The molecular mechanisms involved in the development of pulmonary hypertension (PH) remain unclear, although many investigators have demonstrated that abnormalities in gene expression in pulmonary vascular fibroblasts, smooth muscle cells, and endothelial cells are involved in the pathogenesis of PH. The control of gene expression is a complicated process, involving multiple layers of regulation. There are 3 distinct mechanisms of epigenetic regulation, DNA methylation, histone modifications, and gene silencing mediated by microRNAs (miRNAs). DNA methylation occurs on cytosine residues in CpG regions and is regulated by DNA methyltransferases (DNMTs). DNA methylation is essential for normal development, and 60% to 80% of the human genome CpGs are methylated. Methylation of most CpGs is constant, changing only in response to different cellular processes. In cancers and other diseases, hypermethylation of so-called CpG islands, which are CG-dense regions close to transcription start sites, found in tumor suppressor genes has been reported, leading to gene silencing. These data demonstrate that DNA methylation status is a frequently altered epigenetic modification in human diseases. In addition to DNA methylation, histone modifications represent another layer of regulation of gene expression. For the transcription machinery to be recruited to their target genes, the DNA needs to be accessible. The ability of the transcription machinery to reach the DNA is mainly controlled by histone acetyltransferases and histone deacetylases (HDACs). Histone acetyltransferases acetylate lysine residues and relax the chromatin structure, allowing for transcription factors to bind to the DNA and activate transcription. HDACs remove acetyl residues from histones, resulting in a condensed chromatin structure and transcriptional repression. The last layer of gene expression regulation is controlled by miRNAs, which are small noncoding RNAs that bind to their complementary sequence in the 3’ untranslated regions of their target mRNAs, resulting in gene silencing.

These pathways of gene regulation are often altered in many human diseases, such as cancer, leading to uncontrolled cell growth, migration, and invasion. In fact, changes in epigenetic modifications have recently been associated with PH, a disease that is associated with increased cell proliferation and decreased cell death of pulmonary arterial smooth muscle cells, pulmonary arterial endothelial cells, and adventitial fibroblasts. Recent studies of fawn-hooded rats, which spontaneously develop PH, have demonstrated that superoxide dismutase 2 (SOD2) expression is decreased in pulmonary arteries and plexiform lesions because of hypermethylation of CpG islands in the SOD2 gene. Reversal of the methylation status via DNMT1 inhibition rescued SOD2 expression and inhibited proliferation and increased cell apoptosis of fawn-hooded rat pulmonary arterial smooth muscle cells. Histone acetylation has also been shown to play an important role in the development of PH. Increased HDAC expression has been reported in lung tissues from patients with idiopathic pulmonary arterial hypertension as well as in lung tissues from hypoxia-induced pulmonary hypertensive (HPH) rats. The HDAC inhibitors valproic acid and suberoylanilide hydroxamic acid attenuated and reversed the development of HPH in rats and decreased proliferation in human pulmonary arterial smooth muscle cell and bovine fibroblasts. Additionally, a small-molecule HDAC inhibitor, which selectively inhibits class I HDACs, has been shown to suppress hypoxia-induced cardiopulmonary remodeling in rats. Recent studies have also implicated miRNAs in the development of PH. miR-204 expression has been shown to be decreased in animal models of PH and in human patient samples, and rescue of miR-204 reverses PH in rats. Additionally, miR-17 has been shown to be upregulated in HPH mice and monocrotaline-induced PH rats, and inhibition of miR-17 improved the PH phenotype of HPH mice and monocrotaline-induced PH rats. Several other miRNAs and mRNA targets have been identified to be involved in the development of PH. miRNAs have also been shown to regulate the expression of DNMTs and HDACs, adding on to the multitude of layers of regulation of gene expression.

A study by Wang et al published in the current issue of Circulation Research is a good example of the complex relationship between gene regulation and gene expression. The authors used a large animal model of neonatal calves with HPH and focused their studies on adventitial fibroblasts, the most abundant cell type in the adventitia. In neonatal HPH calves, adventitial fibroblasts have undergone phenotypic changes, resulting in increased proliferation, migration, and inflammation activities. Wang et al identified that miR-124 expression is decreased in adventitial fibroblasts isolated from calves and humans with severe PH. Interestingly, miR-124 expression remained unchanged in fibroblasts isolated from control rats and mice compared with experimental HPH rodent models, indicating that the function of miR-124 in PH is species-specific. miR-124 expression has also been shown to be decreased in several...
cancers, resulting in increased cell proliferation and migration, 2 phenotypic changes that also occur in PH. Using miR-124 inhibitors and mimics in human and bovine fibroblasts, Wang et al6 set out to determine whether miR-124 also regulates cell growth and migration in PH fibroblasts. The authors found that overexpression of miR-124 decreased the proliferation and migration rate of PH fibroblasts. Similarly, inhibition of miR-124 in normal control fibroblasts increased cell proliferation and migration, demonstrating that miR-124 regulates cell proliferation and migration in fibroblasts. These data suggest that loss of miR-124 in PH fibroblasts is a major contributor to the constitutively activated phenotype of the adventitial fibroblasts.

To elucidate the mechanism by which miR-124 regulates cell proliferation and migration, the authors screened transcript levels of cell cycle–related genes and found that miR-124 positively regulates Notch1, PTEN, FOXO3, p21/Cip1, and p27/Kip1, all of which were reduced in PH fibroblasts (Figure). Next, the authors sought to determine the target of miR-124 upstream of the cell-cycle regulator genes. Previous published data identified that miR-124 targets polypyrimidine tract–binding protein (PTBP)-1 and that PTBP1 affects Notch1 signaling.7,8 Therefore, Wang et al6 focused their studies on PTBP1, which is an abundantly expressed RNA-binding protein involved in several post-transcriptional regulation events, such as repressing RNA alternative splicing events, activation of translation driven by internal ribosomal entry sites, and RNA localization and stability. Wang et al6 determined that PTBP1 expression is increased in bovine and human PH fibroblasts as well as in pulmonary artery adventitia from humans and calves with severe PH compared with control fibroblasts and control tissue. Overexpression of miR-124 (using miR-124 mimics) in human and bovine PH fibroblasts inhibited PTBP1 expression, whereas inhibition of miR-124 (using anti-miR-124) in control cells increased PTBP1 expression. Additional luciferase assays were conducted to prove that PTBP1 is a direct target of miR-124. Furthermore, Wang et al6 showed that PTBP1 is upstream of Notch1 and negatively regulates the cell-cycle–related genes Notch1, PTEN, FOXO3, p21, and p27. The authors then elegantly designed an experiment to prove that PTBP1 expression is responsible for the increased proliferation rate of PH fibroblasts and that it is the action of miR-124 on PTBP1 that inhibits cell proliferation in PH fibroblasts. They designed a PTBP1 overexpression vector, which encodes the full-length mRNA without the 3′ untranslated region and hence the binding site for miR-124. Cotransfecting

Figure. Proposed mechanism contributing to the constitutively activated phenotype in pulmonary hypertensive fibroblasts. In healthy subjects, microRNA (miR)-124 is acetylated (AC) and transcribed, inhibiting HIF-2α, polypyrimidine tract–binding protein (PTBP)1, and MCP-1 gene expression, thereby maintaining a normal balance between cell growth, migration, and inflammation in the adventitia. In idiopathic pulmonary arterial hypertension patients and in hypoxia-induced pulmonary hypertensive calve fibroblasts, miR-124 expression is inhibited by class I histone deacetylases (HDACs). A decrease in miR-124 expression increases PTBP1, HIF-2α, and MCP-1. PTBP1 inhibits cell-cycle regulators Notch1 and PTEN. PTEN has been shown to be both a direct and indirect target of Notch1, resulting in different expression levels. The decrease in PTEN subsequently leads to an increase of AKT1/2 and decreased FOXO3/p27/p21, resulting in increased cell proliferation and migration. Additionally, PTEN inhibition leads to the activation of AKT/mTOR pathway, which increases cell growth by inhibiting 4EBP1/2 and activating p70S6K. The exact function of Notch1 inhibition in the diseased phenotype is not clear and requires further investigation. Upregulated expression of MCP-1 leads to β-integrin activation, causing migration and infiltration of monocytes and macrophages into the adventitia, thereby increasing inflammation and contributing to the activated fibroblast phenotype, which is characterized by enhanced cell proliferation, migration, and inflammation.
the miR-124–resistant PTBP1 vector with miR-124 mimics did not reduce PH fibroblast proliferation, demonstrating that miR-124 exerts its antiproliferative effects in PH fibroblasts by negatively regulating PTBP1. The novel findings in this study demonstrate that miR-124 is inhibited in PH fibroblasts, and this inhibition results in an increase of an RNA-binding protein, which posttranscriptionally regulates gene expression, adding to the complexity of gene regulation in PH.

Another interesting finding in this study is that Notch1 is downregulated in PH fibroblasts. The authors demonstrated that miR-124 inhibits PTBP1, resulting in increased Notch1 and PTEN expression, and suggested that in PH fibroblasts where miR-124 is inhibited, PTEN downregulation may be dependent on Notch1 inhibition (Figure). In the absence of Notch signaling, CBF-1 (also called RBP-Jκ) is bound to the promoter region of the target gene in a repressor complex. On activation of Notch signaling, the Notch intracellular domain binds to CBF-1, displacing the corepressors, and results in transcriptional activation of target genes. Based on previous studies that identified PTEN as a direct target of Notch1, the authors suggest that PTBP1-dependent inhibition of Notch1 results in decreased PTEN expression.9 However, several other studies suggest that PTEN is negatively regulated by HES1, a transcriptional repressor and major downstream target of Notch1.10,11 Notch signaling has been shown to be proproliferative in some cancers, and increased Notch3 expression has been demonstrated in PH patient samples and is required for the development of PH in murine models.12 The authors note that Notch activation is highly dependent on cellular and environmental context, and previous studies involving Notch3 have been performed in pulmonary arterial smooth muscle cells. It is, therefore, likely that Notch1 may have different functions in adventitial fibroblasts. The authors clearly demonstrated that a decrease in PTEN leads to the development of the activated phenotype of fibroblasts. This is consistent with recently established findings showing that the AKT/mTOR pathway, which is inhibited by PTEN, is activated in PH12 (Figure). It is then possible that PTBP1-mediated inhibition of Notch1 and PTEN are independent of each other, resulting in 2 different downstream pathways. Although the authors established the role of PTEN in PH fibroblasts, further studies need to be performed to determine how PTBP1 inhibits PTEN and Notch1 signaling and whether PTBP1 directly inhibits the expression of these proteins or whether PTBP1 regulates the expression of an intermediate protein, which in turn inhibits PTEN and Notch1.

Wang et al6 also demonstrated in their study that HIF-2α levels are elevated in PH fibroblasts compared with control fibroblasts and that HIF-1α levels remained unchanged. Furthermore, they demonstrated that miR-124 controls HIF-2α expression in these fibroblasts, thereby additionally contributing to increased cell proliferation. Most studies in the pulmonary circulation have focused on the upregulation of HIF-1α, but recent studies have shown that both isoforms have tissue-specific and cell-specific expression patterns and both contribute independently to cell growth.6,14 It would be interesting to determine whether HIF-1α is important for the initial switch to the activated fibroblast phenotype and which mechanisms control HIF-2α activation in PH fibroblasts, because the authors do not provide any evidence showing that HIF-2α is a direct target of miR-124. It is possible that HIF-2α is an indirect target of miR-124 and that other effectors controlled by miR-124 increase HIF-2α.

Increased inflammatory activity is another hallmark associated with the activated phenotype of PH fibroblasts, and Wang et al6 demonstrated that miR-124 is directly involved in this process. The authors show that PH fibroblasts from calves and humans have elevated levels of MCP-1, a chemotaxant for macrophages and monocytes. Transfection of miR-124 mimics in PH fibroblasts decreased MCP-1 transcript and protein levels, whereas transfection of anti-miR-124 in control fibroblasts increased MCP-1 expression. The application of luciferase assays further confirmed that MCP-1 is a direct target of miR-124, thereby directly contributing to the activated phenotype of PH fibroblasts.

Finally, the authors discovered the mechanism behind the decreased expression of miR-124. Because it has previously been shown that miR-124 itself is subject to epigenetic modifications, the authors investigated whether miR-124 is epigenetically silenced in PH fibroblasts. Interestingly, they discovered that treatment of PH fibroblasts with the HDAC inhibitors suberoylanilide hydroxamic acid, Apidicin, and OSU42, led to a significant increase of miR-124 while decreasing the direct targets of miR-124, PTBP1, and MCP-1 (Figure). This suggests that miR-124 expression is decreased in PH fibroblasts through epigenetic modifications, specifically through the removal of acetylation marks on histones, resulting in a more condensed chromatin structure and inhibition of transcription. Such an epigenetic event would explain the constitutively activated phenotype of PH fibroblasts, which has been shown to be reversible through the application of HDAC inhibitors. The authors discuss that similar observations have been made in synoviocytes from patients with rheumatoid arthritis as well as in cancer cells. Further studies are needed to determine whether HDAC inhibitors are able to prevent and reverse PH in HPH calves and whether other miRNAs or mRNAs are epigenetically modified in activated PH fibroblasts.

In summary, Wang et al6 provide compelling evidence that loss of miR-124 is directly involved in the development of activated PH fibroblasts, leading to PH. miR-124 expression is lost in PH fibroblasts from calves and idiopathic pulmonary arterial hypertension patients, causing increased expression of HIF-2α and the RNA-binding protein PTBP1, which in turn inhibits cell-cycle regulators Notch1/PTEN/FOXO3/p21 and p27, which ultimately leads to an increase in cell proliferation and migration (Figure). Additionally, inhibition of miR-124 increases MCP-1 expression, resulting in increased inflammation. The study by Wang et al6 reveals multiple targets for the development of therapeutic strategies (eg, HDAC inhibitors, PTBP1 inhibitors, or miR-124 mimics) for the treatment of patients with idiopathic pulmonary arterial hypertension.

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References


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