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Epigenetics and miRNA emerge as key regulators of smooth muscle cell phenotype and function

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Abstract

Regulation of phenotypic plasticity in smooth muscle requires an understanding of the mechanisms regulating phenotype-specific genes and the processes dysregulated during pathogenesis. Decades of study in airway smooth muscle has provided extensive knowledge of the gene expression patterns and signaling pathways necessary to maintain and alter smooth muscle cell phenotype. With this solid foundation, the importance and complexity of inheritable epigenetic modifications and mechanisms silencing gene expression have now emerged as fundamental components regulating aspects of inflammation, proliferation and remodeling.

Keywords

Airway smooth muscle; Asthma; Epigenetics; Inflammation; microRNA; Remodeling

1. Introduction

Epigenetics is often defined as the study of heritable changes of a phenotype, including altered patterns of gene expression within a specific cell type that result from changes in a chromosome without alterations in the DNA sequence [1]. In its natural state DNA is packaged into chromatin, an organized and dynamic protein-DNA complex consisting of DNA, histones and non-histone proteins. Epigenetic changes alter the accessibility of the proximal promoters and enhancer/silencer regions within the chromatin to transcription factors and may therefore regulate global gene activation or localized gene-specific transcription. In general, conformationally relaxed chromatin, known as euchromatin, is a

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hallmark of potentially active genes while compact chromatin, known as heterochromatin, is associated with transcriptionally silent genes. These epigenetic changes are mediated by a variety of molecular mechanisms, including post-transcriptional modification of the histone proteins, the involvement of small and other non-coding RNAs and DNA methylation. These changes can result in modifications to the structure of DNA itself upon DNA methylation or can alter the structure of chromatin through distinct modifications to scaffolding proteins, such as histones (See Figure 1).

Although DNA methylation and histone modifications are regulated by different enzymes and chemical processes, there is evidence linking the two systems in co-operatively regulating patterns of gene expression. It appears that the relationship between these two epigenetic mechanisms can function in both directions, with histone methylation directing DNA methylation patterns and DNA methylation acting as a template to facilitate histone modifications following DNA replication [2]. microRNAs (miRNAs) most likely function independently of the other two epigenetic mechanisms. However, gene expression may be controlled by co-incident changes in DNA methylation and histone modification within a promoter region, accompanied by binding of miRNAs to mRNA transcripts targeted for gene-silencing. Individually, each of these regulatory mechanisms is complex, but when combined the complexity of the potential interactions provides unique challenges in dissecting and understanding the pathways involved. In recent years, understanding the mechanisms regulating the initiation, maintenance and heritability of epigenetic changes in cells that may undergo a phenotypic switch from a “normal” to “diseased” state has become a research focus for a variety of diseases.

In the lung, the role of epigenetic changes and miRNA-mediated gene-silencing has recently emerged as an important regulatory mechanism affecting smooth muscle cell phenotype and function. In this review, we will summarize general mechanisms of regulation by epigenetic factors and miRNA, followed by a discussion about what is known in smooth muscle cells using *in vitro* and *in vivo* models of asthma and the importance of these findings to future studies.

2. General mechanisms of epigenetic and miRNA-mediated regulation of gene expression

2.1. DNA methylation

The first epigenetic mechanism to be recognized was DNA methylation, a reversible modification of DNA structure in which a methyl group is added to cytosine residues. The majority of vertebrate genome methylation occurs at regions where a cytosine is followed directly by a guanine in the DNA sequence, also known as CpG sites. Methyl-C is prone to spontaneous deamination to thymine [3] and therefore CpG methylation frequently leads to mutation of these sites to TpG. As a result, the number of CpG occurrences in the genome is depleted from the value expected from random distribution [4]. Within the globally CpG poor genome are regions of, on average, 1000 base pairs with elevated C+G base composition, termed CpG islands (CGIs). In contrast to globally distributed CpGs, CpGs within CGIs are frequently unmethylated. Around 50% of all CGIs contain transcription

start sites and coincide with gene promoters. Methylation of cytosines within these regions can lead to stable silencing of the associated promoter via direct inhibition of transcription factor binding and recruitment of methyl-binding domain containing chromatin remodeling complexes, including histone deacetylases. Histone deacetylases remove acetyl groups from histones, resulting in a closed chromatin structure. The remaining islands (“orphan CGIs”) are either intragenic or at the gene termini. These islands can be initiation sites for the transcription of non-coding RNAs (ncRNA) which, upon transcription, result in imprinting which is a silencing of a paternal/maternal allele such that only a single allele functions. For example, transcription of the ncRNA *Air* initiates from a CGI within exon 2 of the *Igf2r* gene and is only transcribed from the paternal chromosome. The resulting *Air* nc-RNA silences three protein-coding genes *Igf2r*, *Slc22a2* and *Slc22a3* only on the paternal allele. It is postulated that the *Air* promoter is silent on the maternal chromosome due to DNA methylation but this requires confirmation [5]. Furthermore, genome wide correspondence of “orphan CGIs” with RNA polymerase II binding and histone H3 lysine 4 trimethylation (H3K4me3) association suggests them to be novel promoters [4, 6, 7].

The enzymes responsible for DNA methylation are the DNA nucleotide methyltransferases (Dnmts). There are two families of Dnmts that are structurally and functionally distinct. The Dnmt3 family consists of Dnmt3a, 3b and 3L. Dnmt3a and Dnmt3b are active and establish the initial CpG pattern *de novo*, while Dnmt3L has no enzyme activity but immunoprecipitates with Dnmt3a/b and enhances their DNA methylation activity. The Dnmt1 family consists of Dnmt1 and Dnmt2. Dnmt1 maintains the *de novo* DNA methylation pattern during chromosome replication. Dnmt2, while having high sequence and structural similarity to DNA methyltransferases, does not methylate DNA [8]. Its exact role is still controversial but appears to involve methylation of tRNA [9, 10].

2.2. Histone modifications

The fundamental unit of chromatin is the nucleosome, which is composed of an octamer of four core histones (H3, H4, H2A and H2B) around which 147 base pairs of DNA are wrapped. The core histones are predominantly globular but also have unstructured N-terminal tails which contain multiple residues susceptible to modification. At least eight distinct types of post-translational histone modifications have been identified including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation [11]. The combination of different N-terminal modifications, although generated by diverse molecular mechanisms, appear to interact to stabilize epigenetic changes and regulate gene expression [12]. Histone acetylation and histone methylation are the best characterized, with the modifications being regulated by histone acetyltransferases (HATs)/histone deacetylases (HDACs) and histone methyltransferases (HMTs)/histone demethylases respectively [11].

These enzymes are being increasingly implicated as both direct and indirect regulators of gene expression in a wide variety of cells types. In particular, hyperacetylation of histone lysine residues by HATs results in the open chromatin conformation which facilitates DNA interactions with the basal transcription machinery and increases gene transcription. The counterbalancing action of HATs/HDACs and HMTs/demethylases appears to fine tune gene expression to regulate important cellular process such as proliferation and

differentiation, that when dysregulated can contribute to the pathogenesis of disease. As epigenetic changes are reversible, targeting and modifying the function of these enzymes has potential as a therapeutic target in a range of diseases.

2.3. miRNA-mediated gene silencing

miRNA are double-stranded RNA molecules found individually or as polycistronic clusters in introns, exons or in non-coding regions between protein-coding genes [13, 14]. They are members of a growing family of ncRNAs functioning throughout the genome to regulate epigenetic modifications, transcriptional and post-transcriptional events, maintain genomic integrity and to provide a defense from transpositional elements [15]. Indeed a large portion of non-coding sequences, once thought to be 'junk' DNA, are now known to contain ncRNA. miRNA themselves were first described in *C.elegans*, where *lin-4* and *let-7* were identified as repressors of regulatory proteins key to developmental timing [16, 17]. There are now more than 1900 mature human miRNA sequences [18], each predicted to target more than 100 mRNA for post-transcriptional regulation [13].

As shown in Figure 2, miRNA are initially transcribed by RNA polymerase II as a primary miRNA transcript and processed into a stem-loop structure of 70-100 nucleotides by the double-stranded-RNA specific ribonuclease (RNase) III Droscha [19]. This precursor miRNA is exported into the cytoplasm via a GTP-dependent mechanism [20], where it is further cleaved by a second RNase III Dicer [21]. This yields a mature miRNA duplex of 21-23 nucleotides bound by a miRNA-associated RNA-induced silencing complex (miRISC). A core catalytic component of this complex is Argonaute (AGO), a family of endonucleases with RNaseH-like activity [21] that ultimately use miRNA as a guide to bind complementary sequences in mRNA, leading to degradation of the mRNA transcript when binding in the seed sequence is complementary or translational repression when binding occurs with mismatches in seed sequence recognition [22]. In the canonical pathway, base-pairing is recognized between the 2-8 nucleotide seed sequence of the miRNA and the mRNA 3'untranslated region (UTR). In the non-canonical pathway, miRNA binding to its mRNA targets relies less on seed sequence homology and more on complementarity in coding regions or the 5'-UTR of the target transcript [23]. The importance of miRNA-mediated gene-silencing in smooth muscle cell phenotype is underscored in animals with smooth muscle targeted knock-out of Dicer, which impairs contractile differentiation of vascular smooth muscle cells [24] and inhibits smooth muscle cell development in the gastrointestinal tract [25].

3. Phenotypic switching in smooth cells as a product of epigenetic dysregulation

Smooth muscle cells possess remarkable phenotypic plasticity in response to changes in the local environment and these phenotypic changes may be responsible for the development or progression of diseases including asthma [26], atherosclerosis [27] and pulmonary arterial hypertension (PAH) [28]. Although the mechanisms underlying these changes are still uncertain, evidence is emerging that epigenetic mechanisms may be at least partly responsible for the phenotypic changes seen in smooth muscle cells in these disease states

including altered secretory potential, proliferation, extracellular matrix (ECM) production and vascular/airway remodeling.

3.1. Inflammatory mediators

Chronic inflammatory events are known to alter the phenotype of airway smooth muscle (ASM) cells [29, 30]. Differential expression of inflammatory mediators from diseased ASM cells has been primarily reported in the context of asthma using *in vitro* culture models. In the studies reported to date, ASM cells isolated from asthmatics secrete higher levels of inflammatory cytokines and chemokines than cells from non-asthmatic individuals, with no reports of repressed expression documented [31-33]. Descriptions of epigenetic mechanisms regulating inflammatory mediator expression in ASM cells from asthmatic patients are limited. However, these reports do suggest the importance of epigenetic dysregulation to smooth muscle cell phenotype in asthma.

CXCL8/IL8 is a chemokine involved in the initiation and maintenance of an inflammatory response via the recruitment of neutrophils. CXCL8 secretion from asthmatic ASM cells is increased compared to non-asthmatic cells. The mechanism involves increased binding of the transcriptional regulators NF- κ B, C/EBP β and RNA polymerase II to the unstimulated CXCL8 promoter [31]. Recent conference reports suggest that aberrant HAT binding and resultant histone H3 acetylation at the asthmatic CXCL8 promoter may be responsible for the increased binding of these transcriptional regulators [34]. In further support of histone acetylation dependent regulation of the CXCL8 promoter, Keslacy *et al* showed that the HDAC inhibitor trichostatin A (TSA) prevented the inhibitory effect of IFN γ on TNF α induced CXCL8 secretion [35].

The CC chemokine eotaxin is also hypersecreted from ASM cells derived from asthmatic donors compared to those from non-asthmatic donors, under unstimulated conditions [32]. Eotaxin is a potent eosinophil chemoattractant, critically involved in eosinophilic inflammatory diseases. The signaling responsible for asthmatic ASM cell hypersecretion is dependent on enhanced autocrine fibronectin secretion and subsequent signaling via α 5 β 1 integrin. However, histone acetylation is also regulated at the eotaxin promoter in ASM cells. Specifically, TNF α induces histone H4 lysine 5 and lysine 12 acetylation (H4K5/14ac) at the eotaxin promoter [36]. It would be interesting to determine whether the enhanced autocrine fibronectin signaling of asthmatic ASM cells changes the acetylation status of histones at the eotaxin, and other cytokine promoters. Furthermore, the question remains whether changes in ECM composition alter epigenetic processes that contribute to altered inflammatory mediator secretion and disease pathogenesis? Interestingly expression of the eotaxin receptor CCR3 is also increased in asthmatic ASM cells [37]. The molecular mechanism in ASM cells is not reported, however CCR3 transcription in peripheral blood eosinophils is regulated by negative and positive GATA elements, with DNA methylation of CpG sites regulating occupancy of the sites [38]. Thus, speculatively, DNA methylation of the CCR3 gene may regulate its overexpression in ASM cells.

Finally, the CXC chemokine, CXCL10/IP10, is a potent chemoattractant for mast cells and T lymphocytes and is implicated in mast cell migration to ASM bundles [39]. A combined IFN γ /TNF α /IL-1 β stimulus induces higher CXCL10 secretion from ASM cells isolated from

asthmatic patients cells from non-asthmatic individuals. Differential basal expression was not reported [33]. Clarke *et al* have shown that both TNF α and IFN γ induce histone H4 (but not histone H3) acetylation at the CXCL10 promoter but whether this is exaggerated at the asthmatic CXCL10 promoter is unreported [39]. Future determination of how specific genes are epigenetically regulated may provide novel therapeutic targets.

3.2. Proliferation

ASM phenotype plasticity, characterized by reversible switching between contractile and proliferative phenotypes, contributes to increased ASM mass and airway hyperresponsiveness in asthma [40]. There is currently no direct evidence of a role for epigenetic regulation of ASM cell proliferation. However, vascular smooth muscle cells have been shown to undergo profound phenotypic changes during neointima formation within atherosclerotic plaques and in the development of PAH and there is some evidence suggesting that proliferation is under epigenetic control in these cells. Serum response factor (SRF) is an important mediator of transcriptional activation of genes that exhibit smooth muscle cell-restricted expression. Recent studies have determined that binding of SRF to DNA is associated with post-transcriptional histone modifications including di-methylation of lysine residues 4 and 79 on histone H3, acetylation of lysine 9 on histone 3 and acetylation of histone H4 [41]. As modulators of histone acetylation at the site of genes which control the cell-cycle, HDACs have also been implicated in regulating the proliferation of smooth muscle cells [42] with the HDAC inhibitor TSA being shown to inhibit proliferation [43].

3.3. Remodeling

Inappropriate remodeling of airways or blood vessel walls in asthma or vascular disease may result from an imbalance in the production of ECM proteins and their degradation by matrix metalloproteinases. In asthmatic ASM cells, altered patterns of ECM production including increased collagen I deposition, appear to contribute to increased proliferative responses [29, 44, 45] but there is as yet no evidence for an epigenetic mechanism regulating ECM production. Histone acetylation modifications have been implicated in the regulation and/or functional expression of a number of matrix metalloproteinases including MMP1, MMP2, MMP3, MMP9 and MMP13 suggesting that epigenetic changes may be intrinsically involved in airway or blood vessel remodeling. However, these observations have primarily been made in tumor cell lines and evidence is not yet available from primary smooth muscle cells [46].

Dysregulation of genes involved in smooth muscle cell proliferation and remodeling in asthma has received little attention, however we have recently demonstrated a role for epigenetics in the regulation of VEGF expression in this disease[47]. VEGF is a key angiogenic molecule that is overexpressed in asthmatic sputum and tissue and contributes to the increased number and size of airway blood vessels in asthma [48]. VEGF secretion in ASM cells from asthmatic patients is greater than from non-asthmatic controls [49] due to differential histone H3 lysine 9 methylation (H3K9me3) modulating Sp1 and RNA polymerase II binding to the VEGF promoter[47].

There is much more information known about epigenetic dysregulation of vascular remodeling. Endothelin-1 (ET-1) is a potent vasoconstrictor and co-mitogen for vascular smooth muscle (VSM), where it plays a well-known role in vascular remodeling in PAH. ET-1 is overexpressed in the lungs of patients with PAH [50, 51] but this has not been pinpointed to the smooth muscle cells and little attention has been paid to the molecular mechanism regulating the increased expression. However, cooperative acetylation of NF- κ B and histone H4 at the preproET-1 promoter are responsible for the synergistic expression of ET-1 in response to TNF α and IFN γ , therefore it could be speculated that aberrant histone acetylation mediates ET-1 overexpression in PAH.

Bone morphogenetic protein (BMP) signaling represents another example of epigenetic dysregulation in PAH. BMP receptor 2 (BMPR2) mutations are causal of familial PAH and an important target of BMP signaling are the *Id1* genes. Hypoxia is a potential “second hit” in familial PAH and is implicated in the pathogenesis of non-familial PAH. In both familial and non-familial PAH, hypoxia can contribute to the observed vascular remodeling [52]. Hypoxia suppresses BMP signaling and *Id1* expression in pulmonary artery smooth muscle (PASM) cells, via increased HDAC1 recruitment to the BMP response element (BRE) region of the *Id1* promoter. Furthermore, hypoxia induced repression of *Id1* expression is abrogated by the HDAC inhibitor TSA [52]. Whether hypoxia regulates transcription of other remodeling genes in PAH or in other lung diseases via regulation of HDACs is unclear.

Extracellular superoxide dismutase (EC-SOD) appears critically involved in the development of PAH *in vivo*. Its overexpression attenuates the development of PAH secondary to pulmonary fibrosis in a bleomycin model via protection against medial, adventitial and intimal layer remodeling of the pulmonary vessels wall [53]. EC-SOD overexpression also attenuates chronic hypoxia induced PAH [54] and EC-SOD gene transfer ameliorates monocrotaline induced PAH, reducing medial wall thickening and pulmonary microvessel muscularization [55]. EC-SOD is more highly expressed in human PASM cells than pulmonary artery endothelial (PAEC) cells, due to increased methylation of the 5'-flanking region of the EC-SOD gene in PAECs causing repressed transcription [56]. It is possible that repression of EC-SOD synthesis by differential CpG methylation of the EC-SOD gene in the PASM cells may contribute to the development of PAH.

The epigenetic mechanisms contributing to the regulation of genes involved in remodeling of smooth cells are summarized in Fig 3.

4. miRNA mediating ASM cell functions

The role of miRNA-mediated gene silencing mechanisms in regulating smooth muscle cell functions was first described in vascular smooth muscle cells. These studies described upregulation of miR-21 following balloon injury, established that miR-21 stimulates vascular smooth muscle cell proliferation and determined that knockdown of miR-21 expression negatively affects neointimal formation [57]. From these results, it quickly became apparent that miRNA represented a key mechanism regulating smooth muscle cell phenotypic plasticity. This was solidified by the identification of miR-143 and miR-145 as

modulators of vascular smooth muscle cell plasticity [58] and response to injury [59] that directly target the network of transcription factors important for smooth muscle-specific gene expression including SRF, myocardin and Krüppel-like factor (KLF) 4 [58]. Subsequent studies have now described miRNA expression and its role in phenotypic regulation of smooth muscle cell fate in airway smooth muscle [60, 61], smooth muscle from the gastrointestinal tract [25] and in uterine smooth muscle [55]. The implications of miRNA functions to pulmonary vascular disease have recently been thoroughly reviewed [28]. The purpose here will be to highlight the studies in ASM cells, summarized in Table I, and how the miRNA identified to date can provide insight into normal and pathogenic ASM functions.

4.1. miR-25 in ASM cell phenotype

Work by Kuhn *et al* was the first to identify a set of miRNA regulated by inflammatory mediators in human ASM cells [60]. Using the combined pro-inflammatory stimulus consisting of IFN γ /TNF α /IL-1 β , miRNA array profiling demonstrated global downregulation of miRNA expression. This suggests that, at least *in vitro*, the initial inflammatory response of ASM is a widespread increase in gene expression, as a result of repressed gene-silencing and perhaps epigenetic modifications. Further analysis of specific miRNA in this study identified miR-25 as having a broad role in regulating ASM cell phenotype. Inhibition of miR-25 reduced expression of myosin heavy chain, decreased eotaxin and RANTES release, and had a broad impact on expression of genes involved in ECM turnover and remodeling. One target of miR-25's actions is KLF-4, which is also targeted by miR-145 [62], miR-146a [63] and miR-1 [64]. KLF-4 acts as a transcriptional repressor of smooth-muscle specific gene expression by recruiting histone H4 deacetylase activity to smooth muscle cell genes, thereby blocking SRF association with methylated histones and CA β G box chromatin [41]. Recent conference reports further established the role of miR-25 in phenotypic plasticity by demonstrating that miR-25 expression reduces proliferation of ASM cells, possibly through inhibition of cyclin D1 [65]. The role of miR-25 *in vivo* has yet to be reported. However, recent profiling studies of miRNA expression have shown that, as expected, miR-25 expression is significantly downregulated with prolonged OVA-sensitization and challenge [66].

4.2. miRNA targeting RhoA

Up-regulation of RhoA is associated with enhanced ASM contractility in animal models of asthma [67] and has gained momentum as a potential therapeutic target [68]. In cardiac myocytes, upregulation of miR-133 during hypertrophy corresponds to a decrease in RhoA expression [69] and binding sites for miR-133 in the RhoA 3'UTR have been predicted. In ASM, miR-133a targeting RhoA expression was demonstrated both *in vitro* in ASM cells and in OVA-sensitized animals [70]. Antagomirs, which are modified oligonucleotides designed to target specific miRNA sequences, are commonly used as molecular inhibitors of miRNA expression and functions. Expression of a miR-133a specific antagomir upregulated RhoA in ASM cells and provided evidence of direct targeting of RhoA by miR-133a. However, in OVA-sensitized mice both RhoA and miR-133a expression were increased. This may be due to regulation of miR-133a expression by IL-13, which mediates allergic inflammation in the OVA model. The future of this approach to asthma treatment relies on

additional data exploring the efficacy of miR-133a inhibition *in vivo* and in other animal models of asthma.

4.3 miR-206 in ASM innervation

Innervation of the respiratory tract regulates smooth muscle tone by controlling reflex responses necessary for coughing and sneezing [19]. Changes in innervation are associated with allergic airway disease [71] and in a mouse asthma model, expression of the p75 neurotrophin receptor contributes to neuronal hyperreactivity in the airway [72]. Radzikinas et al (2011) recently determined that ASM innervation and branch formation is dependent on brain-derived neurotrophic factor (BDNF) [73]. This work identified targeting of the BDNF transcript by miR-206, which had previously been identified as one of the miRNA induced during skeletal muscle differentiation [74]. miR-206 expression in ASM is blocked by sonic hedgehog signaling, which is activated during innervation to increase BDNF. While many studies to date have focused on role of miRNA expression on ASM remodeling events associated with the inflammatory response, the results from this study underscore the need to not overlook interactions of ASM with surrounding tissues and cell types critical to lung function.

4.3. miR-26a in hypertrophy

In asthmatics, inflammatory events increase epithelial mucosal secretions, induce airway constriction leading to hyperreactivity, and result in clinically relevant airway remodeling evidenced by hyperplasia and hypertrophy of ASM [75]. Mohamed *et al* (2010) used mechanical stretch as an *in vitro* model to identify miRNA candidates involved in ASM cell hypertrophy [76]. Microarray analysis identified miR-16, miR-26a and miR-140 as potential candidates. However, only inhibition of miR-26a affected cell size and protein synthesis, while forced expression of miR-26a promoted hypertrophy. Glycogen synthase kinase 3 β , a recognized mediator of ASM hypertrophy in animal models [77], was consequently identified as a target of miR-26a-mediated silencing. Further studies have verified the role of miR-26a in hypertrophy using ASM cells isolated from desmin null mice, which exhibit hypertrophy and express upregulated miR-26a [78] and have established that the early growth responsive protein-1 activates the miR-26a promoter via ERK 1/2. The notion that mitogenic or inflammatory signaling pathways may transcriptionally or post-transcriptionally regulate miRNA expression in ASM cells is intriguing and is supported by recent studies of miR-146a regulation in ASM cells. Work by Larner-Svensson *et al* (2010) found that IL-1 β stimulated miR-146a expression was regulated transcriptionally by the NF- κ B pathway through I κ B kinase, while the JNK 1/2 and MEK 1/2 signaling pathways regulated miR-126a post-transcriptionally via its primary miRNA sequence [79].

4.4. Interactions with the miR-143/miR-145 cluster in ASM cells

In light of the established roles of the miR-143/miR-145 cluster in phenotypic plasticity and response of vascular smooth muscle cells to injury [58, 59], it is worth noting that a detailed analysis of airway architecture and phenotype in miR-143/miR-145 knockout animals [80] has not been reported. Since these animals are viable and do not display any obvious abnormalities, one can conclude that the miR-143/miR-145 cluster is not essential for

development. Upregulation of miR-143 and miR-145 was reported with long term OVA-sensitization [66] and miR-145 was increased 5-fold in profiling studies using the house dust mite (HDM) model [81], but data in asthmatic patients is lacking. That is not to say that miR-143/miR-145 expression is not an important mediator of ASM phenotype. Both miR-143 and miR-145 are expressed in human asthmatic biopsies containing a heterogeneous cell population, although their expression levels were not significantly different compared to control patients or with corticosteroid therapy [61]. miR-143 and miR-145 were also down-regulated with pro-inflammatory stimulation in human ASM, although again not significantly when compared to non-treated cultures [60].

5. Epigenetics and asthma : potential for therapeutic targeting

Epigenetic regulation affects the transcriptional program of an individual cell without altering the underlying genetic code and importantly, these changes are reversible. A number of studies have suggested that epigenetic changes may be one of the factors responsible for the increasing prevalence of asthma in developed countries [82]. Many of these changes are suggested to be influenced by prenatal exposure to environmental factors including diet, exposure to cigarette smoke and air pollution and have been recently reviewed in detail by Durham et al (2011) and Lovinsky-Desir and Miller (2012) [83, 84].

Although the data relating to epigenetic regulation of gene expression in individual cell types in asthma, such as ASM cells, is limited and is often restricted to the analysis of a single gene in a single cell type, the more global epigenetic changes induced by environmental exposure have the potential to simultaneously regulate expression of a variety of genes across multiple cell types.

Epidemiological studies have identified a role for maternal diet in the development of asthma [85, 86] with *in utero* exposure to folate being associated with increased risk of developing the disease. *In vivo* studies in ovalbumin challenged mice showed increased airway inflammation, Th2 cytokines, serum IgE and hyperresponsiveness in pups of mothers fed a high methylation diet [87]. The dietary change appears to promote an altered pattern of methylation at specific CpG motifs resulting in differential expression of genes which regulate the Th2 immune response and airway remodeling.

There is also continuing interest in the association of prenatal exposure to cigarette smoke with development of childhood asthma [88]. Although not all studies have reported a direct association between smoking and global DNA methylation [89], epigenetic mechanisms have been implicated in global and gene-specific changes in DNA methylation in children exposed to tobacco smoking *in utero* [90]. Smoking is believed to interfere with DNA methyltransferase binding resulting in hypomethylation of DNA [91]. As DNA methylation is often a mechanism for repressing or silencing gene expression, the loss of repression may result in an increase in expression of pro-inflammatory mediators and genes which regulate airway remodeling. In addition to altering DNA methylation patterns, cigarette smoke also downregulates expression of the histone deacetylase, HDAC2. The loss of this deacetylase activity may contribute to the altered HAT/HDAC ratio seen in both children [92] and adults [93] with asthma and, as described in a recent conference report, the elevated global HAT

activity may subsequently lead to increased expression of pro-inflammatory genes including CXCL8 [34].

By assessing the links between environmental exposure and the onset of asthma, DNA and histone methylation as well as histone acetylation have been identified as potential targets for therapeutic intervention in asthma. From a therapeutic standpoint, steroids and β 2 agonists are currently the mainstay of asthma therapy. Although these drugs were not developed with epigenetic targeting in mind, they have been shown to reduce global HAT activity and increase global HDAC activity [94]. In addition, dexamethasone, fluticasone, salbutamol and salmeterol have all been previously shown to inhibit TNF α induced H4K5/12Ac at the eotaxin promoter in ASM cells [36].

There is now clear evidence that histone acetylation plays a role in the transcriptional regulation of cytokine and chemokine expression, and in a recent conference report it is emerging that histone acetylation is altered at the promoters of cells isolated from asthmatic patients[34]. We know that global HAT activity is increased, and global HDAC activity decreased in biopsy tissue from asthmatic patients [94], but it is still unclear whether this results in changes in a single cell type e.g. ASM cells alone or alters gene regulation patterns in numerous cells types in the airways. As discussed above, the epigenetic regulation of a small number of genes with a potential role in asthma has been investigated in ASM cells and all have been found to be hypersecreted in asthmatic cells. If the global HAT/HDAC balance is altered to favor acetylation in asthma, a global increase in transcription would be expected, including increased transcription of anti-inflammatory and anti-proliferative genes. It will therefore be interesting to determine whether the epigenetic mechanisms which promote the asthmatic phenotype result from changes at promoters which regulate pro-inflammatory and pro-remodeling genes while those with anti-inflammatory and anti-proliferative roles are protected from the effects of increased global HAT activity. Although the anti-inflammatory effects of steroids are undoubtedly beneficial in asthma, their inhibitory effects on global histone acetylation, which may have widespread effects on numerous gene promoters including those that are potentially protective in asthma, suggest that in future it may be beneficial to attempt to design drugs capable of targeting therapy to specific promoters.

Although most observations of inflammatory mediator epigenetic modulation involve changes in histone acetylation, the importance of other epigenetic modifications should not be overlooked. As an example, VEGF hypersecretion in asthmatic smooth muscle cells is regulated differently and appears to be dependent on histone lysine methylation and a loss of repression of VEGF [47]. Thus, drugs which modulate histone methylation may also prove to be an important therapeutic target for treating asthma.

6. miRNA in asthma

Expression profiling has been used as an approach to identify differential miRNA expression in disease and has proved to be particularly useful in generating data from mouse models of asthma. A summary of the key miRNA identified in these studies can be found in Table II. Garbacki and colleagues(2011) performed one of the most thorough studies to date

profiling miRNA expression in the lung following short, intermediate and long-term exposure to OVA [66]. Both induction and repression of miRNA expression was noted at each time point with miR-146b the only miRNA remaining upregulated throughout the entire time course. In contrast, miR29c was progressively downregulated with prolonged OVA exposure. miR-29 could be an intriguing candidate for novel epigenetic therapies since the miR-29 family (miR-29a, b, c) targets the DNA methyltransferases Dnmt3a and Dnmt3b and forced expression of miR-29s restores normal DNA methylation patterns in lung cancer cell lines [95]. miRNA profiling studies are most useful when changes in expression can be functionally verified with identification of specific mRNA targets. Garbacki *et al* (2011) experimentally verified targeting of miRNA to the 3'UTR of potential mRNA candidates and used bioinformatics analysis to categorize the types of regulatory pathways affected during the progression of disease in this mouse model [66]. Their analysis highlighted the differences in cell cycle signaling, inflammatory response and ECM regulation at each time point with changes in matrix metalloproteinases, IL-13 and TGF β signaling common to all stages of the disease. miRNA expression has also been profiled using house dust mite (HDM) as a model of human disease. Studies by Mattes *et al* (2009) observed a significant increase in miR-16, miR-21 and miR-126 in the airway wall 24 hours after HDM challenge [96]. Silencing miR-126 with intranasal administration of antagomirs suppressed that asthmatic phenotype by diminishing IL-5 and IL-13 secretion, reducing airway hyperreactivity and eosinophilia. This was one of the first reports to suggest that an inhaled targeted delivery of miRNA inhibitors could affect lung function. This effect, however, appears to be specific to short term HDM exposure since inhibition of miR-126 in a chronic model of OVA sensitization suppressed eosinophil recruitment but did not affect airway remodeling [81]. Longer term exposure to HDM in a subsequent study identified upregulation of let-7b, miR-21 and miR-145 [81]. However, only inhibition of miR-145 with intranasal antagomirs suppressed allergic inflammation and reduced airway remodeling. These effects were comparable to dexamethasone treatment. Thus, it remains to be seen whether targeting miRNA expression can improve existing treatment paradigms or stand-alone as alternatives.

Targeting IL-13 expression in mouse models of asthma has also proven successful using miRNA-based mimics. Kumar *et al* (2011) reported that increased IL-13 production in human T cells was inversely correlated with expression of the let-7 family of miRNA and identified targeting of let-7 to the IL-13 3'UTR [97]. Intranasal administration of chemically modified oligonucleotides designed to mimic let-7 reduced IL-13 levels in mice acutely sensitized to OVA and reduced hallmarks of allergic inflammation, including mucus secretion, fibrosis, airway inflammation and hyperreactivity. Thus, let-7 represents a promising target for miRNA-based interventions, although the importance of let-7 expression to other animal models of asthma or to human disease remains to be determined. In a transgenic model of IL-13-induced allergic asthma, overexpression of miR-21 was again identified and found to target IL-12p35 [98]. Targeted ablation of miR-21 in OVA-sensitized mice leads to increased Th1 responses with increased IFN γ and reduced eosinophilia consistent with suppressed IL-12p35 expression [98]. Regulation of Th1 and Th2 responses by miR-21 in the lung represents a paradigm shift in what is known about polarization of T cells during the allergic immune response.

In human asthmatic airways, miRNA expression has been measured in lung biopsies from mild asthmatics but no significant changes in miRNA expression were noted in expression of 227 profiled miRNA [61]. This same study analyzed the effect of inhaled budesonide treatment and after 1 month of treatment did not find any significant difference in miRNA expression despite improved lung function in the patients. This result was surprising but perhaps not all that unexpected since asthma is a complex, multi-cellular, progressive disease and miRNA expression data was obtained from airway biopsies containing a mixture of cell types at only one time point. Indeed, the authors went on to analyze miRNA expression in individual cell types from the lung, including epithelial cells, ASM cells, alveolar macrophages and lung fibroblasts and reported distinct miRNA expression profiles in each cell type. This suggests that cell-specific gene silencing may be a significant mechanism used to regulate gene expression in a heterogeneous population. Analysis of miRNA expression in severe asthmatics or *in vitro* in individual cell-types, such as ASM cells, from asthmatic patients will provide much needed data on the importance of cell-type specific miRNA expression to the pathogenesis of asthma. As an example, studies in alveolar epithelial cells and macrophages have shown that miR-146a expression negatively regulates IL-8 and RANTES release [99], while evidence in ASM cells does not support a role for miR-146a in IL-6 or IL-8 release [79].

Another approach to understanding the role of miRNA expression in asthma is to identify miRNA binding sites in known or potential susceptibility genes. This can be done *in silico* using miRNA target site prediction algorithms such as TargetScan [100], MiRanda [101] and PicTar [102]. In this way, miRNA binding sites have been identified on the class 1 histocompatibility antigen G (HLA-G). HLA-G was described as an asthma susceptibility gene in families with a history of asthma where the child's *HLA-G* genotype correlated with hyperresponsiveness of the mother [103]. Prediction algorithms identified binding sites for miR-148a, miR-148b and miR-152 in the HLA-G 3'UTR and this result was verified experimentally to demonstrate that these miRNA target HLA-G for gene-silencing [55]. Subsequent experiments identified miRNA binding sites in single nucleotide polymorphisms of HLA-G and suggest that miRNA binding at these sites contributes to HLA-G expression [104].

7. Future directions

Epigenetic and miRNA research is already contributing to an improved understanding of the mechanisms that regulate the development of many diseases including asthma, cancer and vascular disease [105]. In many disciplines, these studies are still in their infancy, focusing on the changes which regulate expression of a single gene within a single diseased cell type. Future studies will need to build on these early observations to assess epigenetic and miRNA-mediated changes across a range of genes and cells types within diseased tissues as well as gaining clearer insights into the more complex interactions which occur when multiple mechanisms contribute to altered patterns of gene expression within diseased cells.

A number of research tools have been developed to characterize and functionally analyze the wide variety of epigenetic changes which occur throughout the entire genome. This research, known as epigenomics, should provide insights into the importance of epigenetic

modifications in both normal development and the transition to diseases states and has the potential to detect quantitative changes, multiplex modifications and also identify regulatory sequences that all combine to control gene expression [106]. Investigating epigenetic changes throughout the genome is likely to be a complex process given the large number of potential modifications and the dynamic nature of the epigenomic landscape, as this may result in the epigenomic profile changing during disease progression. The same can be said for miRNA-mediated changes in gene expression since one miRNA is predicted to target hundreds of mRNA transcripts [13]. There is also evidence that the enzymes responsible for epigenetic modifications are themselves targets of miRNA-mediated gene silencing [95, 107], and interactions between these processes are an opportunity for new avenues of investigation in the lung. The few examples of cell-type specificity that have been highlighted here suggest that global miRNA expression profiling in heterogeneous tissues may not prove useful in delineating individual effects of miRNA on specific cell types.

The currently available epigenetic therapies target one specific type of epigenetic abnormality, but it is becoming clear that many complex diseases may result from a combination of diverse epigenetic modifications. DNA methyltransferase inhibitors or HDAC inhibitors may be effective therapies in diseases such as cancer which result from abnormal silencing of gene expression but these drugs would be ineffective in diseases resulting from an increase in gene expression. Although data on disease-associated epigenetic changes in smooth muscle cells is limited, most of the functional and phenotypic changes in these cells have been associated with gene activation rather than gene repression, and therefore drugs which inhibit histone acetylation or promote histone methylation to silence hyperactive genes may be more effective.

In contrast, the promise of developing viable therapeutic interventions based miRNA-mediated gene silencing is quickly becoming a reality and the lung represents an ideal target for localized delivery. One of the first examples of RNA interference therapy delivered antisense oligonucleotides intranasally to inhibit respiratory syncytial virus replication in mice [88]. Indeed, miRNA represent a versatile target for therapeutic intervention with means that either inhibit or mimic miRNA function. Pre-clinical studies targeting inhibition of miR-208 and miR-15 in heart failure[108] and ischemic injury [109] are currently underway, while miRNA replacement therapy for either let-7 or miR-34a is a strategy being explored in non-small cell lung cancer. The identification of small-molecule inhibitors is as yet an unexplored option that may yield compounds effective in modifying epigenetic changes and/or miRNA expression. Continued study of these processes in ASM using both *in vitro* and *in vivo* methods, coupled with future additional drug discovery and development may yield exciting new therapeutic approaches to lung disease.

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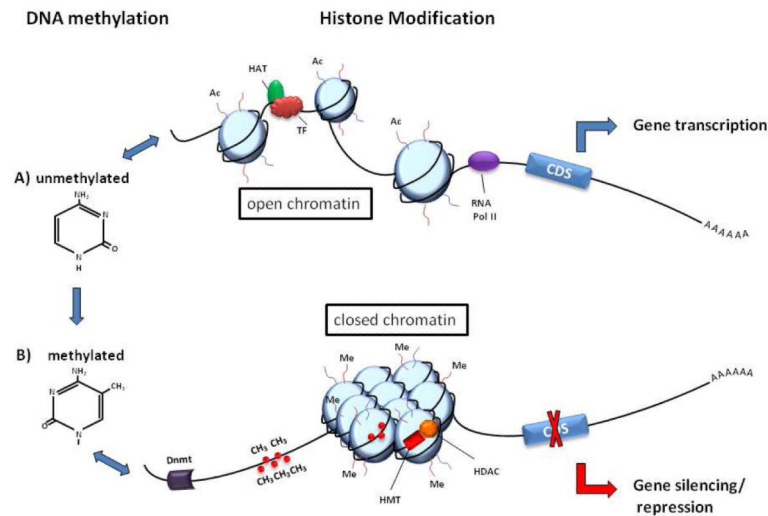


Fig 1. Epigenetic regulation of gene expression

A) Histone modifications occur on the N-terminal tails of the four core histones. Histone acetylation (Ac) by histone acetyltransferases (HATs) stabilizes the unmethylated DNA in an open chromatin conformation to allow for transcription factor (TF) binding, recruitment of RNA polymerase II (RNAPol II), mRNA expression and the synthesis of proteins coded by the coding DNA sequence (CDS). B) DNA methylation occurs when DNA nucleotide methyltransferase (Dnmt) adds methyl groups (CH₃) to cytosine residues at CpG sites. Association of methylated DNA with histone deacetylases (HDAC) and histones methylated (Me) by histone methyltransferases (HMT) closes the chromatin structure to inhibit TF binding and silence gene expression.

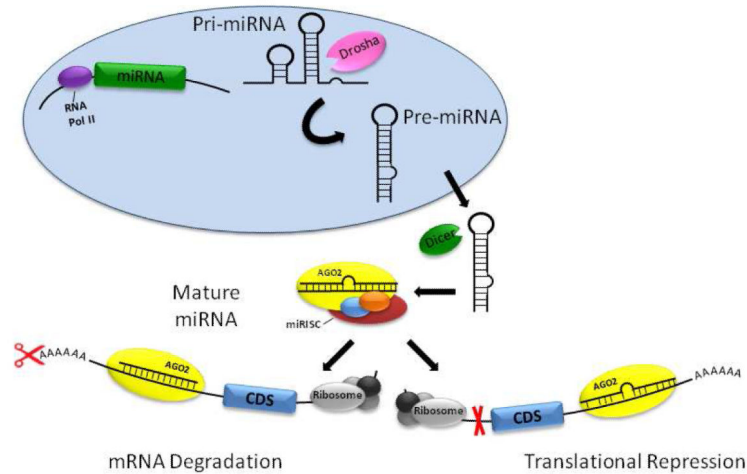


Fig. 2. miRNA biogenesis and function

miRNA are transcribed by RNA polymerase II (RNAPol II) as primary miRNA (pri-miRNA). The transcripts are cleaved by the ribonuclease Drosha to form precursor miRNA (pre-miRNA). Pre-miRNA are exported out of the nucleus and further cleaved by Dicer to yield a mature miRNA duplex bound by a miRNA-associated RNA-induced silencing complex (miRISC). Binding of complementary mRNA sequences by one strand of the miRNA leads to Argonaute (AGO2 in mammals) mediated cleavage, resulting in decreased mRNA stability and transcript cleavage or translational repression and reduced synthesis of proteins coded by the coding DNA sequence (CDS).

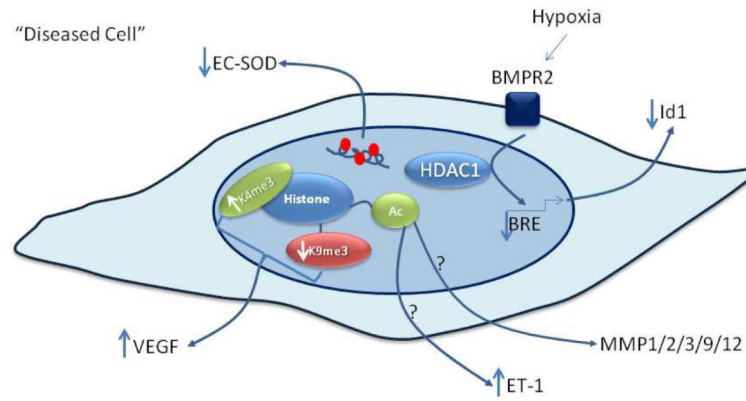


Fig. 3. Epigenetic regulation of genes associated with remodeling

Histone lysine methylation regulates differential VEGF expression from ASM cells isolated from non-asthmatic donors compared to non-asthmatic donors. Histone acetylation is involved in the transcriptional regulation of Endothelin-1 (ET-1) and MMPs but its involvement in dysregulation of these genes expression in disease has yet to be investigated. Increased CpG methylation of the 5' flanking region of the extracellular superoxide dismutase (EC-SOD) gene represses EC-SOD expression and may be implicated in pulmonary arterial hypertension pathogenesis.

Table I

Functions of miRNA Reported in Airway Smooth Muscle

Inflammatory Response	Proliferation	Remodeling	Innervation
let-7	miR-25	miR-16	miR-206
miR-25		miR-25	
miR-133a		miR-26a	
miR-143/miR-145		miR-133a	
miR-146a		miR-146a	

Table II

miRNA identified in Animal Models of Asthma

	IL-13	HDM		OVA	
		ST	LT	ST	LT
let-7	↓		↑		
miR-16		↑			
miR-21	↑	↑	↑	↑	
miR-29c				↓	↓
miR-126		↑			
miR-145			↑		↑
miR-146a				↑	↑

ST, short-term; LT, long-term; ↓ downregulated; ↑upregulated