Epigenomics

Epigenome-modifying tools in asthma

Asthma is a chronic disease which causes recurrent breathlessness affecting 300 million people worldwide of whom 250,000 die annually. The epigenome is a set of heritable modifications and tags that affect the genome without changing the intrinsic DNA sequence. These marks include DNA methylation, modifications to histone proteins around which DNA is wrapped and expression of noncoding RNA. Alterations in all of these processes have been reported in patients with asthma. In some cases these differences are linked to disease severity and susceptibility and may account for the limited value of genetic studies in asthma. Animal models of asthma suggest that epigenetic modifications and processes are linked to asthma and may be tractable targets for therapeutic intervention.

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Asthma is a chronic disease of the airways that causes reversible difficulty in breathing through bronchoconstriction, mucus hypersecretion and airway remodeling and afflicts over 300 million people worldwide [1,2]. Asthma is a heterogeneous disease that can be atopic or nonatopic, and demonstrates various subclinical phenotypes [3]. The variety in asthma phenotypes provides challenges to treatment as phenotyping asthma is neither easy nor readily affordable. This may account for the failure of many drugs to proceed beyond early Phase II studies as patients are not adequately phenotyped [4]. Until better matching of phenotypes to driver pathways or molecules is achieved, phenotype-specific treatment using expensive biologicals for example will not be cost effective [5].

The adaptive immune response in asthma is regulated by CD4$^+$ T-cell subsets. The principle two subtypes of T helper cells are type 1 (Th1) and type 2 (Th2) which drive the cellular and humoral immune responses respectively [6]. Asthma is characterized at the cellular level by hyper-responsiveness of Th2 in both atopic (allergic) and nonatopic asthmatics [7,8]. In susceptible individuals, the Th2 response is stimulated by environmental effectors including allergens, temperature, humidity and air pollution [6]. This process of activation in allergic asthma begins with the airway epithelium, which upon stimulation releases factors which subsequently activate phagocytic cells and together they enable the activation of Th2 cells [6]. Th2 cells are able to self-propagate by the release of IL-4; drive infiltration of eosinophils by releasing IL-5 and activate B cells, which release antibodies against the allergens, by releasing IL-13 [6]. IL-13 also plays a major modulating role on airway epithelial cells increasing the production of mucins, periostin and other mediators [9].

Other T-cell subtypes also play a role in asthma, including the balance of the IL-17-producing Th17 cells and Treg. Th17 cells are associated with neutrophilic inflammation [10] and have been shown to contribute to severe asthma and relative corticosteroid insensitivity [11]. By contrast, Treg cells are able to repress cytokine release and proliferation from other T-cell subtypes [12], including

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Th17 cells. The balance of Th17/Treg cells in peripheral blood of asthmatics is skewed toward Th17 [13,14] which inhibits the resolution of inflammation and increases neutrophilic infiltration.

Generally asthma is well controlled by inhaled corticosteroids and bronchodilators; however, 10% of patients suffer from 'severe' asthma that is poorly controlled even by high doses of inhaled and oral steroids and other treatments such as theophylline, anti-IgE or leukotriene receptor agonists [5]. While asthma can be controlled it cannot be cured, therefore the development of new treatments and identification of novel drug targets are a priority in asthma research.

Asthma has been shown to have a heritable component in large twin studies [15], and by using polygenic heritability estimates [16]. A heritable phenotype can be the product of many different mechanisms; these can be divided into two categories: those that change the DNA sequence and those that do not. To date much of the research into the heritability of asthma has focused on the changes to the DNA sequence, however large-scale genome-wide association studies (GWAS) have only identified a handful of genetic changes or SNPs linked to asthma which are highly significant on a population scale but not predictive at the individual level [17,18]. It is also unknown whether these changes are causative and it is likely that causality will be linked to different environmental exposures in selected sub-phenotypes of asthma.

Research is therefore becoming more focused on heritable characteristics that are not due to altered DNA, termed epigenetic modifications, and the sum of these modifications termed the epigenome. These epigenetic processes include modifications to DNA-binding histones, applying methylation marks to cysteine in DNA and noncoding RNAs such as miRNA [19].

To date studies of the epigenome in asthmatics have demonstrated changes in monocytic DNA methylation [20] and histone modification [21], blood leukocyte [22] and eosinophil [23] methylation, CD4+ T-cell histone modifications [24–26] and smooth muscle and T-cell miRNA expression [27,28] and histone acetylation at distinct residues [29] compared with their healthy controls. What these data are unable to show is whether these changes cause, or are a result of, asthma although animal models may help resolve this issue.

Various techniques have been developed to investigate epigenetic regulation of gene expression. These include using methods such as chromatin immunoprecipitation (ChIP) to confirm specific histone modifications at single gene promoters or at a genome-wide level, or to map the location of specific histone modifications with chromatin structure (using ChIP-Seq [24] and DNase 1 hypersensitivity [30]), measuring DNA methylation (bisulfite sequencing using array or next-generation sequencing [31]) and expression profiles of noncoding RNA (gene array or PCR-based analysis [32]). There is growing number of available tools which can alter the extent to which cells modify histones and methylate DNA which means we can begin to investigate the role of epigenetic modifications in asthma and identify potential new therapeutics. It is important to note that each cell type has a distinct epigenome and it is important to examine changes with disease in single cell types [33] or to use bioinformatic tools to deconvolute data to allocate to single cells [23].

The encyclopedia of DNA elements (ENCODE [34]) a project to catalog the regulatory elements in human cells and its follow-up of the Epigenome Roadmap [35] are two projects that have built reference epigenomes for 127 tissue and cell types [36,37]. The Epigenome Roadmap has been able to gather reference epigenomes for a variety of T-cell subtypes and B cells and this information is ripe for analysis if compared with more asthmatic samples.

Histone modifications

Nucleosomes are octomeric complexes of histone proteins which bind to DNA. Each octomer comprises two pairs of the histones H2A and H2B and two pairs of the histones H3 and H4 [38]. 146bp of DNA is wrapped and stored around each nucleosome which are tethered together by histone H1 [39].

Histones serve multiple functions in normally functioning cells: they store DNA safely [38], they can stop transcription by tightly coiling DNA [40] and they can encourage transcription by forming a suitable local structure to enable accessibility of transcription factors [41]. When the DNA is loosely bound to the histones in what is known as the euchromatin or 'open' state, transcription may readily take place [42]. However, when histones are tightly bound to DNA, known as the heterochromatin or 'closed' state, DNA polymerase II and transcriptional activators are unable to readily access DNA and transcription is limited. The strength of binding between histones and DNA can be controlled by post-transcriptional modifications to the N-terminal tail of the histone proteins, usually pertaining to the addition of acetyl or methyl groups to amino acids such as lysine and arginine [43].

These modifications can change the charge of the histones which either repels DNA if more negatively charged, or attract DNA if more positively charged [44]. Alternatively, these modifications on histones residues form epitopes that enable other proteins including transcription factors and transcriptional co-factors to be recruited to the site of original driver modification in a temporal manner. The site-specific and time-

dependent formation of these histone modifications, including acetylation and methylation, is termed the histone code or language. The histone code is 'read' by proteins termed readers which in turn help recruit the transcription factors. Although the histone code is a useful concept, the myriad numbers of different combinations of modifications that can occur means that this has not been proven. In addition, phylogenetic analysis of histone deacetylases (HDACs) and histone acetyltransferases (HATs) suggests that these existed before histones implicating that acetylation of nonhistone proteins will play an important role in regulating cellular function [45]. Indeed, it is now clear that many cytoplasmic and nuclear proteins including transcription factors, signaling molecules and structural proteins are acetylated.

Histone acetylation
The role of histone acetylation
Histone acetylation is primarily associated with the euchromatin state (loosely packed DNA) and increased transcription as acetylation is associated with a positive electrostatic charges of the histones, repelling the DNA and increasing the DNA's accessibility and ability to be transcribed [44,45]. Multiple lysine residues on a single histone subunit can be acetylated. For example acetylation on histone 3 lysine 9 (H3K9) and lysine 27 (H3K27) have been shown to increase transcription [45,46].

Histone acetylation in asthma
HAT activity is increased in asthmatic biopsies in both adults [47] and in children [48] and the HAT/HDAC ratio alters according to asthma severity. Histone 3 lysine 27 (H3K27) acetylation (H3K27Ac) is associated with the enhancer regions of genes that are actively being expressed [49]. H3K27 is acetylated by the HAT Gcn5. This tag has been shown to be an important component of the histone code and re-analysis of asthma GWAS achieved greater predictive power by only using SNPs in coding or regulatory regions and linking this to H3K27Ac and H3K4me1 marks in different cell types [25]. These two marks are critical for TH2 cell accumulation and differentiation in asthma although the H3K4me2 mark was the most highly enriched at sites encoding transcription factors and microRNAs [24].

Th2 cells and follicular helper T cells (Tfh cells) are the major producers of IL-4 but require distinct and overlapping, molecular mechanisms [50]. The DNA methylltransferase Dnmt3a also regulates Th2, particularly IL-13, expression. Loss of Dnmt3a leads to decreased DNA methylation and changes in the H3K27Ac status at the IL-13 locus and is associated

with increased lung inflammation in a mouse model of asthma [51].

The deposition of acetyl tags appears less selective than the removal of these marks by HDACs as determined in a genome-wide acetylome analysis [52] HDACs remove acetylation marks from both histones and from nonhistone proteins such as the glucocorticoid receptor (GR) and other transcription factors. A combination of GR deacetylation and changes in histone acetylation status are involved in the mechanisms by which corticosteroids reduce the expression of NF-κB-activated inflammatory genes [53]. Defective HDAC2 expression and activity is found in some corticosteroid-insensitive disease cells/tissues and models and corticosteroid sensitivity can be restored by increasing HDAC2 expression [40,54]. This is highly relevant to severe asthma, the form of asthma that remains steroid insensitive.

HDAC1 function is vital to the repair and remodeling of the airway epithelium and epithelial cell growth stops upon HDAC1 inhibition [55]. Epithelial cells form the principle barrier between the lung and the environment and induce the first stages of the immune response during an asthma attack. Increased airway epithelium and remodeling is associated with asthma severity [56] and correspondingly increased HDAC1 is found in patients with severe asthma compared with normal patients and may represent a biomarker to distinguish between severe and nonsevere asthma [57].

In addition to enabling general switching between the eu- and hetero-chromatin states, site-specific histone acetylation also promotes transcription factor binding. One key example for asthma is the binding of the pro-inflammatory transcription factor NF-κB. Acetylation of histones at H3K9 and K27 is recognized by histone code readers including the bromodomain containing family of proteins. One such protein, Brd4, contains two bromodomain regions and is able to bind acetylated histones and acetylated p65 (part of the NF-κB complex). NF-κB p65 activation is controlled by its acetylation status, for example, on K310 and binding of Brd proteins is important for controlling full NF-κB activity in cell-dependent manner [58]. Thus, Brd proteins can bring together nucleosomes and nuclear transcription factors to increase proliferation and inflammation [59,60]. Acetylated histone's ability to bring together DNA and transcription factors positions it as vital pivot for the expression of specific inflammatory genes regulated by NF-κB.

Potential asthma therapies targeting histone acetylation
As histone (de)acetylation plays a role in the activation of inflammation and its resolution, drugs that target histone acetylases and deacetylases have been

investigated in asthma (Table 1). Pan-histone deacety-lase inhibitors, such as Trichostatin A (TSA) and Vorinostat, have been used as cancer treatments and have been investigated in asthma although their efficacy at reducing inflammation remains controversial probably due to their lack of selectivity [61]. TSA reduces inflammation in human precision cut lung slices and in *in vivo* mouse models [61]. In two recent studies TSA treatment reduced inflammation, IL-17 and T-helper cell number while increasing Treg-cell activation [62]. Furthermore, the expression of TGF-β in the bronchoalveolar lavage fluid was increased following HDAC inhibition in a mouse model of asthma [62,63]. These changes were associated with increased acetylation at the TGF-β promoter. It is also of interest to note that this study also showed that severity of asthma was linked to HDAC9 [62].

Vorinostat was the first HDAC inhibitor approved by the US FDA for cutaneous T-cell lymphoma. It was demonstrated to be beneficial in graft-versus-host disease (GVHD) as it increased activation of Tregs [63]. HDAC inhibition reduced plasma cytokine levels without inhibiting T-cell responses to nonspecific stimuli allowing the immune system to continue to function.

While pan-HDAC inhibitors show anti-inflammatory effects in some studies, other studies are unable to replicate these effects [78–81]. There is also evidence that HDAC inhibitors can in fact enhance inflammation through NF-κB-driven inflammatory gene transcription [60–62]. The use of HDAC inhibitors as a potential treatment for asthma is further complicated by the role of HDAC proteins in the anti-inflammatory response to glucocorticoids [61]. As reduced HDAC2 activity is associated with steroid insensitivity, the activation, rather than inhibition of HDAC2, would be beneficial. The drug theophylline has been associated with improved steroid sensitivity in several studies and has a putative mechanism of action which involves restoring HDAC2 activation [82]. It has been shown to improve steroid sensitivity in COPD which may be similar in mechanism to asthma [83].

The multiple roles of histone acetylation in regulating cell function and cell division [84] mean that pan-HDAC inhibitors or activators will almost certainly have large negative side effects if used clinically. More recently, a link between HDAC inhibition of STAT5-activated gene expression and Brd functions has been reported [85]. The consensus is that more work is needed to explore how epigenome modifying drugs will effect acetylation mechanisms in airways disease [86,87], in order to overcome the principle issue of targeting the change in acetylation status to the relevant genes.

Targeted modifications to histone acetylation have been undertaken in neurones using modified transcription factors to increase acetylation at the Fosb gene [88]. If targeted transcription factors can be used to increase acetylation then it is possible that they can be used to decrease acetylation as tools and potentially therapies. Many histone modifications including H3K9 acetylation H3K27me3, H3K9me1 and H4K8ac have been regulated *in vitro* by using light-inducible transcriptional effectors (LITEs). These are a set of blue-light activated restriction enzymes that have been developed for high level temporal and spatial control of target gene expression and may be used to target specific histone effectors and thereby modify the epigenome. For example, H3K9 acetylation was reduced twofold at the target gene Grm2 using this method resulting in repression of Grm2 [89]. This approach may pave the way for epigenome modification *in vivo*.

The bromo and extraterminal (BET) family of proteins recognise lysine acetylation on many proteins including histones [90], which aids the recruitment of transcription factors and the RNA polymerase transcription complex to enable gene transcription [91]. The BET protein Brd4 binds to acetylated histones in the euchromatin state [92] but can also interact with other nonhistone proteins [60] such as NF-κB [93]. Mimics of BET proteins such as JQ1 [94] reduce asthma relevant processes including IL-1β-induced inflammation [95] and proliferation [96]. JQ1 and similar compounds are currently being investigated as anticancer treatments in man [64] but the broad action of the BET family inhibitors may require the development of novel compounds with different selectivity. Alternatively, the use of inhaled delivery to the airways, linked to improved lung retention and rapid systemic breakdown, may reduce the side effect profile to enable thier use in asthma.

Histone methylation

Lysine residues in histone tails can be modified to have between one and three methyl groups: mono, di or trimethylation. The functional effect of the modification depends upon the residue targeted and the number of methyl groups added.

The role of H3K4 methylation

Genes have pretranscriptional regions before transcriptional start site and by modifying this region control can be exerted over gene expression. Histone modifications in this region can either inhibit or activate transcription; known as a bivalent gene or effect. When two contradictory acting histone modifications are bound at the same promoter the inhibitory marker takes precedence [97], leading

Table 1. List of epigenetic modifying tool compounds.

Epigenetic mark	Target	Tool compound	Affected process
Histone acetylation	Pan histone deacetylase inhibitors	Trichostatin A	Cancer treatment reduced IL-17 and T helper cell number, reduced TGF-β in mouse BAL [62,63]
		Vorinostat	Cancer treatment and graft vs host disease [63]
	BET domain mimics – block readers of acetylation	JQ1	Prevent cell cycle progression and investigated as an anticancer treatment [64,65]
Histone methylation:			
– Histone 3 lysine 4 (H3K4)	SETD7	PFI-2	H3K4 is linked to activation of inflammatory responses [26,66]
– Histone 3 lysine 9 (H3K9)	G9a (H3K9 methyltransferase)	UNC0642	Macrophages and dendritic cells of asthmatics undergoing allergen exposure may benefit from the inhibition of H3K9 methyltransferases, to prevent activation of the inflammation
	JMJD2 H3K9 demethylase	ML324	Decrease the effects of Herpes virus in mice [67]
– Histone 3 lysine 27 (H3K27)	JMJ3D (H3K27 demethylase)	GSK-J1 + GSK-J4	Inhibits LPS-induced macrophage inflammation [68]
	Lysine-specific demethylases	Compound 12d	Additive effects with HDAC inhibitors on inhibiting cell proliferation and may inhibit inflammatory cytokines [69]
DNA methylation	DNMT1 complexes		DNMT inhibitors may return T cells to a Th1 phenotype in asthma [70]
	DNMT1/PCNA	Peptide inhibitor - 163–174	Target DNA methylation at specific regions [71]
	DNMT1/USP7	Peptide inhibitor - 561–567	Target DNA methylation at specific regions [71]
	DNMT1/STAT3	Peptide inhibitor - 683–174	Target DNA methylation at specific regions [71]
	DNMT1/CFP1	Peptide inhibitor - 1081–1097	Target DNA methylation at specific regions [71]
miRNAs	miR-34 mimic	MRX34	Inhibits tumor growth [72]
	miR-122		Hepatitis C is supressed by the inhibition of miR-122 [73]
	miR-150	Nanovesicles containing miR-150	Enter effector T cells and suppressing allergic contact dermatitis and promoting antigen-specific tolerance in mice [74]
	miR-9	miR-9 antagamirs	Inhibition of miR-9 increased PP2A activity and GR nuclear translocation in macrophages and restored steroid sensitivity in multiple mouse models of steroid-resistant AHR [75]
	miR-145	miR-145 antagamir	miR-145 antagamir inhibited eosinophilic inflammation, mucus hypersecretion, Th2 cytokine production and AHR in murine model of asthma [76]

AHR: Airways hyper-responsiveness; BET: Bromo and extraterminal domain; GR: Glucocorticoid receptor; LPS: Lipopolysaccharide.

Table 1. List of epigenetic modifying tool compounds (cont.).			
Epigenetic mark	**Target**	**Tool compound**	**Affected process**
miRNAs (cont.)	miR-126	miR-126 antagamir	Blockade of miR-126 suppressed Th2 responses, inflammation, AHR, eosinophil recruitment and mucus hypersecretion in mouse model of asthma via suppression of GATA3 expression [77]
AHR: Airways hyper-responsiveness; BET: Bromo and extraterminal domain; GR: Glucocorticoid receptor; LPS: Lipopolysaccharide.			

to transcriptional pause where the transcriptional complex is bound to the promoter but is halted and unable to generate RNA. Elongation will begin as soon as the inhibitory mark is removed and the RNA polymerase II is able to proceed. Di-methylated histone 3 lysine 4 (H3K4me2) marks are commonly found at poised and active enhancer regions [98].

H3K4 methylation in asthma

Histone methylation has been linked to both T-cell differentiation and function, particularly for the control of CD4+ T-cell differentiation [26]. T-cell fate is principally controlled by the transcription factors T-BET (Th1 cells) and GATA3 (Th2). The ability of these transcription factors to regulate their target genes is dependent on their associated methyltransferase activity [66].

IL-4, IL-5 and IL-13 are all coded for in a single stretch of DNA on chromosome 5 and are separated by the *RAD50* gene. *RAD50* encodes a DNA repair protein [99] and has four conserved enhancer regions in its introns. H3K4me1 modifications at these enhancer regions, are increased in T cells from asthmatic patients [24] and are associated with transcriptional pause. Further environmental signals, such as antigen recognition, trigger other transcription factors to resolve the pause and enable transcription [100]. H3K4me3 is linked to increased transcription of both *IFNG* and *IL-4* [26].

Combining H3K4me2 ChIP-Seq with GWAS in subsets of human peripheral blood T cells (naive, T_H1 and Th2) has shown that the differentiation of Th2 cells is marked by an increased enrichment of H3K4me2 at SNPs within the promoters and cis-regulatory regions of asthma-associated genes including *CCR4* and *CCL5* [24]. CCR4 receptors have shown to be vital in the recruitment of Th2 cells to the lung [101] and CCL5 is chemotactic for T cells [102]. T-cell studies have found patterns of H3K4 dimethylation at enhancers during Th2-cell differentiation that support a pathogenic role in asthma [24]. Using gene ontology software, it was shown that genes associated with mitosis and regulation of apoptosis were most differentially enriched in asthmatics.

Potential asthma therapies targeting H3K4 methylation

At present no therapeutically licenced drugs exist that target histone methylation, although new compounds, such as PFI-2 that target histone methylation have been developed [103]. PFI-2 competitively inhibits the SET domain containing (lysine methyltransferases) 7 (SETD7), a methyltransferase for H3K4 [104], which may play a role in cell stress and inflammation as SETD7 is able to activate expression at NF-κB binding sites.

As H3K4 methylation is associated with the activation of inflammatory and proasthmatic cytokine production preventing histone methyl-transferase activity may be of future benefit to patients. However, as with histone acetylation the ability to target histone modifications at specific sites, such as the asthma SNPs would be the ultimate goal of therapeutic research [24].

The role of H3K9 methylation

The presence of H3K9me3 at gene promoters is associated with gene repression including that of inflammatory genes [105]. H3K9me3 acts by preventing RNA Pol II binding to target gene promoters.

H3K9 in asthma

Airway remodeling is a cardinal feature of asthma and the control of it is mediated in part by VEGF which in asthmatics is hypersecreted by human airway smooth muscle cells (HASM). In asthmatic HASM there is a decrease in the H3K9me3 repressive complex at the promoter of the VEGF gene. The methyltransferase G9a is vital for repression of VEGF in healthy patients HASM [106].

JMJD2D is an H3K9me3 demethylase which removes H3K9me3 repression complexes, activating transcription [107]. In dendritic cells and macrophages, JMJD2D is induced by external stimulus and is required for *Mdc* and *Il12b* transcription. This is an example of how H3K9me3 is able to broadly control functional enhancers linked to cell-type-specific gene expression [105].

Potential asthma therapies targeting H3K9

Inhibitors of both H3K9 methyltransferases and demethylases have been recently developed. These

tools that target the enzymes G9a and JMJD2D prevent activation of the inflammation in macrophages and dendritic cells of asthmatics undergoing allergen exposure [106]. Similar treatments may be of use to limit airway remodeling in HASM cells [106]. UNC0642, a recently discovered inhibitor of G9a, may be a useful tool in future studies [108]. However, G9a inhibition may result in detrimental side effects. For example, while knockout of G9a reduces inflammation in cell culture it is essential for embryogenesis [106,109]. Therefore further research will be required to allow more targeted inhibition of histone methylation at specific gene loci, for example understanding how H3K9 methylation is targeted to specific genes.

The role of H3K27 methylation

H3K27me3 can have different functional effects on gene transcription depending on the location of the histone relative to the gene [110]. First, when the modified residues are located within the body of the gene, H3K27me3 inhibits gene expression; however, when H3K27me3 is found at the transcriptional start site it is associated with the expression of bivalent genes and transcriptional pausing. Finally H3K27me3 in the promoter region of a gene is associated with an increase in transcription [110].

H3K27 methylation in asthma

Trimethylation of H3K27 can be catalyzed by EZH2, which is a subunit of the polycomb repressive complex 2 [97] and has been associated with gene repression. EZH2 is a histone lysine methyltransferase which is highly specific to K3K27, and is vital for CD4+ differentiation and activation [111]. H3K27me3 blocks the production of IL-4 in Th1 cells while in Th2 cells the gene body of the repressed *IFNG* was marked by H3K27me3 [111].

The ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) and JMJD3 are H3K27-specific demethylases [112] which remove the H3K27me3 repressive marks [113]. JMJD3 and UTX regulate chromatin complexes [114], macrophage plasticity in mice, pro-inflammatory gene regulation [115] and T helper cell development [112]. JMJD3 knockdown in THP-1 monocyte cell line and macrophages decreased the effect of inflammatory signaling pathways as measured by gene array, by increasing H3K27me3 at the promoters of NF-κB induced genes and many members of the CD40 and chemokine signaling pathways [113,114].

In a mouse model of asthma, Th2 cells from and *Ezh2-/-* mice were adoptively transferred to wild-type mice which underwent a subsequent acute ovalbumin (OVA) challenge. The transfer of *Ezh2*-KO Th2 cells resulted in in eosinophilia, IL-4, IL-5 and IL-13 and

mucus hyperproduction and enhanced asthma-like pathology [111]. There was also overproduction of IFN-γ indicating activation of both the Th1 and Th2 pathways which is often seen in human asthma. However, other studies suggest that blockade of *Ezh2* is not sufficient to prevent the production of inflammatory cytokines [111].

Epithelial cells respond to IL-4, by demethylating H3K27me3 at the *ALOX15* gene promoter increasing its transcription [116]. *ALOX15* oxygenates polyunsaturated fatty acids to synthesize potent signaling mediators and has been shown to increase in expression with increasing severity of asthma [117]. The *ALOX15* promoter is associated with H3K27me3 and its expression is regulated by UTX [117]. Whether this is causal in asthma, however, is unknown. Tool compounds for this demethylase include the nonselective inhibitor GSK-J4 [68].

Potential asthma therapies targeting H3K27 methylation

As the loss of EZH2 is linked to the asthma phenotype inhibiting the demethylases JMJD3 or UTX may be beneficial in asthma (Table 1). There is currently a selection of new tools and potential drugs available which will target the H3K27me3 modifying enzymes (JMJD3, UTX, EZH2) such as 1-substituted cyclopropylamine [118] an irreversibly binding, nonspecific H3K27 demethylase inhibitor. Blocking JMJD3 in macrophages with the inhibitors GSK-J1 and GSK-J4 prevents LPS-induced inflammation [68].

The phenelzine analog compound 12d is a lysine-specific demethylase inhibitor, it has been shown to have additive effects when used with HDAC inhibitors in inhibiting cell proliferation and may be useful to inhibit inflammatory cytokines as well [69]. Compounds targeting H3K27 methylation have not been therapeutically assessed, nor are all their effects understood. However they do provide potential tools to investigate role of histone methylation in inflammatory gene expression.

Generally histone demethylation is essential for dramatic changes in epigenetic states such as in cell differentiation of T cells; however, demethylase proteins that antagonize repression such as JMJD3 are expressed in terminally differentiated cells. JMJD3 has a separate role in chromatin remodeling which is independent from its H3K27-demethylase function. This demonstrates a role for JMJD3 outside of epigenetic modifications and must be taken into account when using drugs to modify the effects of them [112].

DNA methylation
The role of DNA methylation in asthma

DNA methylation is generally associated with gene repression and occurs at complementary pairs of cys-

teine residues which are directly followed by guanine (CpG) in mammals [119]. DNA methylation regulates many processes including cell fate, inactive X chromosomes and gene-specific activation and silencing. During cellular replication, the methylation marks are maintained by the DNA methyltransferase DNMT1 which converts hemimethylated DNA into fully methylated DNA [120]. During germ cell development, cell-specific methylation is totally removed and is then re-established by DNMT3A and DNMT3B, which can give rise to specific maternal and paternal gene expression profiles, termed genetic imprinting [119].

The importance of DNA methylation in allowing transcription is vital in the activation of the asthmatic immune response and restricting the DNA methylome in some cells may be of therapeutic benefit to patients. Naïve CD4+ T cells show methylated CpG regions in the *IL-4* promoter which limits transcription. After house dust mite stimulation, DNA demethylation increases at the *IL-4* promoter in cells from patients with bronchial asthma, but not in controls [31]. To enforce differentiation to Th2 type CD4+ T cells, Th1 type gene promoters, such as *IFNG*, are inhibited by enhancer DNA methylation. This is linked to decreased expression of IFN-γ in CD4+ T cells [70]. Th2 cells can, however, reactivate the production of IFN-γ by demethylating the IFN-γ promoter, demonstrating the cell's epigenetic plasticity. The changes are mediated by GATA3 and T-bet, both of which are vital to T-cell development and differentiation [121].

DNMT1, the DNA methyltransferase that maintains DNA methylation, appears to be self-regulating in mouse asthma models. Following allergic stimulation, DNMT1 expression in the lung, trachea and bronchoalveolar lavage fluid cells was decreased in tandem with DNMT1 promoter DNA hypermethylation [122]. Studies examining zebrafish development have highlighted the cross-talk that occurs between epigenetic processes. The gene *lef1* is controlled by a network that includes Dnmt3 and G9a, the H3K9 methyltransferase. G9a and Dnmt3 seem to function simultaneously to silence critical regulators of cell fate [123].

The DNA methylation status at asthma-relevant SNPs within the IL-4R gene is associated with an increased risk of asthma at age 18 if the site was more highly methylated [22]. There are significant links between methylation status at 36 loci in peripheral blood eosinophils and the presence of serum IgE [23]. The implicated loci included eosinophil products and phospholipid inflammatory mediators and provide a list of potential new biomarkers and target genes for drug development against allergy and potentially allergic asthma.

Potential asthma therapies targeting DNA methylation

No DNA methylation targeting compounds have been investigated in relation to asthma *per se* (Table 1), however cancer drugs that target DNMT1-CFP1 and DNMT1-Stat3 complexes are able to demethylate specific regions of DNA associated with tumor suppressor genes without causing global DNA hypomethylation [71]. Drugs have been designed which are able to block DNMT1 complexes forming with HDAC1, STAT3, PCNA, CFP1 and USP7 which gives a broad scope for many other investigations into DNA methylation in T-cell differentiation. In addition to this targeting of DNMT at specific genes, knock-in mice that caused promoter hypermethylation at the INSL6 and the p16 genes and transcriptional suppression by adding a cis-acting regulatory element that attracts DNMT have been developed. This method could be applied to other genes as well [124].

Sections of RNA have been shown to modify the effects of DNMT. For example a region of RNA from the CEBPA locus (ecCEBPA) is able to interact with DNMT1 and prevent the methylation of its gene and increase the transcription of CEBPA. If this effect can be shown in other genes it may suggest a novel mechanism by which expressed RNA controls DNA methylation and will be a model to base new treatments off [125].

It is possibly that hyper and hypomethylation is modifiable so it is important that DNMT inhibitors may have beneficial effects in reducing allergen sensitization in asthmatics through the reversal of IFN-γ repression and the return of T cells to a Th1 phenotype [70]. At present DNMT1 inhibitors have been considered as an anticancer treatment, however making sure that they only inhibit hypermethylation of tumor suppressor genes without demethylating oncogenes is a technical challenge. The development of asthma therapeutics will also contain similar challenges. Excitingly as DNMTs act in complexes with other transcription factors, and their targeting is linked to the complexes they are associated with, such G9a [126], HDAC1 [127] and PCNA (proliferating cell nuclear antigen) [128], DNMT inhibitors have the potential to inhibit specific DNMTs that are associated with inflammation while leaving global DMNT activity unaffected.

miRNA in asthma

Noncoding RNAs (ncRNA) are functional RNAs which are not transcribed and may be important in respiratory disease [129]. They can be broadly subclassified into three groups; housekeeping RNAs (ribosomal, transfer, splicesomal), long noncoding (pseudogenes, intronic, intergenic) and the small noncoding

RNAs (PIWI-associated RNA, endogenous siRNA, microRNAs). microRNAs (miRNAs) are the most studied in respiratory disease, including asthma, cystic fibrosis [130] and lung carcinoma [131].

miRNAs are ~20 nt in length, are highly conserved across species and act as regulators of both genes and gene networks [132]. They induce mRNA degradation and/or inhibit mRNA translation, and it is predicted that as many as 60% of mRNAs are targets for miRNAs [133]. Conversely, long noncoding RNAs (lncRNAs) are greater than 200 nt in length and their mechanism of action can include regulation of both mRNA transcription and/or translation [129], and acting as 'sponges' for miRNAs [134]. Currently, the majority of studies of lncRNAs in the lung have been in relation to lung cancer [129].

The study of ncRNAs in disease typically involves examining the differential expression of the ncRNAs between different patient subsets (e.g., nondisease vs disease), concentrating upon a single ncRNA that is significantly changed in expression, and targeting said ncRNA to inhibit or overexpress its action (extensively reviewed by Booton and Lindsay 2014 [129]). A large number of miRNAs are differentially expressed in asthma in a predominantly cell-specific manner. These include miR-19 in T cells [24], miR-18 in epithelial cells [135] and miR-221 in HASM cells [136]. Work examining miRNA profiles show these are dramatically different in the bronchial epithelium of asthmatics compared with healthy subjects with 217 differentially expressed miRNA genes [137]. The use of corticosteroids (a standard asthma treatment) only had a limited effect in restoring normal miRNA expression in the asthmatic population, which still retained 200 differentially expressed genes compared with healthy subjects [137].

The role of miRNA in asthma

In addition to studies on primary human cells, many current studies defining the potential role of miRNAs in asthma utilize mouse models (Table 1). Intranasal administration of miR-1 inhibits inflammatory responses to ovalbumin (OVA) and house dust mite (HDM) in mouse models of asthma by inhibiting the effect of VEGF [138]. VEGF is able to lead to Th2 type gene expression and recruitment [138]. In addition, miR-145 is as effective as dexamethasone in preventing airway hyper-responsiveness and inflammation in an HDM model of asthma [76]. The same group has reported that antagamirs to miR-126 are able to overcome inflammation and airway hyper-responsiveness (AHR) in mouse models of Th2-driven asthma [77] and that miR-9 antagamirs can reverse steroid insensitivity by targeting protein phosphatase (PP)2A and

the glucocorticoid receptor in various mouse models of steroid-insensitive asthma [75].

MicroRNAs have also been used as tools to suppress target genes in vivo. Intranasal delivery of a siRNA against suppressors of cytokine signaling (SOCS3) reduces lung eosinophil and airway hyper-responsiveness to methacholine following OVA challenge [32]. Furthermore, a siRNA directed against CD86, involved in T-cell-dendritic cell interactions, inhibit OVA-induced hyper-responsiveness, lung eosinophilia and serum IgE in mice [139].

Potential asthma therapies targeting miRNA

Utilizing noncoding RNA molecules as novel therapies for treating disease in vivo, is problematic. At present there are only two ncRNAs in clinical trials. A microRNA mimic (MRX34) which inhibits tumor growth and increases overall survival in mouse models, and is currently in Phase I testing in patients with primary or metastatic liver cancer [72]. A miR-122 mimic is in human clinical trials for hepatitis C [73].

The problems when utilizing miRNAs as a therapy, are numerous. For example, modifying miRNAs with a locked nucleic acid (LNA) structure, although making the miRNA more biologically stable, also activates distinct mechanistic pathways, suggesting that toxicity issues may vary drastically with different miRNA sequences [140]. To address this issue, attaching ZEN (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine) to both ends of a miRNA considerably enhances its binding affinity, results in a greater degree of miRNA inhibition and has low toxicity in vitro [140].

Additionally, another limiting factor is determining how to reach a sufficient dose within the cell in order to achieve efficient miRNA targeting. An imaging-based analytical method using fluorescence and electron microscopy to track intracellular transport and release of ncRNAs demonstrated that lipid nanoparticles (LNPs) enter cells through clathrin-mediated endocytosis and macropinocytosis, and that less than 2% of the ncRNAs escaped from the endosomes, suggesting that the efficiency of internalization and release of ncRNAs is extremely low [141].

This problem may also be overcome, by utilizing nanovesicles as delivery vectors [142]. For example, nanovesicles containing miR-150 are capable of entering effector T cells and suppressing allergic contact dermatitis (ACD) and promoting antigen-specific tolerance in mice [74]. At the time of writing, the human application remains unknown.

Finally, the mechanism of action of ncRNAs (both miRNAs and lncRNAs) may not be as simple as first thought. Evidence suggests that miRNAs may have a range of functions, including regulation of transcrip-

tion through epigenetic mechanisms; of translation by acting as decoys and of acting as inhibitors of other long noncoding RNAs [134,143]. Also, lncRNA are thought to have a range of mechanisms including regulation of both mRNA transcription and/or translation [129], and acting as 'sponges' for miRNAs [134]. Therefore, using ncRNAs as a potential therapy may produce numerous 'off-target effects'. That said, improved techniques for targeting ncRNAs, and increasing knowledge of their biological function could lead to the production of specific gene expression modulators for respiratory disease in the future.

Conclusion

Asthma is a heterogeneous disease of the airways, which in cases of severe asthma is currently untreatable. Asthma has both environmental and heritable components, which has not been directly linked to specific genes, implying control through epigenetic mechanisms, which can regulate cell fate, secretion profiles, inflammation and proliferation.

At present study into the role of epigenetics in the development and maintenance of asthma is in its infancy. As new epigenetic tools and techniques become available, the role of different epigenetic mechanisms in asthma is becoming better understood, and may potentially allow for the future development of novel treatments for asthma. The dynamic nature of epigenetic modifications, which unlike DNA modifications can be reversed, offers hope that as some patients 'grow out' of asthma, a similar cure for asthma could be developed in the future.

The biggest challenge facing the development of epigenetic-based therapies for asthma, and other diseases, is the broad actions of the epigenetic modifying enzymes, which have multiple potential targets. Many of the existing early tool compounds are broadly active and therefore will have many undesired side effect proteins, however as our understanding of these enzymes increases so will specificity of the drugs which are available.

Recent developments in genome targeting tools, such as the development of CRISPR (clustered regularly interspaced short palindromic repeats) – Cas9, to provide highly selective RNA-guided endonucleases to modify the genome at specific sites [144], is overtaking standard genetic knockdown approaches in mice. This technology has been used successfully *in vivo* and allows specific targeting of DNA sequences and their associated epigenetic marks which can be directed at potentially clinically useful sites.

Many of the targets for epigenetic therapeutics need to be targeted to specific genes and areas of the genome. Drugs that inhibit DNA methylation are able to target specific regions of the genome as the methylases bind to transcription factors that bind specific regions of DNA. If a similar mechanism could be found to target genes such as histone modifiers then the degree of usefulness of many of these drugs would increase. While there is a long way to go, the future looks bright for new treatments for asthma based on epigenetics. New compounds will offer the possibility to investigate epigenetic pathways and may lead to a novel therapeutic direction of research.

Future perspective

Knowledge of the role of epigenetics in asthma will continue to grow and the links between asthma and specific combinations of epigenetic modifications will become clearer. As the cost of ChIP-sequencing and next-generation sequencing decreases, the affordability of investigating multiple epigenetic modifications and how they relate to gene and protein expression will increase. Investigating the role of epigenetic modifications will also become easier due to technological advances allowing much more complicated studies to be conducted with fewer primary cells from each patient.

Studies are beginning to allow the investigation of how the epigenome changes over time particularly in response to environmental exposures. Changes in DNA methylation linked to SNP analysis (EWAS, epigenome-wide association studies) and deconvolution of transcriptomic data in single cell types may allow for greater determination of important targets for subsets of asthma. Linking these studies to longitudinal methylation analysis (LEWAS) will allow even greater insight into how environmental challenges may affect disease onset. Similar studies in blood, airway epithelial cells or smooth muscle cells focused on histone modifications or chromatin structure using ChIP-seq or ACAT-seq approaches may be of even greater benefit and help define new animal models or targets that will define the potential therapeutic benefits of drugs that target epigenetic mechanisms.

In terms of therapeutics, the technology for delivering miRNAs and other epigenetic drugs to the lungs will improve and provide a greater therapeutic window. Currently, targeting of epigenetic drugs to specific sites is more advantageous with DNA methylation but it is hoped that selective targeting will enable higher specificity of next-generation epigenetic drugs.

Current epigenetic drugs are really tool compounds which have off-target effects which limit their options for clinical trials. Animal models will be useful for pharmacokinetic studies but may lack the correct epigenetic components of human cells and tissues to allow predictive studies. The option of topical delivery to the airways and the development of highly lung resident drugs with very low systemic exposure may improve the chances of successful clinical studies in asthma.

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Executive summary

- Asthma is a heritable heterogeneous disease of the airways and severe asthma currently has no treatment.
- Epigenetics may account of the heritability of asthma through histone modifications, DNA methylation and interfering RNA.
- There are now a variety of tools available to target many epigenetic mechanisms.
- Modifying acetylation and methylation of histones and DNA methylation can change expression of inflammatory genes.
- DNA methylation modifiers can be targeted at particular regions of DNA as they are bound to specific complexes which allow specificity of drugs.
- miRNA treatments such as suppressors of cytokine signaling (SOCS3) siRNA are able to be targeted specifically to the lung and can reduce eosinophilia.
- Further work is required before these drugs progress into human clinical trials.

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