Review Article

Epigenetic mechanisms of pulmonary hypertension

Gene H. Kim, John J. Ryan, Glenn Marsboom, and Stephen L. Archer

Department of Medicine, University of Chicago, Chicago, Illinois, USA

ABSTRACT

Epigenetics refers to changes in phenotype and gene expression that occur without alterations in DNA sequence. Epigenetic modifications of the genome can be acquired de novo and are potentially heritable. This review focuses on the emerging recognition of a role for epigenetics in the development of pulmonary arterial hypertension (PAH). Lessons learned from the epigenetics in cancer and neurodevelopmental diseases, such as Prader-Willi syndrome, can be applied to PAH. These syndromes suggest that there is substantial genetic and epigenetic cross-talk such that a single phenotype can result from a genetic cause, an epigenetic cause, or a combined abnormality. There are three major mechanisms of epigenetic regulation, including methylation of CpG islands, mediated by DNA methyltransferases, modification of histone proteins, and microRNAs. There is substantial interaction between these epigenetic mechanisms. Recently, it was discovered that there may be an epigenetic component to PAH. In PAH there is downregulation of superoxide dismutase 2 (SOD2) and normoxic activation of hypoxia inducible factor (HIF-1α). This decrease in SOD2 results from methylation of CpG islands in SOD2 by lung DNA methyltransferases. The partial silencing of SOD2 alters redox signaling, activates HIF-1α) and leads to excessive cell proliferation. The same hyperproliferative epigenetic abnormality occurs in cancer. These epigenetic abnormalities can be therapeutically reversed. Epigenetic mechanisms may mediate gene-environment interactions in PAH and explain the great variability in susceptibility to stimuli such as anorexigens, virus, and shunts. Epigenetics may be relevant to the female predisposition to PAH and the incomplete penetrance of BMPR2 mutations in familial PAH.

Key Words: CpG islands, DNA methyl transferases, histone acetylation, small inhibitor RNA, superoxide dismutase 2

INTRODUCTION

This review focuses on the emerging recognition of a role for epigenetics in the development of pulmonary arterial hypertension (PAH). Epigenetics refers to changes in phenotype mediated by altered gene expression, which are not the result of alterations in DNA sequence. Epigenetic mechanisms can be acquired and/or heritable and constitute a means by which gene-environment interactions occur. To date there are few examples of epigenetics contributing to PAH;[1] however, there are many unexplained observations in PAH that may have an epigenetic component. For example, although most cases of familial PAH are associated with mutations of the bone morphogenetic protein receptor (BMPR2),[2,3] it is still unclear why only 20% of BMPR2 carriers develop PAH during their lifetime. Typically this reduced penetrance is attributed to gender, genetic modifiers and/or the environment.

Address correspondence to:
Prof. Stephen L. Archer
Section of Cardiology, Department of Medicine,
University of Chicago, 5841 South Maryland Avenue, (MC6080),
Chicago, Illinois, 60637.
Phone: 773/702-1919, Fax: 773/702-1385
Email: sarcher@medicine.bsd.uchicago.edu

While genetic studies search for modifier genes that may be important determinants of penetrance,[4] experiments in fawn-hooded rats, a strain with heritable, spontaneously developing PAH, suggest an important role for environmental factors in determining the time to onset and severity of PAH. Exposing fawn-hooded pups to small reductions in inspired oxygen that does not affect other rat strains leads to a more rapid onset of PAH.[5] Environmental factors also determine the prevalence of pulmonary hypertension in broiler fowl (a strain of chicken intercrossed for meat production).[6] Cold temperatures and differences in diet and increased rates of weight gain have been described to worsen the onset of PAH in broiler fowl.[7] Such environmental factors could predispose to PAH by as yet undiscovered epigenetic mechanisms.

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In this review, we first give a general overview of the exponential increase in scientific articles in epigenetic research. We then review the epigenetic mechanisms of gene regulation, with examples from the fields of oncology and neurodevelopment. Finally, we highlight recent publications that study epigenetics in pulmonary hypertension.

Trends in biomedical epigenetic research publications

There has been a recent, near exponential increase in the number of research articles that discuss epigenetics (Fig. 1a). Since 1997, the first year with more than 100 published research articles with either "epigenetic" or "epigenetics" in the title or abstract, publication numbers have increased annually, with more than 2,000 in 2010. Based on the publications to date in 2011 (2,099 as of 18 August 2011), this trend continues. When reviews and editorials are included in the analysis, it becomes obvious that the scientific community is interested in epigenetics. Arguably there is more interest than information, with review articles (such as this one) accounting for approximately one-third of all publications with the key words "epigenetic" or "epigenetics" in their title or abstract (Fig. 1b).

Epigenetics research is currently focused on cancer (70% of published research articles in 2010) with some interest in stem cells and neuronal disease and much less attention to cardiopulmonary diseases (including asthma) (Fig. 1c).

Mechanisms of epigenetic regulation

There is a large body of evidence that epigenetic modifications are involved in the pathological mechanisms of many diseases including cancer,[8,9] asthma,[10] and several human syndromic disorders, such as Prader-Willi, Angelman, Silver-Russell, and Beckwith-Wiedermann syndromes.[11-13]

Relevant to PAH, many of these conditions have both genetic and epigenetic mechanisms which can independently produce the same phenotype. Prader-Willi syndrome (hypotonia, insatiable appetite, obesity, developmental impairment) and Angelmann's syndrome are neurobehavioral syndromes resulting from paternal or maternal imprinted genes or genetic deletions within the chromosomal 15q11-q13 region.[14] While deletions of chromosome 15q11-q13 can give rise to Prader-Willi syndrome, so too can uniparental disomy and imprinting mutations.[14] The epigenetic or "imprinting" disorders create a syndrome that is phenotypically indistinguishable from genetic deletion. However, in patients where uniparental disomy (two copies of a chromosome or segment thereof from one parent) causes Prader-Willi syndrome, there is no DNA sequence abnormality.[11] Epigenetic and genetic mechanisms targeting the same gene can yield a similar phenotype. As an example, there is an imprinting center (epigenetic target) near the promoter for the small nuclear ribonucleoprotein N (SNRPN) gene. This is a gene which, if deleted, causes Prader-Willi syndrome.[11] In a further variation, Beaudet et al. have pointed out that there are Prader-Willi patients with imprinting defects in whom a small deletion in the imprinting center results in a larger epigenetic defect. [11] Thus Prader-Willi syndrome provides an important precedent when one considers the interplay between genetic and epigenetic causes of PAH. It demonstrates that an abnormality which can be genetically encoded can also be epigenetically mediated and that either or both mechanisms may be at play in a given patient.

Epigenetic mechanisms of altered gene expression

Epigenetic modifications provide a mechanism that allows the stable propagation of gene expression states from one generation of cells to the next.[15] Mechanisms of epigenetic

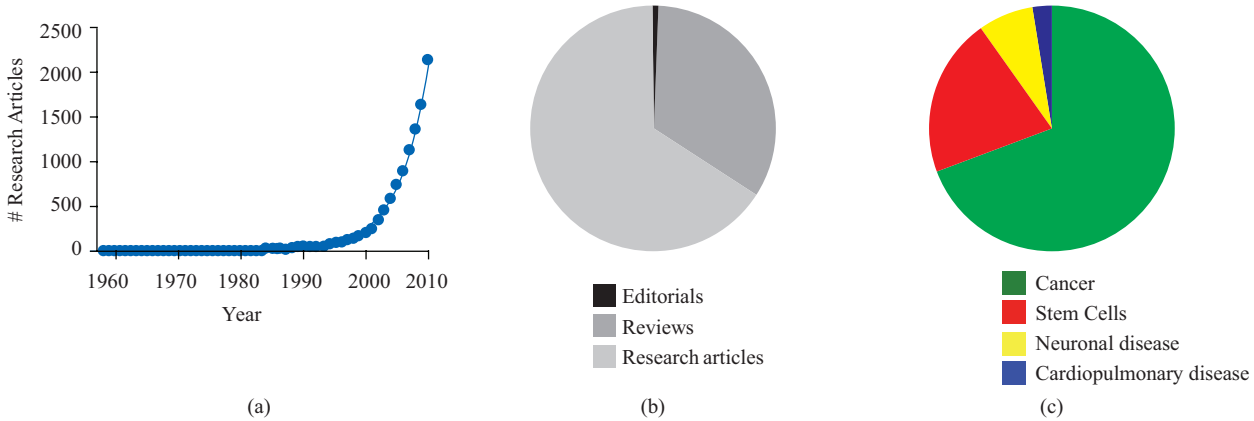

Figure 1: Trends in biomedical epigenetic research publications. PubMed citations containing the words epigenetic or epigenetics in the title or abstract were counted (www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed). (a) Research articles, excluding review articles and editorials, were counted for each year up to 2010. (b) All publications covering epigenetic(s) are categorized to editorials, reviews and research articles. (c) Distribution of research articles published in 2010 for cancer, stem cells and their differentiation, neuronal diseases and cardiopulmonary diseases including asthma.

regulation include DNA methylation, histone modification, and RNA interference represented in (Fig. 2).[16]

DNA methylation

In the context of epigenetics, DNA methylation refers to the covalent attachment of a methyl group to the C5 position of cytosine residues in CpG dinucleotide sequences that are called CpG islands. DNA islands are often in the promoter or enhancer regions of genes and methylation of these sites can alter gene transcription. DNA methylation is involved in normal cellular control of gene expression and is dynamically regulated.[17] However, changes in DNA methylation are also relevant to disease. Hypermethylation of CpG islands can lead to silencing of tumor-suppressor genes, promoting the development of cancer;[9,18] conversely, hypomethylation can lead to gene overexpression, which may also promote cancer.[19-23] CpG methylation is an important mechanism to ensure the repression of transcription of repeat elements and it plays a crucial role in imprinting and X-chromosome inactivation.[24] Transcriptional gene silencing by CpG methylation also restricts the expression of some tissue-specific genes during development and differentiation by repressing them in non-expressing cells, thus providing another layer of important temporal-spatial control of expression. Throughout the genome, areas of increased CpG dinucleotides, or CpG islands, in the promoter regions of many genes have been intensively studied for changes in methylation status.[25] Generally, it has been found that hypermethylation of CpG islands is associated with epigenetic silencing.[26] CpG methylation can suppress transcription by several mechanisms. First, the presence of the methyl group at a specific CpG dinucleotide site may directly block DNA recognition and binding by some transcription factors.[27] In other instances, some proteins may preferentially bind to methylated DNA, thereby blocking transcription factor access to these regulatory elements.[28] Taken together, DNA methylation represents an important mechanism in the regulation of gene expression.

Critical to understanding this means of regulating gene expression is the mechanism by which DNA is methylated. A family of DNA methyltransferase enzymes (DNMTs) is involved in de novo DNA methylation and the maintenance of methylation. Thus, epigenetic control of gene expression by cytosine methylation is facilitated by the activity of DNMTs. The regulation of the expression of the DNMTs (whether mediated transcriptionally or by post-translational means) represents an additional mechanism of epigenetic control.[29] Cell- and tissue-specific activation of DNMTs may lead to localized changes in gene expression. In brief, DNMTs recognize CpGs within double-stranded DNA as substrates. Maintenance DNA methylation is generally performed by DNMT1

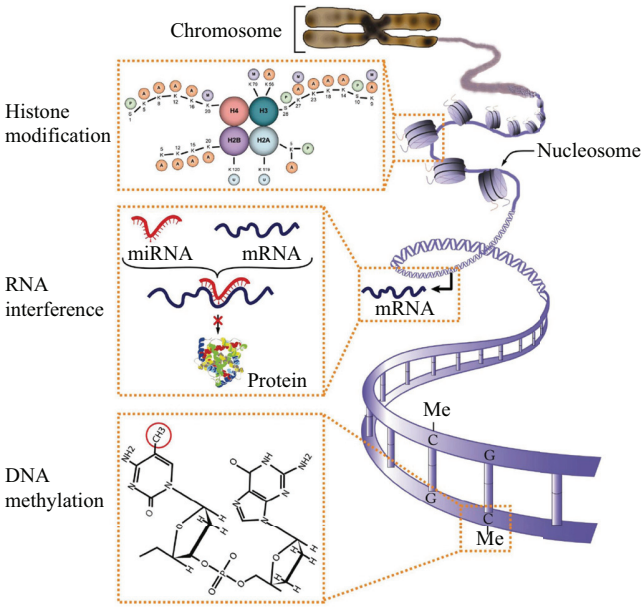

Figure 2: Schematic of the mechanisms of epigenetic regulation. DNA methylation, histone modifications, and RNA-mediated gene silencing constitute three distinct mechanisms of epigenetic regulation. DNA methylation is a covalent modification of the cytosine (C) that is located 5' to a guanine (G) in a CpG dinucleotide. Histone (chromatin) modifications refer to covalent post-translational modifications of N-terminal tails of four core histones (H3, H4, H2A, and H2B). The most recent mechanism of epigenetic inheritance involves RNAs. Reproduced with permission from Z. Herzeg.[16]

and occurs in step with DNA replication. Thus, one may consider one of the major roles of DNMT1 as the passing on epigenetic control of gene expression to daughter cells.[30] Interestingly, DNMT3a and DNMT3b do not exhibit a substrate preference between hemimethylated and unmethylated DNA and therefore appear to be critical for de novo methylation.[31] During embryogenesis, de novo methylation is performed by DNMT3A and DNMT3B. Although some studies suggest an ongoing role for DNMT3A and DNMT3B in maintaining methylation status in some cell types, the ubiquitously expressed DNMT1 is predominantly responsible for maintaining cellular levels of CpG methylation.[31]

Histone modification

Genetic information is packaged into higher order structures by nucleosomes. Nucleosomes package approximately 146 base pairs of DNA wrapped around an octamer of core histone proteins. Each core nucleosome consists of two of each histone protein: H2A, H2B, H3, and H4. Aside from the organizational function of DNA in the nucleus, nucleosomes play a critical role in regulating gene activity by controlling their accessibility and therefore their transcriptional activity. Histones can be post-translationally modified to restructure chromatin in many ways, including acetylation, methylation, phosphorylation, ubiquitination, poly-ADP-ribosylation, biotinylation, and sumoylation.

Acetylation is one of the most frequent epigenetic modifications. Histone acetylation occurs at the many lysine residues in both histones H3 and H4 (Table 1). Increased levels of histone acetylation are highly correlated with increased transcriptional activity, whereas decreased levels of acetylation repress gene expression. This process is catalyzed by histone acetyltransferases (HATs) that utilize acetyl-CoA as a cofactor. Recruitment of these HATs to promoters is generally associated with activated transcription. Unlike histone acetylation, histone methylation can activate or inhibit transcription, depending on where the modification occurs. The methylation of histones is carried out by a large family of histone methyltransferases (HMTs). Just like DNMTs, HMTs utilize S-adenosylmethionine (SAM) as a cofactor to methylate their target amino acids and produce S-adenosylhomocysteine (SAH) as a byproduct (Table 1).

Acetylation and methylation of histone tails are not permanent modifications. Histone acetylation and deacetylation are dynamic processes determined by the balance between histone acetyltransferases and histone deacetylases (HDACs). Similarly, histone demethylases remove methylation from histones. Regulation of chromatin structure directly affects the transcriptional process. The extensive combinatorial post-translational modifications to histones represent yet another layer of complexity to understanding the "histone code." More importantly, this dynamic nature of acetylation and methylation provides flexibility to the epigenetic control of gene expression.[32]

RNA interference

MicroRNAs (miRNAs) are small RNA molecules, approximately 22 nucleotides long that can negatively control their target gene expression posttranscriptionally. First described in *Caenorhabditis elegans* in 1993, miRNAs have now been identified throughout the plant and animal kingdoms. To date, miRNAs have been shown to inhibit translation or decrease mRNA stability by binding to specific sites usually in the 3' untranslated region (3'UTR) of target messages, thus providing another layer of control of gene expression. MicroRNAs are initially transcribed as primary microRNAs by endogenous RNA polymerases and undergo a series of processing steps and incorporation into the RNA-induced silencing complex (RISC).[33] MicroRNAs interact with mRNA through sequence specific interactions with key areas of sequence homology at the 5' end of the microRNA, particularly at bases 2-8 which is termed the "seed" region. This control is sequence specific, and changes in just a single base within the miRNA target site can abolish this regulation. It is this region which forms the basis for virtually all computational algorithms predicting mRNA targets. However, beyond Watson–Crick base pairing, the efficiency of repression depends on the number and configuration of mismatches between the miRNA and the target mRNA, the secondary structure of the surrounding region, and the number of target sequences on the mRNA.[34] Up to one-third of human genes are predicted to be regulated by one or more microRNAs. Despite this vast regulatory network and hundreds of microRNAs, few targets have been validated.[35]

As the functions of individual microRNAs are being studied, it has become clear that microRNAs can be regulated by epigenetic mechanisms including DNA methylation and histone modification such as let-7a, miR-9, miR-34a, miR-124, miR-137, miR-148 and miR-203.[36-40] Conversely, another subset of miRNAs controls the expression of important epigenetic regulators, including DNA methyltransferases and histone deacetylases.[41] MicroRNA-29b can reduce the expression of DNMT enzymes and thereby affect global methylation status.[42] This complex network between miRNAs and epigenetic pathways appear to form an epigenetics–miRNA regulatory circuit intertwined with the transcriptional

Table 1: Summary of epigenetic histone modifications

Tri-methylation of histone H3 lysine-4	Associated with transcriptional activation and creates a binding site for proteins containing chromodomain that recruits HATs[74]
Methylation of lysine-36 and lysine-79 of histone H3	Associated with active chromatin and transcriptional activation[32]
Tri-methylation of histone H3 lysine-9	Transcriptional silencing by recruiting heterochromatin protein (HP1) and triggers the formation of heterochromatin[74]
Methylation of histone H3 lysine 27	Associated with transcriptional repression and maintaining silent chromatin through recruiting the Polycomb complex (PRC1)[75]
H3K9 acetylation	Associated with chromatin decondensation and the formation of chromatin loops. These loops separate out actively transcribed genes from more compact chromosome territories[76]
Trimethylation at H3K4, H3K36, or H3K79	Associated with an open chromatin configuration which is characteristic of euchromatin[32]
Methylated H3K9 provides a binding site for the chromodomain-containing heterochromatin protein 1 (HP1)	Induces transcriptional repression and heterochromatinization[32]
H3K4 demethylation through histone demethylase LSD1	Results in to transcriptional inactivation[32]

and post-transcriptional pathways known to regulate epigenetic modulators and to organize the whole gene expression profile.[41] Though not yet demonstrated, one can conceive of situations in which potential microRNA target binding site accessibility is influenced by the methylation of the target region.

Examples of epigenetic regulation of PAH

Superoxide dismutase 2

Superoxide dismutase 2 (SOD2), a candidate tumor-suppressor gene,[43] is silenced in several malignancies.[44,45] In multiple myeloma and pancreatic carcinoma, the epigenetic silencing of SOD2 is caused by hypermethylation of CpG islands within the promoter for SOD2.[45] SOD2 is subject both to DNA methylation and histone acetylation.[46,47] Demethylation of SOD2 in cancer restores SOD2, increases H_2O_2 production and decreases cell proliferation and tumor growth. Production of H_2O_2 is a critical link between SOD2 expression and regulation of proliferation. SOD2 is found in the mitochondria where it regulates production of H_2O_2 (produced physiologically from mitochondrial superoxide during respiration). H_2O_2 is less toxic than superoxide and its greater diffusion radius allows it to serve as a signaling molecule. H_2O_2 modulates the activity of transcription factors such as HIF-1α (which it inhibits)[48] and sulfhydryl rich proteins, including the voltage-gated potassium channel Kv1.5 (which it activates). SOD2 deficiency has been identified in the pulmonary arteries and plexiform lesions of PAH[49,50] and activation of HIF-1α is also evident.[1,49-52] However, the mechanism by which these two abnormalities was linked had been unclear. We found that fawn-hooded rats, the development of PAH is preceded by downregulation of SOD2. Interestingly, in the fawn hooded rat, PAH is heritable and yet sequencing has shown that the SOD2 gene has no mutations.[1,50] However, several regions of the SOD2 promoter and intronic regions contained CpG islands, which could be targets for epigenetic regulation via CpG methylation. We discovered that the selective hypermethylation of CpG islands in the SOD2 gene reduces its expression ~50% compared to PASMCs from genetically matched consomic rats[50] and this contributes to the proliferative, antiapoptotic phenotype in pulmonary artery smooth muscle cells (PASMC) of the FHR.[1]

To demonstrate this, the promoter of SOD2 and the first 2kb after the transcriptional start site were surveyed using genomic sodium bisulfite sequencing (Fig. 3). As shown in other tissues, the region immediately 5' to the transcriptional start site was completely unmethylated. However, differentially methylated CpG dinucleotides within intron 2 (an enhancer region) were found. This methylation pattern was tissue specific as the SOD2 methylation in intron 2 was not seen in aortic smooth muscle cells of FHRs. Moreover, this same region is methylated in various tumors. Interestingly, treatment with the DNMT inhibitor, 5-azacytidine (5-AZA) resulted in a dose dependent increase in SOD2 expression.[1] Both DNMT1 and DNMT3b are significantly upregulated in lung tissue and pulmonary arterial smooth muscle cells compared to control (Fig. 4).[1]

Reversing the methylation status of the SOD2 gene using 5-AZA not only restored SOD2 expression, it also decreased proliferation and slightly increased apoptosis in the abnormally hyperproliferative FHR PASMCs. Likewise, increasing SOD2 in the PASMC, either by administering adenovirus carrying the SOD2 transgene, or by giving a SOD analog (MnTBAP) inactivated HIF-1α (i.e. achieved similar effects as demethylating the gene). The similarities in therapeutic effects of SOD supplementation and 5-AZA in cellular experiments suggests an important role for decreased SOD2 in the mechanism of PAH. *In vivo* studies of MnTBAP, given to FHR with established PAH for 4-weeks, showed a decreased mean pulmonary artery pressure and increased exercise capacity, while reducing the medial thickness of precapillary resistance arteries (Fig. 5).

This study demonstrated that SOD2 methylation is important in the development of PAH and contributes to HIF-1α activation and the development of an apoptotic-resistant state with marked proliferation.[1] From a therapeutic standpoint it is also exciting because of the potential to increase SOD2 either through DNMT inhibition (5-AZA) or administering MnTBAP. It is noteworthy that similar abnormalities in SOD2 expression are epigenetically mediated and are similarly reversible with a resultant decrease in cell proliferation in several cancers.[44,47]

Maternal restrictive diet

The Barker hypothesis suggests that fetal stressors can lead to cardiovascular disease in adults.[53] While the effect of poor maternal diet and low birth weight on the lifetime development of coronary artery disease is recognized,[54] the effects of maternal malnutrition on the right ventricle and pulmonary hypertension has only recently been considered. Rexhaj et al. assessed the pulmonary vascular responsiveness in offspring of pregnant mice fed with a restrictive diet in both normoxic and hypoxic conditions.[55] To detect reversible epigenetic changes, the authors also administered the histone deacetylase inhibitor trichostatin A to male offspring of pregnant mice that had been fed a restrictive diet.

In the offspring of restrictive diet pregnancy, there was exaggerated hypoxic pulmonary hypertension and right ventricular hypertrophy. An epigenetic change was demonstrated by the increased update of radioactive methyl groups in the offspring of the pregnant diet-restricted mice. Of note, the pulmonary

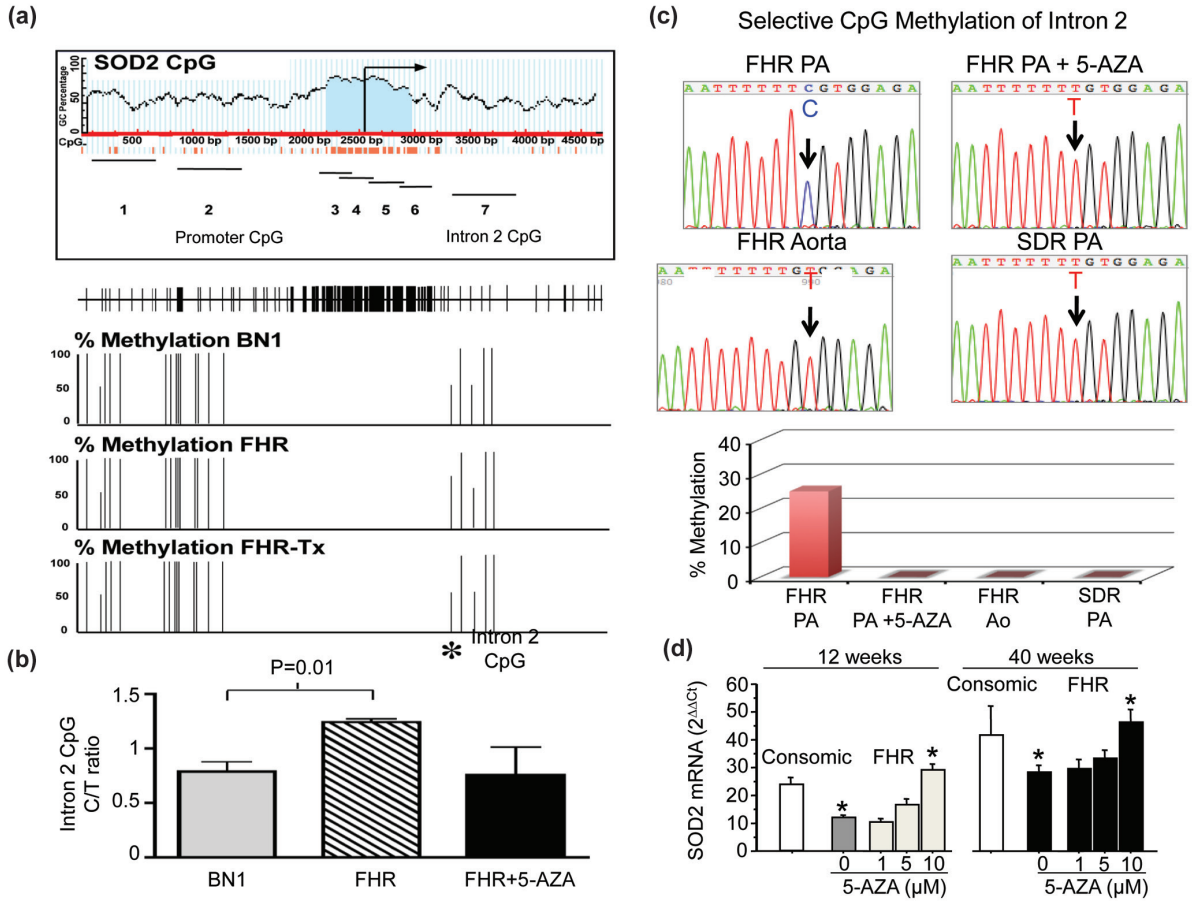

Figure 3: Methylation of SOD2 in FHR PASMC is reversible by 5-AZA. (a) Schematic of the CG dinucleotide percentage and CpG islands within the SOD2 promoter and first 2 kb after the transcriptional start site. Seven amplicons were surveyed within the SOD2 gene. Their approximate locations are represented by solid horizontal lines. The positions of individual CpG dinucleotides are shown as vertical tick marks below the amplicon map. No methylated CpG pairs were identified in amplicons 3 to 6. *Differentially methylated CpG dinucleotides in FHRs within amplicon 7 vs BN1 tissue. (b) Corresponding methylation percentage of the differentially methylated CpG in intron 2. Results are expressed as a frequency of cytosine methylation in PAs from consomic control BN1 rats (n=2), FHRs (n=3), and FHRs treated with 5-AZA (FHR-Tx; n=3). (c) Representative sequencing traces of genomic DNA from cultured PASMCs. Only methylated cytidines are protected against bisulfite-mediated deamination of cytidine into uridine (which is recognized as thymidine when the polymerase chain reaction product is amplified). As indicated by the arrow, the cytidine in FHR PASMCs was methylated (and therefore remains a cytidine; top left); this is reversed by 5-AZA. The site is not methylated in FHR aortic SMCs or in SDR PASMCs. The bar graph shows the mean data indicating the reversibility and tissue specificity of this SOD2 methylation in intron 2 in cultured PASMCs. (d) FHR PASMCs have lower SOD2 mRNA levels vs consomic PASMCs. 5-AZA causes a dose-dependent increase in SOD2 expression. Reproduced with permission from Archer et al.[1]

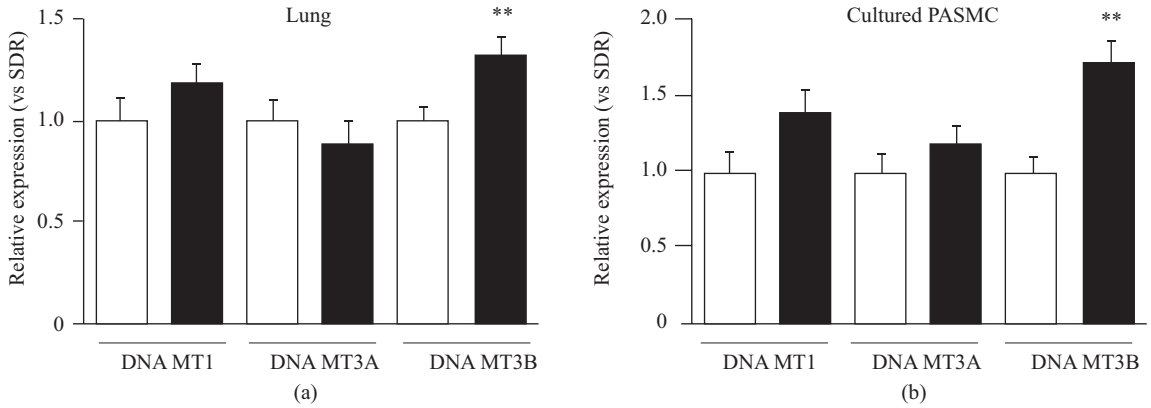

Figure 4: DNA methyltransferase expression is increased in FHR lung and PASMCs. (a) DNA MT1 and 3B mRNA are increased in FHR vs SDR lungs (n=12 each). *P<0.05, **P<0.01. (b) In low-passage (3 to 4) PASMCs (n=8 in each group), FHRs had higher DNA MT3B expression and a trend toward increased DNA MT1. Reproduced with permission from Archer et al.[1] Black bars = FHR; White bars = SDR

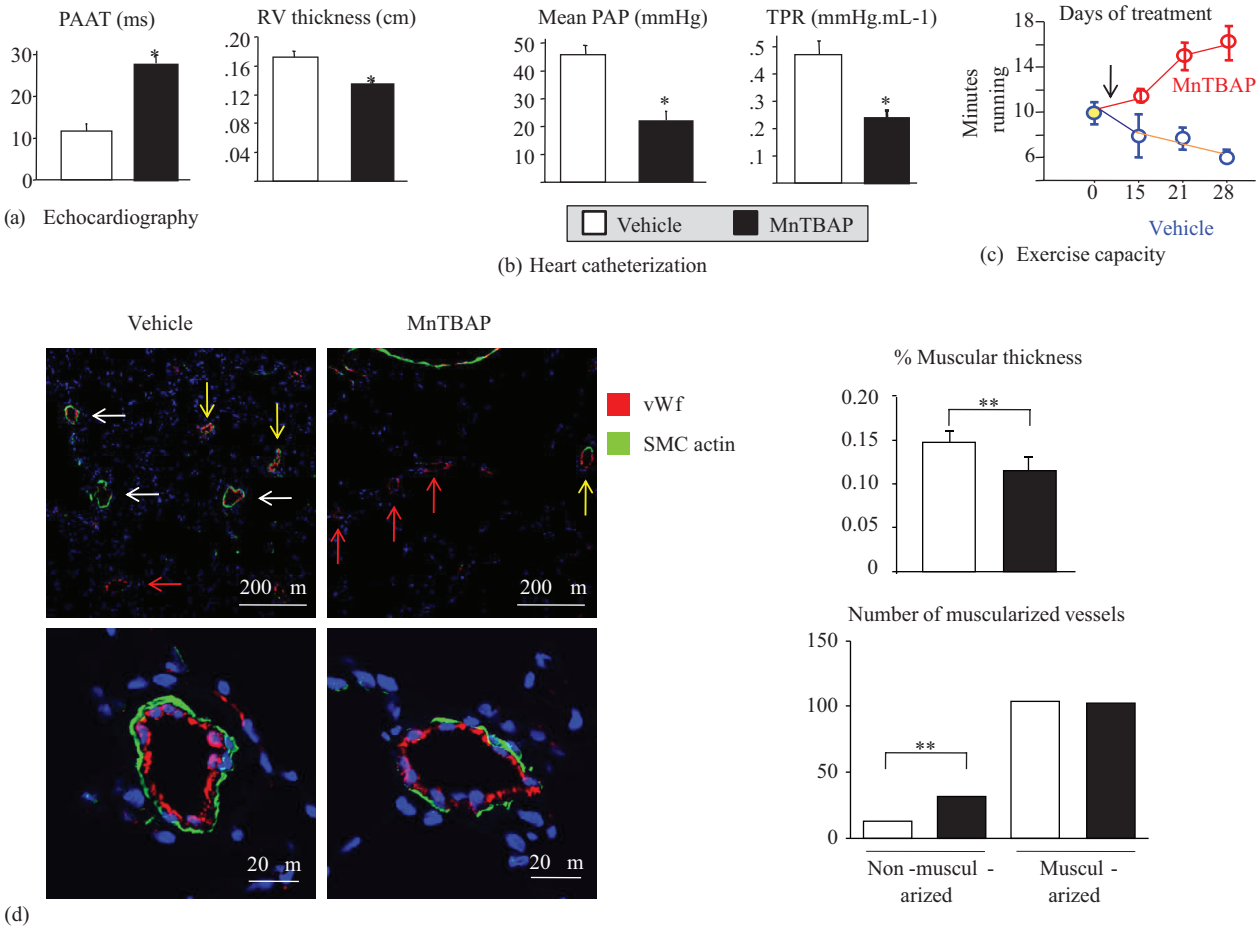

Figure 5: Administration of the SOD analog, MnTBAP, regresses PAH in FHRs. (a) MnTBAP reduces mean PA pressure measured by Doppler (lengthens PA acceleration time [PAAT]) and decreases right ventricular (RV) thickness in FHRs treated for 4 weeks (n=5 per group). *P<0.05. (b) MnTBAP therapy reduces mean pulmonary artery pressure (PAP) and total pulmonary resistance (TPR). (c) FHRs treated with MnTBAP exercise longer on a graded treadmill (n=15 per group). (d) Lung sections were stained for von Willebrand factor (vWF; red), a-smooth muscle cell (SMC) actin (green), and DAPI (blue). Note the fully muscularized (white arrows), partially muscularized (yellow arrows), and nonmuscularized blood vessels (red arrows). Bottom, A representative fully muscularized PA in a vehicle-treated FHR (left) vs MnTBAP (right). The percent medial thickness of precapillary resistance PAs was reduced and the number of nonmuscularized resistance PAs was increased by MnTBAP. **P<0.01 vs control. Reproduced with permission from Archer et al.[1]

DNA methylation induced by dietary restriction, and the epigenetic changes, were reversed by the administration of butyrate and trichostatin A, inhibitors of histone deacteacylation (HDAC inhibitors). In fact, butyrate administration to male offspring prevented the transmission of pulmonary vascular dysfunction to their own offspring, again suggesting reversal of the epigenetic effects. Tempol, a membrane-permeable radical scavenger, administered to the mothers in conjunction with their restrictive diet during pregnancy prevented endothelial dysfunction, RVH and hypoxic pulmonary hypertension implying a key role of oxidative stress in mediating the epigenetic effects of restrictive diets.

Primary pulmonary hypertension in the newborn

Primary pulmonary hypertension in the newborn (PPHN) is defined as a failure of pulmonary vascular resistance to fall at birth and results in severe hypoxemia and PAH.[56] PPHN can be primary or secondary to a variety of clinical conditions, including asphyxia, sepsis, pneumonia, meconium aspiration syndrome and antenatal exposure to non-steroidal anti-inflammatory drugs. Additional intrauterine environmental factors such as large for gestation age and maternal asthma might be also important risk factors for PPHN.[57] Endothelial nitric oxide synthase (eNOS) levels has been increasingly implicated in the development of PPHN[58] and thus the expression of eNOS has been studied from an epigenetic perspective.

In a study by Xu et al., the authors demonstrated a 6-fold upregulation of eNOS expression in pulmonary vascular endothelial cells derived from a neonatal rodent PPHN model induced by intrauterine exposure to hypoxia and indomethacin between the 19th and 21st day of gestation.

In this model, NOS upregulation was associated with increased H3 and H4 histone acetylation in the eNOS promoter.[59] They also noted a mild decrease in eNOS methylation. However, this research did not have a reversal protocol which would be required to definitively demonstrate the role of these epigenetic abnormalities in the pathogenesis of PPHN.

Epigenetics also explains the cellular localization of eNOS expression. It has been shown that endothelial-specific expression of eNOS is regulated by epigenetics.[60] Chan et al. demonstrated that the nine CpG dinucleotides in the promoter region of the eNOS gene were unmethylated or lightly methylated in human endothelial cells.[61] In contrast, in vascular smooth muscle cells, the eNOS promoter was found to be almost completely methylated. In addition to changes in methylation of the eNOS promoter, the eNOS core promoter is highly enriched in acetylated histone H3/K9 and H4/K12, and methylated H3/K4 in endothelial cells.[60] Furthermore, HDAC inhibitor, trichostatin A, may induce eNOS expression in non-endothelial cells, and small RNA may suppress eNOS expression by altering histone acetylation and DNA methylation in endothelial cells.[62] Taken together, the dysregulation of endothelial eNOS, and thereby the development of this particular pulmonary vascular disease, appears to be epigenetically controlled.

Epigenetics and RV hypertrophy

Epigenetic changes occurring in the right ventricle have only recently been studied. Marked changes in the right ventricular morphology coincide with changes in the metabolic profile.[63] Though much focus remains on the changes in the pulmonary vasculature, the adaptive changes in the right ventricle may be even more important a predictor of survival.[64]

Adults with PAH and children with congenital heart disease compensate for RV pressure overload by developing right ventricular hypertrophy (RVH). Why some patients develop RVH that is compensatory while others develop a maladaptive RVH and rapidly develop RV dilatation and failure is uncertain. Whereas left ventricular hypertrophy (LVH) can be regressed with angiotensin converting enzyme (ACE) inhibitors resulting in therapeutic benefit, the benefit of reducing RVH are less clear, as is the optimum way to do this.

Cho et al. studied the effects of an HDAC inhibitor, sodium valproate on RVH in monocrotaline (MCT) rats and pulmonary artery banding (PAB) rats. MCT rats develop a maladaptive form of RVH and tend to die within 6 weeks with RV failure; conversely, PAB rats develop an adaptive form of PH and are much less prone to premature death or RV failure.[65] HDAC inhibition in PAB rats significantly decreased RVH. Pulmonary artery flow acceleration was significantly reduced in PAB rats treated with sodium valproate, suggesting a functional as well as anatomical improvement with epigenetic modification. HDAC inhibition also reduced myocardial fibrosis. The involvement of HDACs in these response was demonstrated by the increased acetylation of histone H3 in the RV in animals that received sodium valproate versus controls. As would be expected with sodium valproate there was no change in HDAC1, HDAC2, HDAC 3 and HDAC8. Similar improvements in RVH were observed in MCT rats treated with sodium valproate. ACE inhibitor therapy did not show this benefit in either models in terms of RVH or fibrosis.

However, not all studies suggest benefits from HDAC inhibition in RVH. In a study by Bogaard et al.,[66] a different HDAC inhibitor, trichostatin A (TSA), was administered intraperitoneally four weeks after PAB. TSA-treated rats developed decreased cardiac output and signs of RV failure. In contrast to the Cho study in RVH and the beneficial effects of HDAC inhibition on LV hypertrophy in aortic banding models, trichostatin A actually increased RVH. TSA-treated PAB rats also demonstrated increased RV fibrosis, capillary rarefaction and rates of cell death. These negative results contrast with the findings of benefit in studies by Cho and Rexhaj, as well as the beneficial effects of HDAC inhibition in LV hypertrophy.[67] Do these differences reflect the different HDAC inhibitors, differences in the models or (in the case of LVH) differences in the RV versus the LV transcriptome response to pressure-overload?[68] There are several classes of HDAC inhibitors and further study is clearly required to determine whether a subclass-specific inhibition of a specific HDAC family is beneficial or harmful.

CONCLUSIONS

The study of the epigenetic regulation of development, cancer, and other diseases has increased at an exponential rate. In the area of pulmonary hypertension, the number of studies remains small, but is on the rise. The evolving technology to study epigenetics is accelerating.[69,70] High throughput means of studying the methylation status of the genome, or the "methylome," are emerging and will likely uncover an extensive network of epigenetically regulated genes. The application of methylomics to the field of pulmonary hypertension may also provide insight into new molecular targets or regulatory pathways not previously recognized.

Epigenetic regulation of gene expression may link known risk factors for the development of pulmonary

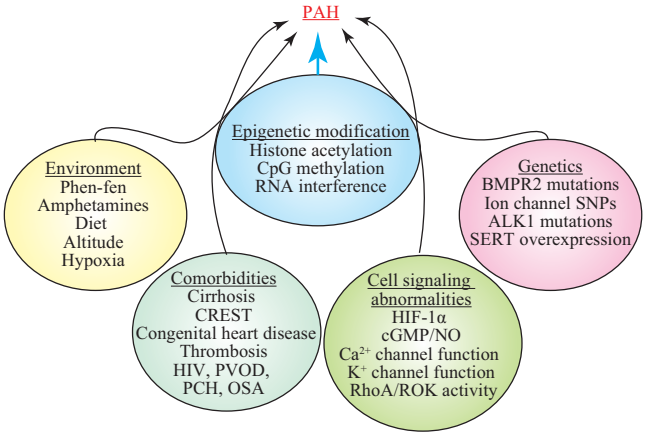

Figure 6: Schematic of pathophysiologic mechanisms leading to the development of PAH.

hypertension and contribute to the net risk of developing overt disease (Fig. 6). Epigenetic changes may be the missing link between genomic sequence variation, comorbid disease states, environmental exposure, and cell signaling events. Epigenetics could also play a role in the phenotypic variability of PAH (severity, time to disease onset). The accumulation of epigenetic "hits" en route to overt disease has been observed in cancer formation.[71] In a similar way, the influences of gender and toxin exposure, on top of genetic polymorphisms and disease conditions may change the methylation status of a network of disease modifying genes and lead to pulmonary hypertension.

Epigenetic modifications may be reversible[72] and it is possible that epigenetic modifications could be targeted by pharmacological intervention,[73] suggesting novel therapeutic options for experimental testing. Inhibition of DNMTs or HDACs have shown both promise and harm. The lack of specificity for epigenetic treatments is an important issue and warrants cautious application given the concerns for "off-target" effects of HDAC inhibition or demethylating agents. Interestingly, changes in the activity or expression of DNMTs have been noted to affect relatively specific outcomes in terms of site-specific methylation and regulation of specific genes in certain tissues. Current models suggest that the specificity of DNMT activity can depend on their expression levels or their interaction with other epigenetic regulators. While the development of selective HDAC inhibitors may have more utility, a more comprehensive understanding of the intricate intersecting pathways between DNA methylation, miRNAs, and chromatin remodeling will be critical. Similarly, investigations into the epigenetic control of right ventricular gene expression and adaptive hypertrophy will be required to understand a disease process which is not isolated to the pulmonary vasculature.

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