliferative effects on PH-Fibs. However, cotransfection with miR-124 mimic and pcDNA3.1-CMV-PTBP1 vector (pcDNA3.1-PTBP1) abrogated the antiproliferative effects of miR-124. B–F, Induction of cell cycle–related gene expression by miR-124 mimic was markedly attenuated on cotransfection with pcDNA3.1-PTBP1 plasmid. Data are presented as fold-change mean±SEM from 3 different experiments on n=5 different cell populations (each, fibroblasts isolated from control calves, fibroblasts isolated from pulmonary hypertensive calves). *P<0.05. MTT indicates 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
We showed that reduced miR-124 is associated with increased expression of HIF2α, but not HIF1α, and that restoring miR-124 expression can reduce HIF2α expression and attenuate cell proliferation. This is interesting because most previous studies in the pulmonary circulation have focused on the overexpression of HIF1α. However, studies from several groups would suggest that HIF1α and HIF2α have distinct tissue and cell patterns of distribution and that both play critical but nonoverlapping roles in disease progression. Eul et al demonstrated that hypoxia-induced fibroblast proliferation requires HIF2α but not HIF1α activation. Ahmad et al showed that, in the remodeling processes observed in PH, HIF2α plays a predominant role in the endothelial growth, whereas HIF1α plays roles in smooth muscle cell growth. Studies in cancer also support the idea that HIF2α is strongly related to the proliferative and invasive cell phenotype. The Simon laboratory showed that HIF2α in contrast to HIF1α promotes cell proliferation by enhancing the activity of c-myc. HIF2α can promote cell cycle progression by further inhibiting the expression in genes encoding p21 and p27, which we have shown in the present study to be decreased in the highly proliferative PH-/IPAH-Fibs. It is also important to note that RNA-binding proteins are known to be regulators of gene expression in response to numerous stresses such as hypoxia. PTBs, including PTBP1, have been demonstrated to associate with mRNAs encoding hypoxia-response proteins such as HIF to modulate their expression under hypoxic and other stressful conditions and to play major roles in establishing a hypoxic gene expression pattern. It will be thus important to define mechanisms controlling HIF2α, the activated adventitial fibroblast.

Adventitial fibroblasts are increasingly recognized to play important roles in orchestrating inflammatory responses in the vessel wall. Our previous studies have documented that the activated PH-Fibs exhibit a stable proinflammatory phenotype, characterized by high expression levels of cytokines and chemokines including MCP-1. MCP-1 is one of the key chemokines that is known to regulate migration and infiltration of monocytes and macrophages, both of which are increasingly thought to play key roles in the pathogenesis of pulmonary vascular disease in humans and animal models. Because MCP-1 is known to be produced in various cell types, it is important to understand the mechanisms that regulate MCP-1 production specifically in adventitial fibroblasts, as regulation could occur in a cell-specific way. Our data show that production of
MCP-1 by PH-/IPAH-Fibs is under the direct control of miR-124, consistent with studies in synoviocytes from patients with rheumatoid arthritis. Our data also support previous observations demonstrating that HDAC inhibitors have beneficial anti-inflammatory effects in animal models of inflammatory fibrotic vascular diseases and cancer. Taken together, these observations suggest that targeting the acetylation state of miR-124, through the use of HDAC inhibitors, is a potentially effective approach to abrogating fibroblast activation.

In conclusion, decreased miR-124 expression directly contributes to the constitutively activated phenotype of adventitial fibroblasts in the hypertensive pulmonary vasculature. Our data support the idea that targeting miR-124 directly or indirectly with specific HDAC inhibitors or agents affecting alternative splicing factors (PTBP1) should be pursued for the pharmacological treatment of PH.

Acknowledgments
We thank Drs Raphael Nemenoff, Rubin Tuder, and R. Dale Brown for their contributions to the manuscript review and Marcia McGowan and Stephen Hofmeister for outstanding help in preparing the manuscript.

Sources of Funding
This work was supported, in part, by National Institutes of Health A114887-01, Program Project grant 5P01 HL014985-39, and Training grant 2 T32 HL07171-36.

Disclosures
None.
References


67. Dotto GP. miR-124 directly regulates the RNA splicing factor polypyrimidine tract–binding protein 1, which, in turn, controls the translation of Notch1/PTEN/FOXO3/p21Cip1 and p27Kip1, thus exerting control on proliferation and migration. J Am Heart Assoc. 2013;6:80–84.


**Novelty and Significance**

**What Is Known?**

- Chronic pulmonary hypertension is characterized by robust fibroproliferative changes in the adventitia of both small and large pulmonary arteries.
- During the remodeling process, the principal cell type of the adventitia, the fibroblast, undergoes profound phenotypic changes characterized by heightened proliferative, migratory, and inflammatory activity, changes that are durable even in culture ex vivo.
- Recent studies have implicated a role for miRNAs in the complex pathology and molecular dysregulation that characterizes pulmonary hypertension.

**What New Information Does This Article Contribute?**

- MicroRNA-124 (miR-124) expression is markedly reduced in fibroblasts derived from bovines or humans with severe pulmonary hypertension, and targeted overexpression of miR-124 suppresses their proliferation, migration, and monocyte chemotactic protein-1 production.
- MiR-124 directly regulates expression of the RNA splcing factor polypyrimidine tract–binding protein 1, which, in turn, controls expression of Notch1/PTEN/FOXO3/p21Cip1 and p27Kip1, thus exerting control on proliferation and migration.
- miR-124 expression is suppressed by histone deacetylases (HDACs), and treatment of hypertensive fibroblasts with HDAC inhibitors increased miR-124 expression and decreased polypyrimidine tract–binding protein 1 and monocyte chemotactic protein-1 production.

Perivascular fibroblasts are increasingly appreciated to play critical roles in fibroising vascular diseases. Previous work raises the possibility of emergence and expansion of fibroblasts that have lost their ability to limit stimulus-induced proliferation and cytokine production through mechanisms that include epigenetic change. We found that miR-124 expression is decreased in human and bovine fibroblasts from hypertensive vessels and that targeted overexpression of miR-124 reversed their highly proliferative, migratory, and inflammatory phenotype to normal. miR-124 regulates proliferative changes in fibroblasts by targeting the RNA-splasing factor polypyrimidine tract–binding protein 1, which, in turn, regulates Notch1/PTEN/FOXO3/p21Cip1 and p27Kip1 signaling. Furthermore, miR-124 directly regulates monocyte chemotactic protein-1 expression through binding to its promoter. Decreased miR-124 expression hypertensive fibroblasts is mediated by increased HDAC activity, and HDAC inhibition increased miR-124 expression and decreased polypyrimidine tract–binding protein 1 and monocyte chemotactic protein-1 expression in hypertensive fibroblasts to near-normal levels. Thus, targeting miR-124 directly or indirectly (eg, small molecule HDAC inhibitors) may abrogate the contribution of activated perivascular fibroblasts to pulmonary hypertensive vascular remodeling.
MicroRNA-124 Controls the Proliferative, Migratory, and Inflammatory Phenotype of Pulmonary Vascular Fibroblasts

Circ Res. 2014;114:67-78; originally published online October 11, 2013;
doi: 10.1161/CIRCRESAHA.114.301633

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/1/67

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/10/11/CIRCRESAHA.114.301633.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

ONLINE TABLES

Online Table I: Demographic Data of Patients with IPAH.

Online Table II: Primers and siRNAs Sequences.

METHODS

Animals
The neonatal calf model of severe hypoxia-induced pulmonary hypertension (PH) has been described previously and includes the development of pulmonary artery pressure equal to, or exceeding, systemic pressure as well as remarkable pulmonary artery remodeling with prominent medial and adventitial thickening and perivascular (adventitial) inflammation, resembling that of human neonatal PH. One-day-old male Holstein calves were exposed to hypobaric hypoxia (P<sub>B</sub> = 445 mmHg) for 2 weeks (experimental group, n = 10), and age-matched controls (n = 10) were kept at ambient altitude (P<sub>B</sub> = 640 mmHg) as previously described. Wistar-Kyoto rats (Harlan Laboratories, Madison WI) and C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were exposed to hypobaric hypoxia (P<sub>B</sub> = 380 mmHg) for 4 weeks (rats, n = 9) or for 16 weeks (mice, n = 6). Age-matched controls (rats, n = 9; mice, n = 6) were kept at ambient altitude. Standard veterinary care was used following institutional guidelines: for rats and mice, at University of Colorado Denver Center for Laboratory Animal Care in compliance with Institutional Animal Care and Use Committee-approved protocols; for calves, at the Department of Physiology, School of Veterinary Medicine, Colorado State University (Fort Collins, CO). All animals were euthanized by overdose of sodium pentobarbital (160 mg/kg body weight).

Patient Characteristics
Human lung tissues from subjects with idiopathic pulmonary arterial hypertension (IPAH) (n = 17) were obtained during lung transplantation. Samples of normal donor (n = 7) lung tissues were taken from lungs that had not been transplanted, as described previously. The study protocol for tissue donation was approved by the ethics committee (Ethik Kommission am Fachbereich Humanmedizin der Justus Liebig Universität Giessen) of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with Good Clinical Practice/International Conference on Harmonisation guidelines. Written informed consent was obtained from each individual patient or the patient’s next of kin (AZ 31/93).

Cell Isolation and Culture
Bovine pulmonary artery adventitial fibroblasts were isolated from normal control calves (n = 10, CO-Fibs) or calves with severe experimental hypoxic pulmonary hypertension (n = 10, PH-Fibs), as previously described. Cell phenotype was thoroughly characterized and all experiments were performed on cells at passages 5–8. Human pulmonary artery fibroblasts were derived from patients with idiopathic pulmonary hypertension (IPAH-Fibs) (n = 5) undergoing lung transplantation at Papworth Hospital, Cambridge, UK. Control human fibroblasts (hCO-Fibs) were derived from patients undergoing lobectomy or pneumonectomy for suspected malignancy (n = 5). All patients provided written informed consent.

Proliferation assay
Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Briefly, cells were plated on 96-well TissueTek plates (2000 cells/well), and after various days in culture, 20 μl of MTT reagent
(MP Biomedicals, Santa Ana, CA) was added to wells and incubated for 4 hours. The cells were dissolved in DMSO, and optical density (OD) was measured at 570 nm.

Migration (“Scrape” assay)
“Scrape” migration assay was performed as previously described6,7. Briefly, cells were grown in full growth media (full DMEM supplemented with 10% calf serum) on 6-well plates until confluence, then media was replaced with serum-free DMEM containing 5 μM hydroxyurea to inhibit cell proliferation, and a 2-mm scrape was performed. The borders of the scrape were outlined on the bottom of the plate, images were acquired at different time points and the number of cells migrating into the scrape area, were counted on the image.

Real-time RT-PCR
Extraction of total RNA was performed using the mirVana miRNA isolation Kit (AB Applied Biosystems) and quantified using the NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA). Identical starting concentrations of total RNA were used for all samples. Total RNA was reverse-transcribed to cDNA using a miScript Reverse Transcription Kit (Qiagen, Inc.) for miRNA and High-Capacity cDNA Reverse Transcription Kits (AB Applied Biosystems) for mRNA. The intron-spanning primers were designed using sequence information from the NCBI database (Online Table II). Changes in expression of various miRNA levels were determined quantitatively using Quantitative-Reverse Transcriptase PCR (qRT-PCR). The miScript SYBR Green PCR Kit (Qiagen, Inc.) containing a QuantiTect SYBR Green PCR Master Mix and the miScript Universal Primer along with the miRNA-specific primer was used for the detection of mature miRNAs. Amplification and detection of the PCR products was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). For mRNAs, the data were normalized using the endogenous HPRT control. U6 snRNA was used as the endogenous control for miRNA since the data did not show any differences in expression levels under normoxic or hypoxic conditions.

Plasmid construction
The calf PTBP1 cDNA without 3’UTR was amplified by PCR with forward primer: 5’-GATATCGGATCCTGATGTGCAAT-3’ and reverse primer 5’-AATTTGATATCGCGGCTTACAGATGATTGA-3’. The plasmids overexpressing PTBP1 from the CMV promoter were constructed by insertion into pcDNA3.1/Hygro (Invitrogen) via its EcoRV site. The 3’UTR of calf PTBP1 fragments were amplified using RNA from CO-Fibs with the forward primer 5’-ACCTCGAGCCACCAGCAGGACGCT-3’ and reverse primer 5’-ACCTCGAGTCTAAGGTCACACTCAGTCTC-3’. Mutant PTBP1 3’UTRs were amplified using forward primer 5’-ACCTCGAGGCCCAGGACCAGCCT-3’ and reverse primer 5’-ACCTCGAGTCTTAAAGTAATGTAATTTTATCAAT-3’ (the seed region was replaced with all A’s) or reverse primer 5’-ACCTCGAGTCTTAAAGTAATGTAATTTTATCAAT-3’ (the seed region was deleted). The 3’UTR of calf MCP-1 fragments were amplified using forward primer 5’-ACCTCGAGGTGACACTGACCTGACCAACC-3’ and reverse primer 5’-ACGATATCTTTTCCACAATAGCAGTGTAAACAAATG-3’. Site-directed mutagenesis was performed to substitute 2 nucleotides in the seed sequences of MCP-1. The primers used for the MCP-1 3’UTR mutation were ACATGAAAAAAAGTAATGTAATTTTAT (forward) and CTGTTTTTTTTTCTGTCATCATCAAT (reverse) (the seed region was replaced with all A’s). LongAmp® Taq PCR Kit (New England BioLabs) was used for PCR amplification.

miR-124 mimic, anti-miR and siRNA transfection
miR-124 mimic and scramble were purchased from Ambion (Applied Biosystems, USA) and anti-miR-124, and short interfering (si)RNAs scramble, (si)RNAs targeting PTBP1, PTBP2 and PTEN (Online Table II) were purchased from IDT (Integrated DNA Technologies). 5X10^5 cells/well in 6-well plates were cultured for 16 hours and transfected with 50 nmol/L of miR-124 mimic, anti-miR or siRNA by DHARMAFECT Transfection Reagents (Dharmacon). After 8 hours, culture medium was replaced without the transfection reagent. Total RNA/miRNA was extracted 48 hours after transfection using mirVana miRNA isolation Kit.

TUNEL staining
48 hours after transfection, TUNEL staining was performed according to the manufacturer’s protocol (DeadEnd Fluorometric TUNEL System; Promega, WI, USA). The cells were fixed in 4% paraformaldehyde in PBS for 25 min at 4°C, and washed three times in PBS. Afterwards, equilibration buffer was added, and equilibration at RT for 10 min was performed. TdT reaction mix was added to the cells and incubated at 37°C for 60 min, after which cells were incubated in 2× SSC for 15 min. To visualize all nuclei, DAPI solution was applied for 5 min.

**Western blot**

Total cell protein was isolated with RIPA lysis buffer (Sigma Aldrich, St. Louis, MO). Protein concentrations were measured via BCA assay (Thermo Scientific, Walthan, MA). Proteins were fractionated on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA), blotted onto nitrocellulose membrane, and probed with antibodies specific for PTBP1 (Acris, GmbH), PTEN (Cell Signaling, Danvers, MA), and β-actin (Santa Cruz Biotechnology, Dallas, TX). Signal was detected using ECL (Thermo Scientific).

**Immunohistochemical staining**

Human lung tissue sections (3-8µm thickness) were mounted on positively charged glass slides (R. Langenbrinck, Teningen, Germany) and incubated with anti-PTBP1 antibody (1:100; Acris Antibodies GmbH). Antibody binding was determined using an ImmPRESS reagent kit (Vector Laboratories, Burlingame, CA).

**Immunofluorescent staining**

Calf lung tissue cryosections (5µm thickness) were fixed with ice-cold acetone:methanol (1:1), incubated with mouse monoclonal PTBP1-specific antibody (1:50, Novus Biologicals, Littleton, CO), and processed via biotin-streptavidin system as previously described. Staining was examined under Zeiss fluorescent microscope and images were acquired with an AxioVision digital imaging system (Carl Zeiss MicroImaging, Thompsonwood, NY).

**Laser-Assisted Microdissection (LAM)**

Microdissection was performed as described previously. Briefly, human lung cryosections (10 µm) were mounted on membrane-coated slides, briefly (45 sec) stained with hemalaun followed by hematoxylin/eosin and processed through ethanol (70%, 96%, 100%). Microdissection of intrapulmonary arteries with 50–100 µm diameter was performed using the Lasermicrodissection device LMD6000 (Leica, Wetzlar, Germany), the microdissected material was collected into RNA lysis buffer, and stored in liquid nitrogen until analysis.

**Cell cycle analysis**

Cells were plated as detailed above and, at 50% confluence, were treated with miRNA mimic Scramble or miR-124 mimic (50 nM). After 48 hr, cells were trypsinized, and cell pellets were collected and washed two times with cold phosphate buffered saline (PBS). Cell were fixed with 70% ethanol at −20°C for at least 12 h. The cells were incubated in RNase A/PBS (100 µg/ml) at 37°C for 30 min. Intracellular DNA was labeled with PI (50 µg/ml) and cell-cycle distribution was then analyzed by flow cytometry by using the fluorescence activated cell sorting analysis core services at the University of Colorado Cancer Center, Denver, CO.

**ONLINE FIGURE LEGENDS**

**Online Figure I. miR-124 expression in whole lung tissues from different rodent (rats, mice) models of PH.**

(A) mRNA expression levels of miR-124 are increased in lung tissue from monocrotaline (MCT)-treated and chronically Hypoxic rats compared to Controls (MCT, n=5; vehicle-treated Control, n=5; Hypoxic, n=9). (B) Real-time PCR analyses demonstrated that there was no significant change in miR-124 expression in [Sugen+Hypoxia, 5 weeks] mouse whole lung tissue compared to Normoxic vehicle-treated controls. (C, D) miR-124 mRNA expression is increased and PTBP1
mRNA expression is decreased in the lungs of mice with Hypoxia-induced PH (n=6) compared to Normoxic controls (n=6), as assessed by qRT-PCR analysis. Data are presented as fold change mean ± SEM of PH versus CO. *P<0.05.

**Online Figure II. miR-124 regulates proliferative capabilities of bovine CO and PH-Fibs via effects on the cell cycle.**

(A) TUNEL staining of PH-Fibs after treatment with miR-124 mimic. Apoptotic nuclei are shown in green. Normal cell nuclei are shown in blue, stained by DAPI. (B to E) The effect of miR-124 mimic on cell cycle in CO and PH-Fibs as evaluated by flow cytometry cell using Propidium iodide DNA staining. miR-124-transfected cells showed a significant decrease in G2/M phase populations. Experiments are performed with n=5 different PH-Fibs and CO-Fibs cell populations from 5 different animals. Data are presented as fold-change mean ± SEM of PH versus CO-Fibs. *P<0.05.

**Online Figure III. miR-124 induces FOXO3 and p21Cip1 expression via a PTEN-dependent pathway in PH-Fibs.**

(A, B) Real-time PCR and Western blot analyses demonstrate that PTEN expression was effectively silenced by siPTEN even upon upregulation by miR-124 mimic. (C, D) Real-time PCR analysis demonstrates that marked upregulation of mRNA expression of the cell cycle-related genes FOXO3 (C) and p21Cip1 (D) is attenuated upon PTEN silencing (via siPTEN). Data are presented as mean ± SEM of mRNA fold-change over scramble. *P<0.05

**Online Figure IV. Effect of PTBP1 on cell cycle-related gene expression, cell proliferation and migration in PH-Fibs.**

(A, B) Real-time PCR and Western blot analyses demonstrate, at both mRNA and protein levels, effective silencing of PTBP1 upon co-transfection with siPTBP1 and empty control vector (pcDNA3.1), as well as upregulation of PTBP1 expression upon co-transfection with the pcDNA3.1-CMV-PTBP1 expression vector (pcDNA3.1-PTBP1) and scrambled siRNA (scramble). (C-G) Real-time PCR analyses of mRNA expression of cell cycle-related genes Notch1 (C), PTEN (D), FOXO3 (E), p21Cip1 (F), and p27Kip1 (G) in PH-Fibs demonstrate that these genes were upregulated upon silencing of PTBP1 (via co-transfection with siPTBP1 and empty control vector pcDNA3.1), and these genes were downregulated upon increased PTBP1 expression (via co-transfection with the pcDNA3.1-PTBP1 and scrambled siRNA). (H) Proliferation rate of PH-Fibs (measured by MTT assay) was attenuated upon transfection with siPTBP1 or miR-124 mimic, compared to Scramble. (I) Migration of PH-Fibs (Scrape Assay) was attenuated upon transfection with siPTBP1. All data are presented as mean ± SEM of fold-change of mRNA expression (A-G) or absorbance (MTT assay, H). *P<0.05.

**Online Figure V. Effects of miR-124 mimic, pcDNA3.1-PTBP1 or miR-124/pcDNA3.1-PTBP1 co-transfection on PTBP1 and miRNA-124 expression in PH-Fibs.**

(A, B) The effect of miR-124 on PTBP1 expression was evaluated by real-time PCR and western blot in PH-Fibs in the presence and absence of the PTBP1 expression vector lacking miR-124 binding site in the 3' untranslated region (pcDNA3.1-PTBP1). PTBP1 expression was downregulated by miR-124 mimic an effect, which was lost in the presence pcDNA3.1-PTBP1, compared with empty vector pcDNA3.1. C) Real-time PCR showed that miR-124 is over-expressed in PH-Fibs after miR-124 mimic transfection and in cells co-transfected with miR-124/pcDNA3.1-PTBP1. Results are expressed as mean ± SEM of fold change. *P<0.05

**Online Figure VI. No significant change of EVI1 expression in PH-Fibs compared with CO-Fibs, the DNA demethylation treatment by 5-aza did not effect miR-124 expression.**

(A) Real-time PCR analyses confirmed that there was no significant differences in EVI1 expression in PH-Fibs compared to CO-Fibs. (B) The DNA demethylation treatment with 5-aza-deoxycytidine (10 μM) for 5 and 7 days did not effect miR-124 expression as assessed by real time PCR analysis in PH-Fibs. Experiments are performed with n=5 different PH-Fibs and CO-Fibs cell populations from 5 different animals. Data are presented as fold change mean ± SEM.

**Online Figure VII. miR-124 regulates MCP-1 expression in CO-, PH-Fibs and HCO-, IPAH-Fibs.**
(A and C) Western blot analyses showed that MCP-1 protein was increased in PH or IPAH-Fibs compared to CO-Fibs. (B and D) The effect of miR-124 mimic and anti-miR-124 on MCP-1 expression in CO, PH-Fibs and HCO, IPAH-Fibs as evaluated by western blot analyses. Expression of MCP-1 is attenuated by transfection with miR-124 mimic, and is augmented by transfection with anti-miR-124 in CO-Fibs and HCO-Fibs.

REFERENCES

Online Figure II

A. Positive control  Scramble  miR-124 mimic

DAPI

Tunel

B. CO-Fibs Scramble  CO-Fibs miR-124 mimic

G1: 80.53 %
S: 11.48 %
G2/M: 7.99 %

G1: 76.4 %
S: 17.71 %
G2/M: 5.89 %

C. Fold change of G2M phase cell

CO-Fibs

Scramble  miR-124 mimic

D. PH-Fibs Scramble  PH-Fibs miR-124 mimic

G1: 81.53 %
S: 10.02 %
G2/M: 8.45 %

G1: 80.83 %
S: 15.18 %
G2/M: 4 %

E. Fold change of G2M phase cell

PH-Fibs

Scramble  miR-124 mimic
Online Figure IV

A. PTBP1

B. Notch1

C. Fold change of mRNA expression

D. PTEN

E. FOXO3

F. p21Cip1

G. p27Kip1

H. Fold change of OD (MIT: A_{570})

I. Migration (Scratch assay)
Online Table I: Demographic Data of Patients with IPAH

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Mean PAP (mmHg)</th>
<th>CI (l/min/m²)</th>
<th>NYHA class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>37</td>
<td>52</td>
<td>1.75</td>
<td>III-IV</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>66</td>
<td>2.1</td>
<td>III-IV</td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
<td>34</td>
<td>2.41</td>
<td>IV</td>
</tr>
<tr>
<td>Male</td>
<td>35</td>
<td>56</td>
<td>2.68</td>
<td>III</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>62</td>
<td>2.1</td>
<td>III-IV</td>
</tr>
<tr>
<td>Female</td>
<td>45</td>
<td>60</td>
<td>2.74</td>
<td>III-IV</td>
</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>43</td>
<td>2.6</td>
<td>III-IV</td>
</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>58</td>
<td>2.1</td>
<td>III-IV</td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>62</td>
<td>2.96</td>
<td>III</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>70</td>
<td>1.2</td>
<td>II–III</td>
</tr>
<tr>
<td>Female</td>
<td>37</td>
<td>52</td>
<td>1.94</td>
<td>III–IV</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>62</td>
<td>2.1</td>
<td>III–IV</td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
<td>73</td>
<td>1.56</td>
<td>III</td>
</tr>
<tr>
<td>Male</td>
<td>45</td>
<td>47</td>
<td>2.18</td>
<td>III</td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>62</td>
<td>2.79</td>
<td>II–III</td>
</tr>
<tr>
<td>Male</td>
<td>29</td>
<td>56</td>
<td>1.98</td>
<td>III</td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
<td>34</td>
<td>2.41</td>
<td>IV</td>
</tr>
</tbody>
</table>

Definition of abbreviations: CI = cardiac index; PAP = pulmonary arterial pressure; NYHA = New York Heart Association
## Online Table II: Primer and siRNA Sequences

<table>
<thead>
<tr>
<th>Gene Bovine</th>
<th>Forward Primer 5' to 3'</th>
<th>Reverse Primer 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTBP1</td>
<td>ATCAGGCCTTCATCGAGATGCACA</td>
<td>TGTCTTGAGCTCCTTGTGTTGGA</td>
</tr>
<tr>
<td>Notch1</td>
<td>TCTGCTCACGCTACTGAGTACAA</td>
<td>AGATATACGACGCCTCAAAAGGGCA</td>
</tr>
<tr>
<td>PTEN</td>
<td>TGTGGTCTGCCCCCTGAAGGGTGGAA</td>
<td>TGATGTCAACCACACACAGGCAATG</td>
</tr>
<tr>
<td>FOXO3</td>
<td>GAATTCCGTCCAGCAACATGGGGCTT</td>
<td>TGGAGGGTTTCGCACTGGTTGAGTA</td>
</tr>
<tr>
<td>p21Cip1</td>
<td>AGAGCGGTGGAACCTCCGACTTTTG</td>
<td>ACAGGTCCAAGTGCTCTCCTGAG</td>
</tr>
<tr>
<td>p27Kip1</td>
<td>ACTTGGAGAACAGACTGCAGAGACA</td>
<td>TTTCTACCTCTCCGTGCAACTG</td>
</tr>
<tr>
<td>HIF 1a</td>
<td>CCACCTCTGGAGCTGTGCTTTT</td>
<td>TTTCTTGTCCGAGGCCGCCC</td>
</tr>
<tr>
<td>HIF 2a</td>
<td>CGCAACTTCCACCGTACCCTG</td>
<td>GCAGGCCGTACTCCGTCTG</td>
</tr>
<tr>
<td>HPRT</td>
<td>CTGGCTCGAGATGTGATGAA</td>
<td>CAACAGGTCGGCAAAGAAC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene Human</th>
<th>Forward Primer 5' to 3'</th>
<th>Reverse Primer 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>TGAGGACTGCGAGCGAGAACAT</td>
<td>ATTAGACAGGGTTGCTGAGCA</td>
</tr>
<tr>
<td>PTEN</td>
<td>TGTTCAGTGGCGAGAACATTCGA</td>
<td>AGGTAAACGCGTCGGCAACCTC</td>
</tr>
<tr>
<td>FOXO3</td>
<td>AGATCTACAGATGAGGGTTG</td>
<td>AGCTCTTGACCAGGTCCCTCAT</td>
</tr>
<tr>
<td>p21Cip1</td>
<td>ACCTATGAGACCTGTCACAGT</td>
<td>AGAAATCTGTCAGTGCTGCTT</td>
</tr>
<tr>
<td>p27Kip1</td>
<td>ACGCGCGCAAGGGTTTGGGA</td>
<td>AATGAGACTGGCAGGAC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miR-124</th>
<th>TAAGGCACGCGGGAATGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6</td>
<td>CGATAACAGAGAGATTAGCTGGG</td>
</tr>
<tr>
<td>Anti-miR-124</td>
<td>GCAUUCACCGCGUGCCUUA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siPTBP1 BOS</td>
<td>CCACAAGGAGCUCAAAGACAGACAGC</td>
</tr>
<tr>
<td>siPTBP2 BOS</td>
<td>GCAGCUCUUACCAGGGUUUAAUACT</td>
</tr>
<tr>
<td>siPTEN BOS</td>
<td>CCAUUAAACCACAGUAGAAGACTT</td>
</tr>
<tr>
<td>siRNA scramble</td>
<td>AACAGTGGCCGTTTGGCAGACTG</td>
</tr>
</tbody>
</table>