REVIEW

Targeting epigenetic mechanisms as an emerging therapeutic strategy in pulmonary hypertension disease

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Abstract

Pulmonary arterial hypertension (PAH) is a multifactorial cardiopulmonary disease characterized by an elevation of pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR), which can lead to right ventricular (RV) failure, multi-organ dysfunction, and ultimately to premature death. Despite the advances in molecular biology, the mechanisms underlying pulmonary hypertension (PH) remain unclear. Nowadays, there is no curative treatment for treating PH. Therefore, it is crucial to identify novel, specific therapeutic targets and to offer more effective treatments against the progression of PH. Increasing amounts of evidence suggest that epigenetic modification may play a critical role in the pathogenesis of PAH. In the presented paper, we provide an overview of the epigenetic mechanisms specifically, DNA methylation, histone acetylation, histone methylation, and ncRNAs. As the recent identification of new pharmacological drugs targeting these epigenetic mechanisms has opened new therapeutic avenues, we also discuss the importance of epigenetic-based therapies in the context of PH.

Key Words

- ▶ PAH
- ▶ epigenetics
- ▶ vascular remodelling

Background

Pulmonary arterial hypertension (PAH) is a vascular remodeling disease characterized by an increase of PVR and an elevation of PAP ≥ 25 mmHg at rest or 30 mmHg with exercise, leading to RV failure and ultimately death (1, 2, 3, 4). Various biological processes contribute to muscularization, obstruction, and constriction of distal pulmonary arteries (PAs) (5) (Fig. 1). It is now well established that vascular remodeling includes multiple cell types of the vessel wall such as pulmonary artery endothelial cells (PAEC) and pulmonary artery smooth muscle cells (PASMC). Vascular remodeling occurs as a result of pulmonary vascular endothelial dysfunction, PASMC proliferation and migration, medial hypertrophy,

inflammation, and thrombosis *in situ* leading to the formation of plexiform lesions, which progressively increase the vascular resistance in PAH (6). In addition, the imbalance between vasodilators and vasoconstrictors contributes to increase PVR and RV hypertrophy and ultimately to RV dysfunction (7).

The World Health Organization classifies PH into five groups based on the underlying etiology (8) (Fig. 2). Group 1 (PAH) refers to idiopathic or inherited PAH, drug or toxins induced, connective tissue and heart diseases, human immunodeficiency virus infection, portal hypertension, congenital tissue and heart disorders, schistosomiasis, chronic hemolytic anemia, pulmonary

Figure 1

Pathogenesis of pulmonary hypertension. PAH is associated with vascular and cardiac remodeling. In PAH, vascular remodeling is characterized by smooth muscle and endothelial cell dysfunction. Indeed, both cell types contribute to muscularization, obstruction, and constriction of distal pulmonary arteries (PAs) which progressively increase the vascular resistance to induce right ventricle (RV) hypertrophy and ultimately RV dysfunction. Representative pictures of the vascular remodeling in pulmonary arterioles have been previously published by Dr Hadri and collaborators (185). PAH, pulmonary arterial hypertension; PASM, pulmonary artery smooth muscle cells; PAEC, pulmonary artery endothelial cell.

capillary hemangiomatosis, pulmonary veno-occlusive disease, and PH of the newborn (8). The term PH is used for all the other groups of the classification: group 2, 3, 4, and 5. Left heart-associated diseases are associated with group 2 (8). Group 3 is associated with lung diseases such as chronic obstructive pulmonary disease and sleep apnea (8). Group 4 develops from thromboembolic diseases (8). Group 5 occurs in response to various disorders related

Figure 2

Updated classification of pulmonary hypertension. The World Health Organization classifies PH into five groups based on the underlying etiology. Group I includes PAH. Group II refers to PH from left sided heart disease. Group III refers to PH caused by chronic hypoxia lung disease. Group IV is associated with chronic blood clots, and Group V includes all other forms of PH associated with unclear multifactorial mechanisms such as sarcoidosis and hematological disorders.

to sarcoid and other rare disorders including blood and metabolic disorders (8). PAH is considered as a rare lung disease and is estimated to affect around 15–60 patients per million population.

Most of the current therapies for treating PH target mostly patients with WHO group I PAH, with a significant pre-capillary component (9). Specific current PAH treatment strategies target mainly three major pathways: Prostacyclin, NO signaling, and Endothelin (ET) receptor; and fall into four categories: Prostacyclin analogues and receptor agonists, Phosphodiesterase 5 inhibitors, ET-receptor antagonists (ERA), and cGMP activators (10). Prostacyclin (also called prostaglandin I₂ or PGI₂) is a prostaglandin member, which acts as a potent vasodilator and platelet-aggregating inhibitor. Clinical studies have shown that continuous administration of a synthetic prostacyclin analog, named Epoprostenol, increased the survival and exercise capacity in patients with WHO Group I PAH by sustaining the RV function but did not prevent the vascular remodeling (11). Endothelial nitric oxide (NO) is synthesized by endothelial nitric oxide synthase (eNOS) and acts as a powerful vasodilator of pulmonary vessels (12). Inhalation of NO has shown beneficial effects in pediatric cases of PAH. However, inhaled NO remains an expensive approach, and not all patients respond to this therapy. Moreover, rapid withdrawal of inhaled NO therapy can also have deleterious effects and drastically increase pulmonary pressure. Finally, ET-1 is secreted by the vascular endothelial cells and potentiates vasoconstriction and vascular remodeling in lungs (13). While the dual ET_A and ET_B receptors antagonist bosentan improved exercise capacity and cardiopulmonary hemodynamics in patients with PAH. However, high liver toxicity remains the most prevalent side effect of oral ERA. Increased hepatic aminotransferase levels have been reported in patients under ERA treatment. For example, sitaxentan was withdrawn from the market due to its acute and irreversible liver toxicity. As some ERAs have been associated with liver failure, its use highlighted the critical importance of monitoring hepatic function in patients under ERAs-based therapies.

Unfortunately, our understanding of the PH pathogenesis remains incomplete, and despite advances in molecular biology, there is no curative treatment as of today. Therefore, the identification and characterization of new therapeutic targets for treating PAH are necessary.

Epigenetic mechanisms

Epigenetics is defined as a heritable change occurring in the genome resulting in a change of gene expression

without affecting the DNA sequence (14). These heritable changes occur through specific epigenetic modifications called epigenetic marks and have a direct impact on the chromatin packaging, accessibility, and gene expression (15). In mammals, epigenetic mechanisms play a key role during the embryonic development, in genomic imprinting, X-chromosome inactivation, and repression of transposable elements, aging, and carcinogenesis (16, 17, 18). There are three major layers of epigenetic modifications: DNA methylation, histone modifications, and ncRNAs (Fig. 3). These epigenetic modifications can turn the genes 'off' and 'on'. The key mediators associated with DNA and histone modifications are divided into three main groups: writers, erasers, and readers (19). Writers refer to the enzymes that are directly responsible for modifying nucleotide or specific amino acid residue on histones. Erasers refer to a group of enzymes able to remove these marks and readers include all the proteins containing a specialized domain capable of recognizing specific epigenetic marks. ncRNAs also play a critical role in the gene expression as a part of the epigenetic machinery (20). They are RNA molecules transcribed from DNA to RNA, but not translated into proteins. They include miRNA, siRNA, piRNA, and lncRNA. In general, they regulate gene expression at the transcriptional and post-transcriptional level (21). miRNAs have been extensively investigated over the past few decades (22). They belong to a class of small noncoding RNAs of

~22 nt in length and are involved in the regulation of gene expression at the post-transcriptional level by degrading their target mRNAs and/or inhibiting their translation. There is increasing evidence showing that dysregulation of these epigenetic modifiers plays an important role in cancer and cardiovascular diseases (23, 24, 25, 26). For instance, abnormal hyper-methylation in the promoter region of the tumor suppressor genes has been described as one of the key mediators in tumorigenesis, while hypo-methylation is associated with chromosomal instability and loss of imprinting (27). Recent findings have indicated that targeting DNA or histone methylation/acetylation might be a promising strategy for epigenetics-based therapy (28, 29, 30, 31).

In this review, we focused on different epigenetic mechanisms and emphasized on how they affect various cellular processes. Over the past years, it has become increasingly recognized that epigenetics may play a critical role in the initiation and progression of PAH, as demonstrated by the increasing amount of reviews recently published in the field (32, 33, 34). Here we present the most comprehensive and up-to-date review on the role of epigenetic mechanisms in the context of PAH. This review not only summarized classical histone or DNA modifications, miRNAs or lncRNAs but also critically discussed some controversial findings in the literature. This review also highlights the relationship between ncRNAs and PSMCs contractility. Finally, as recent developments

Figure 3

Major epigenetic mechanisms. There are three major layers of epigenetic modifications: DNA methylation, histone modifications, and non-coding RNAs. These epigenetic marks regulate the expression of genes. DNA methylation is regulated by enzymes called DNA methyltransferases and DNA demethyltransferases. Histone post-translational modifications occur on the N-terminal tails of four core histones (H3, H4, H2A, and H2B). Among the histone modifications, acetylation and methylation remain the most studied mechanisms. ncRNAs also play a critical role in gene expression by degrading their target mRNAs and/or inhibiting their translation. ncRNAs include miRNA, siRNA, Piwi-interacting RNA (piRNA), and lncRNA.

and identification of new pharmacological drugs targeting these epigenetic tools in cancer have opened new avenues for the treatment of PAH, we have also highlighted the importance of epigenetic dysregulation in the progression of this disease.

DNA methylation

DNA methylation is a process by which methyl groups -CH₃ are added to the DNA molecules on the 5-carbon of the Cytosine ring to form 5-methylcytosine (35, 36). DNA methylation occurs, almost exclusively, at the Cytosines that are located next to Guanines and bond by a phosphate called CpG dinucleotide sites. Enzymes, known as DNA methyltransferases (DNMTs), tightly regulate this process (37). DNMTs are a family of highly-conserved proteins, including five members in mammals: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (38). DNMT1 was the first-identified DNMT and remains the most abundant isoform in adult cells. Another two mammalian members, DNMT3A and DNMT3B mediate the *de novo* methylation. The DNA methylation state is conserved during cell division and transmitted to daughter cells along with the DNA sequence (39). During the replication, DNMT1 is responsible for duplicating the methylation pattern from the parental strand to the daughter strand. This enzyme is described as a maintenance methyltransferase. DNMT1 interacts specifically with hemimethylated DNA. Hemimethylated DNA contains the methylation marks only on one strand. DNMT1 interacts specifically with hemimethylated DNA and contributes to the methylation of the daughter strand to restore fully methylated CpG dinucleotides. Thus, DNMT1 maintains the stability of this epigenetic mark throughout the different generations. Interestingly, recent studies have also associated DNMT1 with methylation of unmethylated human CpG islands in cancer cells. They reported that a majority of the *de novo* methyltransferase activity was mediated by DNMT1 in a gene-specific manner. DNMT2 enzymes do not methylate DNA but methylate small RNA. However, in 2003, Hermann *et al.* have demonstrated that DNMT2 may have a *de novo* CpG methylation activity (40).

DNA methylation at CpG sites represses gene transcription and silences gene expression (41, 42). Methylated CpGs recruit and interact with methylated-CpG-binding proteins known as MeCP1 and MeCP2. These proteins alter the transcription through their transcriptional repression domain. Alternatively, MeCP2 or MeCP1 proteins can form a complex along with other

molecular partners to condense the chromatin (43). Repression of gene transcription is also achieved by structural changes directly associated with the methyl group. Addition of a methyl group directly affects the interaction of the transcription factor on its DNA binding sites and will therefore block the transcription of the gene (44). Indeed, DNA methylation induces structural changes that physically obstruct the binding of transcriptional factors onto the gene promoter region (45). In addition to methyltransferase activity, the interaction of DNMT1 with DNMT1-associated proteins, E2F1 transcription factor, histone deacetylases (HDAC), and methyl-CpG binding proteins (MBD) make DNMT1 a central element of transcription repression complex (46). Yet, methylated DNA may recruit additional proteins known as methyl-CpG-binding domain proteins (MBDs), which can interact with other proteins such as HDAC and other chromatin remodeling proteins, resulting in compact and inactive chromatin (47). This relation between DNA methylation and chromatin arrangement may strongly influence disease development. For example, mutations in the MeCP2 gene caused Rett syndrome, a neuro-developmental disorder characterized by mental retardation; and MBD2 mediates the transcriptional repression of hypermethylated genes in cancer (48, 49).

In PAH, DNA methylation plays an important role in the regulation of superoxide dismutase 2 (SOD2) expression – a critical intra-mitochondrial antioxidant enzyme. SOD2 expression is significantly decreased in PSMCs in PAH patients and Fawn-hooded rats (FHR), a rat model which spontaneously develops PH (50, 51). Mechanistically, downregulation of SOD2 activates the hypoxia inducible factor (HIF-1 α) which increases the expression of the oxygen-sensitive, voltage-gated K⁺ channels (Kv1.5). These changes result in impaired oxygen sensing and reduced cytoplasmic and mitochondrial redox state (50). Inhibition of SOD2 expression by siRNA in normal Sprague–Dawley PSMC recapitulates the PAH phenotype. Conversely, SOD2 overexpression inhibits HIF-1 α and restores Kv1.5 expression in FHR PSMC. Using genomic bisulfite sequencing, Archer and colleagues (51) identified selective hypermethylation of CpG island in the promoter and an enhancer region of intron 2 of the SOD2 gene (51) (Fig. 4). Of particular interest, increased DNA methylation level correlates with increased expression of the DNMT1 and DNMT3B in FHR lungs and PSMCs. Most importantly, inhibition of DNA methylation by 5-aza-2'-deoxycytidine restores SOD2 expression, reduces proliferation, and promotes apoptosis in FHR PSMC. These data showed that epigenetic

Figure 4

Illustration of several underlying epigenetic mechanisms in pulmonary hypertension. DNA methylation plays an important role in the regulation of the expression of superoxide dismutase 2 (SOD2) and bone morphogenetic protein receptor 2 (BMPR2). Low SOD2 and BMPR2 expression is associated with increased proliferation and decreased apoptosis in hPASMC. Several histone deacetylases (HDAC1, 4, 5, and 6) are upregulated in PAH. HDACs inhibitors (VPA, SAHA, MGCD0103, MS-275, and Vorinostat) have been extensively investigated in multiple experimental models of PH. miRNAs (i.e. *miR-17/29*; *-21*; *20a*; *-135a*; *miR-124*; *miR-204*) target key signaling pathways (i.e. BMPR2 and PPAR γ) to influence the proliferation and migration of PASMCs and PAECs. The aforementioned miRNAs regulate several processes such as BMPR2 and PPAR γ and hypoxia and other pro-proliferative signaling pathways implicated in the development of PAH.

attenuation of mitochondrial SOD2 contributes to PAH development.

Heritable PAH represents (HPAH) approximately 6–10% of all PAH. Genetic studies have identified more than 450 mutations heterozygous germline mutations in the bone morphogenetic protein receptor type 2 (BMPR2) gene (52, 53, 54). Accumulated evidence pointed to BMPR2 mutations as being the most important predisposing factors for HPAH, with a penetrance of 27% (55), indicating that a BMPR2 mutation alone is not sufficient to develop HPAH (56). Moreover, Gimelbrant *et al.* (57) have reported a differential allelic expression using a genome-wide approach. These findings demonstrate that the two

copies of many genes are not simultaneously expressed at identical levels (57). This study also suggests that other mechanisms, such as epigenetic marks, may contribute to the diversity in individual cells and clonal descendants. These mechanisms may also affect gene expression of the WT allele and contribute to the early onset of the disease (58).

In 2017, Liu *et al.* have measured the methylation status of the BMPR2 promoter in DNA samples isolated from peripheral blood mononuclear cells (PBMCs) in 12 patients with HPAH using genomic bisulfite sequencing (59). They have demonstrated that the BMPR2 promoter is hypermethylated in patients with HPAH, resulting in

the downregulation of BMPR2 expression. Low BMPR2 expression in lung tissue and endothelial cells increases cell proliferation and promotes the development of PAH. In addition, their results suggest that specific allelic methylation of the WT strand in patients with HPAH may contribute to the penetrance of HPAH and trigger the early onset of disease. However, this study faces several limitations. Only a few patients were enrolled in this study, and the methylation status of the BMPR2 promoter was measured only in PBMCs. Lung tissues or PAEC from patients may be more relevant to decipher the development of HPAH.

On the contrary, a similar study analyzed the methylation status of the upstream region of the BMPR2 gene and its association with BMPR2 mutations in iPAH patients (60). The authors found no significant difference in the methylation of CpG islands of the BMPR2 promoter between iPAH patients and healthy control donors. In another study, Poussada *et al.* analyzed the methylation pattern of the BMPR2 promoter region in PAH patients and controls from genomic DNA obtained from the peripheral blood using methylation-specific PCR (61). Again, no evidence of methylation was detected in this region in patients and controls. Several differences across these studies may explain the heterogeneity of the previous results. First, only few patients were enrolled in these studies. Second, age, gender, stages of the disease, diversity of PAH group, and intra-individual variability may also affect the methylation pattern. Finally, different regions of the BMPR2 promoter or gene were investigated. Additionally, the genetic profile of the enrolled patients was not reported and may explain the lack of reproducibility. Nonetheless, recent technological advances for epigenetic profiling offer now new methodologies to further investigate the methylation pattern of the *BMPR2* gene.

In contrast to DNA methylation, DNA demethylation of a gene promoter is associated with transcriptional activation and gene expression. Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) catalyzes the conversion of the modified DNA base 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and initiates the first step of active DNA demethylation in mammals (62, 63). Colon, breast, prostate, and liver cancer showed a significant decrease of TET1 expression (64, 65, 66). Additionally, increasing TET1 expression levels in colon cancer cells decreased cell proliferation both *in vitro* and *in vivo* through demethylation of promoters of the WNT signaling pathway (67). Degradation of TET1 in hypoxia-induced EMT lung cancer cells decreased metastasis rates and cells (68). By initiating DNA demethylation,

TET1 activates gene transcription of tumor suppressor genes and plays a major role in the regulation of several cellular processes including proliferation, apoptosis, and migration (69, 70). Since PAH shows many similarities with cancer such as proliferation, resistance to apoptosis, and dysregulation of tumor suppressor genes, further investigations of DNA demethylation should be considered in future studies in PAH.

Histone post-translational modifications

Histones are highly-evolutionary conserved proteins and constitute the primary components of chromatin (71). The histone octamer is composed of dimers of each of the four central core histones: H2A, H2B, H3, and H4 (72). DNA is wrapped around the histone octamer and forms the nucleosome, which is defined as the repeating unit of chromatin. Histone post-translational modifications modify and affect gene expression patterns and can either activate or repress gene expression. Among the histone modifications, acetylation and methylation remain the most studied mechanisms so far (73). However, other histone marks such as glycosylation, ubiquitination, ADP-ribosylation, biotinylation, phosphorylation, and SUMOylation can also affect the chromatin compacting (73, 74). Histone acetylation and methylation occur on specific amino acids like Lysine (K) and/or Arginine (R) (75). These chemical processes result, respectively, in the addition of acetyl or methyl groups to a specific amino acid located on the histone.

Histone acetylation

Histone acetylation is found at the amino termini of the four core histones and is mediated by the histone acetyltransferases, commonly named HATs or KATs since only lysine residues (K) can be acetylated (76). There are 18 different types of HATs in mice and human. Among the most active HATs in mammals, we can mention cAMP response element-binding protein (CREB) and its binding protein (CBP), p300, p300/CREB-binding protein-associated factor (PCAF), and HIV Tat interactive 60-kDa protein (Tip60) (77, 78). The histone acetylation level is also regulated by histone deacetylases or HDACs. In contrast, HDACs remove acetyl groups from histone proteins and represses DNA transcription by tightening the chromatin (79). They are classified into four different groups: a- class I HDACs (HDAC 1, 2, 3, and 8) are exclusively expressed in nucleus; b- class II HDACs

(HDACs 4, 5, 6, 7, 9, and 10) are expressed in nucleus and cytoplasm; c- class III HDACs (Sirtuins 1–7) are located in the nucleus (Sirtuin1–6–7); and d- class IV (HDAC 11) are expressed both in the cytoplasm and nucleus. Histone acetylation is universally associated with transcriptional activation and is not mitotically heritable due to the rapid dynamics of histone acetylation and deacetylation. Interestingly, proteins expressing bromodomain, such as BRD4, recognize specific acetyl groups and may recruit specific proteins to form a molecular complex in order to finely modulate gene transcriptional activity (15). Recruitment of chromatin remodelers might affect the structure and level of condensation of the chromatin.

Over the past decade, many studies have shown that HDACs are major regulators in left ventricular (LV) cardiac hypertrophy in response to stress such as transverse aortic constriction, infusion of isoproterenol, DOCA salt, phenylephrine, or Endothelin-1 (80, 81). Conversely, HDAC inhibitors, such as the small compound Trichostatin A (TSA), prevent pressure overload-induced pathological cardiac hypertrophy and fibrosis and improve ventricular function. With respect to PAH, Zhao *et al.* have shown that HDAC1, HDAC4, and HDAC5 protein expression are significantly upregulated in human lung isolated from iPAH patients (82) (Fig. 4). Similar results were found in the lungs and RV of hypoxia-induced PH animal models. In this line, increased levels of histone acetylation on H3 and H4 at the promoter region of the endothelial nitric oxide synthase (eNOS) gene have been found in PAEC from Persistent PH of the Newborn (PPHN) (83). Recent studies illustrate the importance of HDACs in RV failure in several PH experimental models. For example, Cho *et al.* have assessed the therapeutic potential of Valproic Acid (VPA), a histone deacetylase inhibitor, in a rat model of monocrotaline (MCT)-induced PAH and in the rat pulmonary artery banding model (PAB) (84). VPA has been used in the clinic for decades as an anticonvulsant and mood stabilizer. The authors found that VPA administration in drinking water increased histone acetylation in the RV of VPA-treated rats and consequently reduced RV hypertrophy. Echocardiography analysis also revealed that VPA improved RV systolic function in MCT-treated animals (84). In 2015, another study demonstrated that pharmacological inhibition of class IIa HDACs using MC1568 (50 mg/kg; daily intraperitoneal administration) restored MEF2 activity in PAECs, as demonstrated by increased expression of its transcriptional targets such as miR-424 and miR-503 (85). Restoration of MEF2 activity resulted in decreased cell migration and proliferation and rescue of experimental pulmonary hypertension models.

These data indicate that HDACs can be considered as promising therapeutic targets in vascular remodeling disease.

Controversially, a separate study from Bogaard and colleagues investigated the effect of a broad-spectrum HDAC inhibitor TSA on RV function and remodeling using the PAB-induced PH model in rats (86). Surprisingly, their results showed that HDAC inhibition with TSA failed to prevent RV remodeling, but instead exacerbated RV fibrosis and promoted RV dysfunction. Their data also showed that isoform-selective HDAC inhibition might be better tolerated than non-selective pan-HDAC inhibition with compounds such as TSA. This was later confirmed by Cavasin *et al.* who tested two compounds that were highly selective for class I HDACs (HDAC1, HDAC2, and HDAC3): MGCD0103 and MS-275 in the hypoxia-induced PH rat model. MGCD0103 (10 mg/kg) and MS-275 (3 mg/kg) were administered via intraperitoneal injections for 3 weeks (Fig. 4). Both compounds successfully decreased class I HDAC catalytic activity in the RV and the lungs. Although the ability of HDAC inhibitors to reverse PAH was not explored in this study, they demonstrated that class I HDAC inhibitors impaired hypoxia-induced PH and improved hemodynamics including RV cardiac output, pulmonary vascular resistance PA acceleration time (PAAT) and velocity time integral (VTI), RV end-systolic pressure, and pulmonary artery pressure. Finally, Zhao *et al.* showed that VPA and SuberoylAnilide Hydroxamic Acid (SAHA), Vorinostat (HDAC class I, II, and IV inhibitor) reversed hypoxia-induced PH in rats and decreased hPASCs and hPAECs proliferation as well as inflammation *in vitro* (82, 87). As previously described, there is a controversy surrounding the role of HDAC in PH. PH is also characterized by the heterogeneity and severity of RV remodeling, which may be considered either as adaptive or maladaptive, compensated or decompensated. Therefore, inhibition of adaptive RV response by broad-spectrum HDAC inhibitors may be associated with detrimental outcomes while inhibition of maladaptive RV may be beneficial. It is essential to define the type of RV remodeling and function in PAH patients. This is well illustrated by the electrocardiographic abnormalities, pulmonary embolism, and thrombocytopenia that have been reported after the use of broad-spectrum HDAC inhibitors in patients with lymphoma (88, 89). The use of selective HDAC inhibitors may be an alternative approach to limit side effects on off-target genes. For example, Kim *et al.* have demonstrated that selective HDAC IIa inhibition protected against RV dilatation (85). Similarly, Cavasin and colleagues showed that selective HDAC I attenuated

RV hypertrophy in the chronic hypoxia model of PH (90). The use of different PAH models (Sugen-Hypoxia, MCT, and PAB) and protocols, duration of treatment, frequency of treatment, and selectivity of HDAC inhibitors may explain this controversy surrounding the use of HDAC inhibitors as a therapeutic approach.

Contrary to nuclear HDACs implicated in epigenetic regulation of transcription, HDAC6 is exclusively localized in the cytoplasm and displays a deacetylation activity toward 'non-histone' proteins (91). Several studies have demonstrated that HDAC6 is overexpressed in many cancers such as hepatocellular carcinoma, glioblastoma, and prostate cancer. Domain-selective small-molecule inhibitor of HDAC6 inhibits cell growth, cancer progression, and re-sensitizes transformed cells to anticancer agents (92). Boucherat and colleagues reported increased expression levels of HDAC6 in lungs and distal PAs from PAH patients and experimental animal models of PAH induced by MCT and Sugén/Hypoxia (93). They found that HDAC6 was overexpressed only at the protein level in PAH-PASMCs and PAH-PAECs (compared to control cells) as well as RV from PAH patients or Su/Hx and MCT rats. Blockade of HDAC6 activity by either Tubastatin A (TubA) or ACY-775 or siRNA against HDAC6 significantly decreased PAH-PASMC proliferation, resistance to apoptosis, and migration. Pharmacological inhibition of HDAC6 improved pulmonary hypertension in the Sugén/Hypoxia and MCT rat model as demonstrated by decreased RV systolic pressure (RVSP), mean PA pressure (mPAP), cardiac output (CO), total pulmonary vascular resistance (TPR), RV hypertrophy, vascular remodeling, and restoration of the proliferation/apoptosis balance (93). Using the genetically engineered WT (*Hdac6*^{+/+}) and *Hdac6*-knockout (*Hdac6*^{-/-}) mice models, they showed that the loss of *Hdac6* conferred protection against chronic hypoxia-induced PH (93).

Finally, epigenetic readers that recognize histone modification patterns and bind to acetylated lysine residues of histone tails, may also contribute to the development of PAH. This is well illustrated with the BRD4 bromodomain protein, which acts as a cofactor, facilitating transcriptional activation of target genes. BRD4 is upregulated in lungs, distal PAs, and PASMCs of patients with PAH compared with healthy donors (94). Mechanistically, *microRNA-204* upregulated BRD4 expression in PAH. Pharmacological inhibition of BRD4 with JQ1 increased p21 expression and induced cycle arrest in PAH-PASMCs. This effect of BRD4 inhibition was accompanied by decreased expression of several oncogenes such as nuclear factor of activated T cells

(NFAT), B-cell lymphoma 2 (BCL2), and Survivin, all known to be overexpressed in PAH. Inhibition of this oncogenic signature decreased proliferation and increased apoptosis of PAH-PASMC. Finally, blockade of BRD4 activity by either nebulization of siRNA against BRD4 or JQ1 reversed established PAH in the Sugén/hypoxia rat model *in vivo* (94).

Histone methylation

In addition to DNA methylation, histone methylation also regulates the gene expression pattern (95). Histone methylation is a process by which methyl groups are transferred to specific amino acids of histone proteins. Methylation of histones tightly influences gene expression. Gene activation or inhibition status depends on which amino acids in the histones are methylated and how many methyl groups are attached (Fig. 3). This is the only post-translational modification that can add up multiple methyl groups onto Lysine (K) residues resulting in mono (me1), di (me2), and tri-methylated (me3) states (96). As acetylated lysines served as docking sites of proteins expressing a Bromodomain, methylated lysines can interact with several different domain types including Chromodomains, Malignant Brain Tumor (MBT) domain, plant homeodomain (PHD) domains, or Tudor domains. Binding of chromodomain-containing proteins has different functional consequences depending on the docking sites. For example, the chromodomain of CHD1 binds to the H3K4 methylation, heterochromatin protein 1 (HP1) chromodomain interacts with the H3K9 methylation, and CBX2 chromodomain recognizes and binds to the H3K27 methylated (97). CHD1, which binds to the active epigenetic methylation H3K4, belongs to the family of ATP-dependent chromatin remodeler (98). CHD1 stimulates DNA unwrapping and makes the nucleosomes more broad. Histone lysine methyltransferases catalyze the transfer of methyl group from adenosyl-methionine. Due to the complexity and heterogeneity of this process, we have highlighted only several well-characterized modifications. These include the mono- and di-methylation of lysine residues 4 and 9 on histone H3 (H3K4me2/3 and H3K9me2/3, respectively) and the tri-methylation of the lysine residues 27 on the histone H3 (H3K27me3).

H3K4 methylation is restricted to the promoter and enhancer region of genes and is not able to spread over the entire gene (99). H3K4 methylation is associated with an increase of the transcriptional activity while H3K9 methylation represses gene expression and is

mostly associated with constitutive heterochromatin (100). Similarly to H3K9me2/3, H3K27 methylation is a repressive methyl mark and can spread over the entire gene locus (101). Heterochromatin is associated with a different type of histone methylation like the H3K9 or H3K27 methylation and is found in regions with inactive gene transcription. A specific class of H3K9 methyltransferases, including SUV39H1/KMT1A, SUV39H2/KMT1B, SETDB1/KMT1E, dimeric G9a/KMT1C-GLP (G9a-like protein)/KMT1D, and PRDM family, mediates the methylation of H3K9. Similarly, H3K27 or H3K4 methylation is mediated by other lysine methyltransferases such as EZH2 and SET1 proteins, which can specifically recognize the H3K27 and H3K4 residues (102). Methyl groups can also be removed from the histone by specific lysine demethylases, which are known as KDMs and express specific activity toward the methylated H3K9, K4, and K27.

Polycomb group proteins are important chromatin-associated factors contributing to the maintenance of transcriptional repression (103). The Polycomb Repressive Complex 2 (PRC2) is one of the most- and best-investigated epigenetic complexes as of today. The PRC2 complex has a very important role in silencing gene expression (104). PRC2-modified histone tails act as docking sites for other chromatin proteins, which can alter the chromatin packaging or recruit other molecular partners (105). EZH2 is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) and promotes transcriptional gene silencing by trimethylation at lysine 27 of Histone H3 (H3K27me3). EZH2 overexpression has been shown to be important for tumor growth, metastasis and correlates with poor prognosis in several cancer types including melanoma, brain, breast, lung, liver, kidney, prostate, ovarian, gastric, and pancreatic cancer and Diffuse Large B-Cell lymphoma (106, 107). Of particular interest, Aljubran *et al.* analyzed the role of EZH2 in PSMCs isolated from control and hypoxia-induced PAH mouse model (108). Their data showed that EZH2 overexpression induced proliferation and migration of hPSMCs while decreasing apoptosis (Fig. 4). In 2018, Shi and collaborators have demonstrated that pharmacological inhibition of EZH2 with EPZ005687 protects against TAC-induced PAH by preventing ROS production in the lung (109). At the molecular level, EPZ005687 significantly decreased the H3K27me3 enrichment in the promoter region of SOD1, thereby repressing SOD1 transcription and expression.

Another study has focused on the role of the H3K27me3 mark in pulmonary vascular endothelial cell proliferation, apoptosis, and inflammatory response in PAH (110). It is shown that pharmacological inhibition

of JmjC domain-containing protein JMJD3/KDM6B by GSK-J4 significantly decreased proliferation, potentiated apoptosis, and reduced TNF alpha-induced IL-6 release in a concentration-dependent manner in pulmonary vascular endothelial cells. This study strongly suggests that JMJD3/KDM6B may regulate the pulmonary vascular endothelial cell activation in an H3K27me3-dependent manner in PH. Yet, Chen and collaborators investigated the role of the transcriptional modulator megakaryocytic leukemia 1 (MKL1), also known as myocardin-related transcription factor A (MRTF-A) in hypoxia-induced PAH. Loss of MKL1/MRTF-A protected against the development of hypoxia-induced PH (111, 112). Specifically, it is shown that MKL1 coordinates epigenetic alterations on cell adhesion molecules (CAMs) promoters in human umbilical vein endothelial cells. Indeed, MKL1/MRTF-A mediates the recruitment of the H3K4 complex to the CAM promoters (113). In addition, silencing of ASH2 and WDR5, two key components of the histone H3K4 methyltransferase complex, reduced hypoxia-induced PH in mice (113).

Non-coding RNA (ncRNA)

Another layer of epigenetic regulation of gene expression, not taking place on DNA, is ncRNAs (Fig. 3). There are several groups of ncRNA clustered based on their function or length as previously mentioned, but here, we will only review the contribution of micro RNA (miRNA) and long ncRNA (lncRNA) to the development of PAH.

micro RNA

miRNAs are short non-coding transcripts with the length 21–25 nucleotides. Single miRNA can regulate the expression of multiple genes and inversely the expression of a single gene can be regulated by multiple miRNAs (114). miRNAs are transcribed as pre-miRNAs by RNA polymerase II from either intronic or exonic regions (115). Pre-miRNAs are several hundreds of bases long and contain 5' m7G cap and 3' poly-A tail (116) and are then processed in the nucleus by Drosha to yield 70 nt transcript in a form of hairpin (116), which is exported to cytoplasm (117). The pre-miRNA is then spliced by DICER to produce double-strand miRNA, then separated and loaded onto the RNA-induced silencing complex (RISC) (118). miRNAs within the RISC complex then regulate cellular processes such as proliferation, differentiation, or apoptosis, and therefore miRNAs are also implicated in

the pathogenesis of PAH (119). miRNAs play an important regulatory role in the proliferation and migration of both PSMCs and PAECs. These *miRNAs* regulate several pathways such as BMPR2, PPAR γ , Hypoxia, and other pro-proliferative signaling pathways implicated in the development of PAH.

One of the key signaling pathways involved in the regulation of many miRNAs is the hypoxia-regulated pathways through the hypoxia inducible factor (HIF). It has been shown that hypoxia stimulates the expression of *miR-1*; -9; -17/92; -21; -27a; -27b; -138; -190; -199-5p; -210; -322; -361-5p; and -23a (120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132). Expression of most of these miRNAs is upregulated by the action of HIF-1 α . Several of these miRNAs affect multiple signaling modules involved in the development of PAH such as the BMP/SMAD signaling or expression of transmembrane ion channels. Among these miRNAs, *miR-17/92* downregulates prolyl hydroxylase 2 which leads to HIF-1 α stabilization and stimulation of proliferation of PSMC after hypoxia (133). *miR-17/92* also targets proteins such as BMPR2, p21, PTEN, MFN2, and promotes cell survival in PSMCs (122, 134, 135) (Fig. 4). Moreover, *miR-17/92* is stimulated by inflammation and activates NF-KB, thus upregulating the retinoblastoma protein (136). Similar to *miR-17/92*, *miR-138* exerts pleiotropic effects and stimulates cell survival in PSMCs via AKT and Mst1 kinase (137). Additionally, it exerts a profound effect on nitric oxide (NO) production. Indeed, *miR-138* targets S100A1 to promote downregulation of eNOS activation and subsequent inhibition of NO production (126, 138) (Fig. 4). *miR-27b* and *miR-195a-5p* also modulate NO production. *miR-199a-5p* inhibits NO production by targeting SMAD3 in PSMCs and PAECs (128). *miR-27b* targets PPAR γ , thus altering the stability of eNOS/HSP90 complex important for NO production (125).

PPAR γ is another key player in the miRNA network – many miRNAs target PPAR γ and vice versa PPAR γ downregulates several *miRNAs*. *miR-21* is one of the miRNAs that is negatively regulated by PPAR γ . Downstream, multiple targets of *miR-21* have been identified. Indeed, *miRNA-21* downregulates BMPR2, RhoB, PDCD4, SPRY2, PPAR α , PTEN or SATB1, leading to the stimulation of PSMCs and PAECs proliferation, migration and contraction and therefore to the development of PAH (123, 139, 140, 141). Moreover, PPAR γ also downregulates *miR-27a* or *miR-130/301* (124, 142, 143). One of the hallmarks of PAH is the alteration of the bone morphogenetic proteins/SMAD signaling (144). A large body of evidence indicates that miRNAs play an

important role in regulating BMP/SMAD signaling. One of the proteins, whose expression is tightly regulated by miRNAs, is BMPR2. Indeed, *miR-17/29*; -21; -20a; -135a; -23a targets BMPR2 and regulates proliferation and differentiation of PSMC and PAECs (122, 123, 132, 145, 146). Additionally, *miR-322* targets other members of the cascade, namely BMPR1 and Smad5, thus stimulating the proliferation of PSMCs (130).

The development of PAH is also characterized by increased contractility of PSMCs (147, 148). Contractility is dependent on the activation of the Rho/ROCK pathway, which stimulates actomyosin cytoskeleton and the expression of transmembrane transporters (149). It has been shown that miRNA targets several unrelated transporters. One of them is the ATP-binding cassette transporter A1 (ABCA1). ABCA1 is a c-AMP dependent transporter exporting intracellular cholesterol and phospholipids (150). Two miRNAs – *miR-143/145* and *miR-361-5p* – have been shown to downregulate the expression of ABCA1 in PSMCs in hypoxic conditions, therefore stimulating proliferation and survival (131, 151). Moreover, voltage-dependent potassium channels are also targeted by miRNAs – KCNA5 as a target of *miR-1*, KCNQ5 as a target of *miR-190* and TASK1 as a target of *miR-138* (120, 127, 152). Downregulation of these channels alters the membrane potential of PSMCs and stimulates Ca²⁺ influx, thus stimulating the contractility of PSMCs (153).

On the other side, multiple miRNAs inhibit PSMCs and PAECs proliferation and are downregulated in hypoxic and PAH conditions. One of the most studied is *miR-124*. It has been shown that *miR-124* is downregulated after hypoxia in PSMCs (Fig. 4). *miR-124* inhibits the proliferation of PSMCs by inhibiting NFAT and Notch-FoxO3 pathways via stimulation of PTBP1 expression (154, 155). Similarly, in pulmonary adventitial fibroblasts, *miR-124* normalizes the ratio of PKM2/PKM1 via PTBP1, consequently inhibiting the proliferation and the migration of the fibroblast (156). *miR-204* is also downregulated in hypoxia and targets SHP2 phosphatase, thus inhibiting NFAT and Src pathways, PSMCs proliferation and survival (157) (Fig. 4). Moreover, *miR-204* downregulates RUNX2 and inhibits HIF1 α , which inhibits the proliferation and promotes the apoptosis in PSMCs under physiological conditions (158).

Many miRNAs targeting the BMP/Smad signaling are downregulated in hypoxic and PAH conditions. *miR-140-5p* targets the SMAD-specific E3 ubiquitin-protein ligase 1 (SMURF1), which leads to the stimulation of BMP/SMAD signaling and inhibition of PSMCs proliferation (159). Several miRNAs target tyrosine kinase receptors,

resulting in the activation of the downstream signaling and inhibition of proliferation and survival. For example *miR-193-3p* and *miRNA-328* downregulate the IGF1R receptor (160, 161). *miR-424/503* and *miR-339* inhibit FGF-stimulated signaling via targeting FGF2, FGFR1, or FRS2 (162, 163). PDGFRA expression is downregulated by *miR-34a* in PSMCs (164). Finally, contractility of PSMCs is inhibited by several miRNAs. Especially *miR-223* is especially important in the regulation of actomyosin contractility by targeting upstream regulators such as ITGB3, RhoB, and MLC2 (165, 166). Furthermore, calcium levels are important for contractility and are regulated by CaSR, which is targeted by two miRNAs – *miR-429* and *miR-424-5p* (167). An interesting connection between hypoxia, PAH, miRNAs, and DNA damage-stimulated pathways is illustrated by *miR-223*. Hypoxia-stimulated HIF1 α downregulates *miR-223*, which leads to upregulation of *miR-223* target – PARP-1. Increased PARP-1 levels subsequently stimulate PSMCs proliferation and resistance to apoptosis, promoting the development of PAH (168).

Long non-coding RNAs

Long ncRNAs are RNA molecules which are longer than 200 nt but do not encode for any specific and functional protein (169). Based on the DNA element that is being transcribed from, they are classified into sense lncRNAs, antisense lncRNAs, bidirectional lncRNAs, intronic lncRNAs, and intergenic lncRNAs (170). lncRNAs are usually transcribed by RNA polymerase II or III and processed similarly to mRNAs – spliced, 3'-polyadenylated, or 5'-capped (171, 172). lncRNAs can have multiple functions: signal lncRNAs are expressed in specific cellular compartments or at specific times and usually regulate the transcription of other genes. Decoy lncRNAs are used to sequester regulatory proteins or miRNAs to regulate gene expression at transcriptional or translational levels. Guide lncRNAs can recruit transcription activators or repressors to specific genome locations. Scaffold lncRNAs act as a platform for the assembly of various ribonucleoprotein complexes to regulate the chromatin landscape. The most recently discovered class is a circular lncRNAs and acts as transcriptional regulators (173).

Because lncRNAs play an important role in gene expression, they are prime candidates to be involved in the development of PAH. Similar to miRNA regulation, hypoxia is an important stimulus altering the expression of lncRNAs. One of the important pleiotropic lncRNA stimulated by hypoxia and shared with cancer cells is

MALAT1. This lncRNA controls the phenotypic switch and stimulates the proliferation of PAECs (174). *H19* lncRNA is stimulated by hypoxia via increased PDGF-BB expression (Fig. 4). *H19* then increases AT₁R expression via sponging *let-7b* miRNA, which targets AT₁R. Increased AT₁R expression then leads to the stimulation of PSMCs proliferation (175). Similarly, hypoxia promotes the expression of lncRNA *PAXIP1-AS1*. *PAXIP1-AS1* alters the focal adhesion signaling and upregulates paxillin, which leads to increased proliferation and migration of PSMCs (176). Another hypoxia-induced lncRNA is *UCA1* (urothelial carcinoma-associated-1). The mechanism of *UCA1* action is sequestering ING5 from hnRNP I, which induces the stimulation of PSMCs proliferation and apoptosis resistance (177). Finally, hypoxia also induced the expression of the lncRNA *Hoxaas3*, located within the HoxA gene cluster. lncRNA *Hoxaas3* was shown to stimulate the expression of HoxA3, which increases the expression of Cyclins A, D, and E, thus stimulating the cell cycle progression and proliferation (178).

Several other lncRNAs are downregulated under hypoxic stress. One of them is lncRNA *MEG3* and has been shown to be downregulated in the lungs of patients with PAH (Fig. 4). Inhibition of *MEG3* in PSMCs results in the induction of PCNA, cyclin A, and cyclin E expression, thus leading to faster progression through the cell cycle (179). It has been shown that *MEG3* is important for p53 activation and therefore cell cycle inhibition (179). Moreover, hypoxia-mediated downregulation of *MEG3* promotes *miR-21* expression and downregulates PTEN expression, leading to the stimulation of PSMC proliferation (180). lncRNA-p21, a transcriptional target of p53, feeds back to enhance p53 transcriptional activity by physically interacting with the E3 ubiquitin-protein ligase called mouse double minute 2 (MDM2). The physical interaction of lncRNA-p21 with MDM2 releases MDM2 repression of p53, which then allows p53 to interact with p300 and to bind the promoters/enhancers of its target genes (181) (Fig. 4).

Hypoxia also represses the expression of lncRNAs *TCONS_00034812* and *CASC2*. *TCONS_00034812* stimulates the expression of the Stox1 transcription factor, which inhibits the activity of major effectors of the MAPK pathway – ERK, JNK, and p38. After hypoxia, downregulation of *TCONS_00034812* results in the stimulation of MAPK pathway activity, PSMCs proliferation, and apoptosis resistance (182). lncRNA *CASC2* inhibits proliferation, migration, and stimulates apoptosis of PSMCs. It has been shown that *CASC2* inhibits the expression of phenotype switch-related

marker alpha smooth muscle actin (α -SMA) (183) in PASMCs.

Finally, it is now well-known that altered PDGF signaling contributes to the PAH pathogenesis. However, the underlying mechanism remains not fully understood. Chen and colleagues have shown that PDGF-BB exerts its function partially by repressing the lncRNA *LnRPT*. *LnRPT* inhibits the proliferation of PASMCs by decreasing the expression of Cyclin A and by inhibiting the Notch signaling via the downregulation of Notch3 and Jag1 (184).

Conclusion

Considerable progresses have been made in our understanding of gene expression regulation. In the past decades, numerous studies have demonstrated the critical role of epigenetics in cancer and other diseases. New advances in epigenetic drugs have led to the development of new pharmacological drugs for treating several cancers and are now under clinical investigation in phases II and III. Since PAH shows many common features with cancer, new opportunities to investigate the current therapeutic strategies used in cancer should be considered to treat PAH. Indeed, increasing evidence have identified DNA methylation, histone post-translational modifications (acetylation, methylation), and ncRNAs as critical in the development, severity, and progression of PAH. Although several unknown epigenetic mechanisms in PAH are yet to be elucidated, strong evidence suggests that targeting these epigenetic mechanisms may represent an emerging and very promising therapeutic strategy in PAH. Moreover, recent technological advances in epigenetic profiling may help to further investigate and decipher the underlying molecular mechanisms in PAH to identify new therapeutic targets.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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