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Epigenetic Mechanisms and the Development of Asthma

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Abstract

Asthma is heritable, influenced by the environment, and is modified by *in utero* exposures and aging; all of these features are also common to epigenetic regulation. Furthermore, the transcription factors that are involved in the development of mature T cells that are critical to the Th_2 immune phenotype in asthma are regulated by epigenetic mechanisms. Epigenetic marks - DNA methylation, modifications of histone tails, and non-coding RNAs – work in concert with other components of cellular regulatory machinery to control spatial and temporal level of expressed genes. Technology to measure epigenetic marks on genomic scale and comprehensive approaches to data analysis have recently emerged and continue to improve. Alterations in epigenetic marks have been associated with exposures relevant to asthma, particularly air pollution and tobacco smoke, as well as asthma phenotypes in a few population-based studies. On the other hand, animal studies have begun to decipher the role of epigenetic regulation of gene expression associated with the development of allergic airway disease. Epigenetic mechanisms represent a promising line of inquiry that may, in part, explain the inheritance and immunobiology of asthma.

Keywords

asthma; atopy; epigenetics; gene expression; DNA methylation; histone marks; noncoding RNAs

Introduction

Asthma is a complex, heritable disease affecting more than 8% of the U.S. population, ~7 million children and ~18.7 million adults[1, 2]. This disease has been increasing in prevalence, incidence, and severity[3] although recent evidence suggests that the prevalence of asthma and allergies may have come to a plateau in developed countries[4]. Asthma accounts for over $10 billion of direct health care costs in the U.S.[3]. In 2008, persons with asthma missed 10.5 million school days and 14.2 million work days due to their disease[1]. Gender and ethnic differences exist for women and African American asthmatics, both having a significantly higher rate of outpatient asthma visits, emergency room evaluations, and hospitalizations than non-Hispanic males[1]. Consistent with these data is an increased mortality rate in women and African American asthmatics, 45% and 200% higher respectively, than non-Hispanic white males. What is most disturbing is that ongoing increases in disease prevalence, incidence, and severity are occurring despite the intense national and international investigation into the pathobiology, genetics, and treatment of asthma.

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Consequently, it is essential to consider alternative explanations for the growing health problems associated with the development and persistence of asthma.

Several separate lines of evidence support a role for epigenetics in asthma (Figure 1). First, asthma, like epigenetic mechanisms, is heritable. While asthma is a strongly familial condition (36–79% heritability) with a non-Mendelian pattern of inheritance and polymorphisms in more than 100 genes[5–8], these associations have infrequently been replicated and genetics has explained only a small portion of the etiology of this disease[6]. Second, asthma, like epigenetic mechanisms, shows a parent-of-origin transmission of inheritance with an affected mother significantly more likely to transmit the disease than an affected father[9]. These parent-of-origin effects may result from immune interactions between the fetus and the mother[10]. Alternatively, the maternal effect may be the result of epigenetically regulated genomic imprinting[11]. Several known genes show parent-of-origin effects on allergic disease; these genes include the *FcεRI-β* locus[12], and the *Spink5* gene[13]. Third, asthma, like epigenetic mechanisms[14, 15], is affected by *in utero* exposures[16, 17]. Prenatal exposure to maternal and grand-maternal cigarette smoke[18–20] and traffic-related air pollution[21, 22] are among *in utero* exposures that contribute to the development of this disease. On the other hand, higher maternal fruit and vegetable intake and oily fish consumption during gestation have been associated with a lower risk of asthma [23]. Fourth, asthma, like epigenetics, is influenced by the general environment[24]. Environmental factors are known to play important roles in the pathogenesis of asthma; both in terms of main effects, and those exerted indirectly through complex interactions with gene variants[25]. The dramatic increase in the prevalence, incidence, and severity of asthma over the last 20 years provides strong evidence that exposures, including diet, play an important role in the development of this disease; these changes have occurred too rapidly to be accounted for by changes in primary DNA sequence alone. While allergens are classically associated with asthma[26], many other exposures, including smoking behavior[25, 27], agents in the workplace[28], indoor and outdoor air pollution[29], viruses[30], domestic[31] and occupational[32] exposure to endotoxin, and immunization against certain infectious diseases[33] are associated with the development and progression of this disease, and several of these agents have been shown to alter epigenetic marks. Finally, asthma is an immune-mediated disease characterized mainly by skewing toward a Th_2 phenotype although other T cell subtypes may be involved[34]. Epigenetic mechanisms regulate expression of transcription factors that are involved in T cell differentiation (Th_1, Th_2, and T_{regs})[35–42].

Epigenetic Mechanisms

Epigenetics is traditionally defined as the study of heritable changes in gene expression caused by molecules that bind to DNA rather than changes in the underlying DNA sequence (Table 1)[43]. Recent evidence suggests that the epigenome is dynamic and changes in response to the environment, diet, and ageing[44]. In addition to a set of inherited epigenetic marks, there are likely non-heritable epigenetic marks that are more dynamic and change in response to environmental stimuli. Three main classes of epigenetic marks are DNA methylation, modifications of histone tails, and non-coding RNAs (Figure 2).

Methylation of cytosine residues in CpG dinucleotides (5-methylcytosine) within the context of CpG islands is the simplest form of epigenetic regulation in eukaryotes with hypermethylation of CpG islands in gene promoters leading to gene silencing and hypomethylation leading to active transcription. CpG island methylation has long been studied in cancer with findings that hypermethylation of tumor suppressor genes and hypomethylation of oncogenes contribute to the process of carcinogenesis[11, 45]. More recent studies have demonstrated that methylation of less CpG dense regions near CpG islands (‘CpG island shores’) controls expression of tissue specific genes as well as genes relevant

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to carcinogenesis and lineage-specific cell differentiation[46, 47], suggesting that DNA methylation outside of CpG islands is an important mechanism that controls gene transcription. Additionally, recent evidence suggests that DNA methylation is more prevalent within gene bodies than in promoters[48]. Intragenic DNA methylation functions at least in part by regulating transcription from alternative promoters[49] but it is likely that other mechanisms are also involved. The DNA 'methylome' of the H1 human embryonic stem cell line uniquely revealed that nearly one-quarter of all methylation is in non-CpG context[50], suggesting that embryonic stem cells may use different methylation mechanisms to control gene expression. 5-methylcytosine can be oxidized to 5-hydroxymethylcytosine by the recently discovered TET family of enzymes[51]. Although the role of 5-hydroxymethylcytosine in epigenetic regulation of gene expression is not fully elucidated, it has been suggested that 5-hyroxymethycytosine is a mark of demethylation[51] and that it potentially plays a role in the regulation of specific promoters and enhancers[52].

Methylation, acetylation, phosphorylation, and ubiquitylation [43] of histone tails occur at specific sites and residues, and controls gene expression by regulating DNA accessibility to RNA polymerase II and transcription factors. H3K4 trimethylation (H3K4me3), for example, is strongly associated with transcriptional activation whereas H3K27 trimethylation (H3K27me3) is frequently associated with gene silencing[53]. Similarly, histone tail acetylation leads to active gene transcription while deacetylation is a repressive mark and leads to gene silencing. Histone acetyltransferases (HATs) are enzymes that acetylate histone tails while histone deacetylases (HDACs) remove acetyl groups from histone tails. Analogous to DNA methylation, deregulation of these histone modifications has been linked to misregulation of gene expression in cancers[54].

MicroRNAs (miRNAs) are the most studied class of noncoding RNAs that control gene expression by binding to the 3′ untranslated regions (UTRs) of messenger RNA (mRNA), which leads to either mRNA degradation or inhibition of protein translation[55]. Almost 2000 mature miRNAs have been identified in the human genome (http://www.mirbase.org/) but it is expected that more miRNAs will be identified in the near future. Alterations of expression of miRNAs contribute to pathogenesis of most malignancies with miRNAs acting as both oncogenes and tumor suppressor genes[56] but miRNAs also have well-established roles and are therapeutic targets in cardiovascular disease[57] and liver injury[58]. More recently, ncRNAs, such as PIWI-interacting RNAs (piRNAs), small nucleolar RNAs, (snoRNAs), transcribed ultraconserved regions (T-UCRs) and large intergenic non-coding, RNAs (lincRNAs) are emerging as a key component of deregulated transcription not only in tumorigenesis but also in many non-malignant diseases[59].

An emerging paradigm for epigenetic regulation of gene expression is the relationship between DNA methylation and histone modifications. One example of these interactions is binding of DNMT3L, a regulatory factor related in sequence to mammalian *de novo* methyltransferases DNMT3A and DNMT3B, to the N terminus of histone H3 tail[60, 61]. DNMT3L recognizes unmethylated H3 tails at lysine 4 and induces *de novo* DNA methylation by recruitment or activation of DNMT3A2; these findings establish the N terminus of histone H3 tail with an unmethylated lysine 4 as a chromatin determinant for DNA methylation. Similarly, DNA methyltransferases preferentially target nucleosome-bound DNA[62]. The relationship of histones and DNA methylation is bidirectional; in addition to histones playing a role in the establishment of DNA methylation patterns, DNA methylation is important for maintaining patterns of histone modification through cell division[63]. Cross-talk between DNA methylation and miRNAs has also been identified[64, 65].

In addition to cross-talk between different epigenetic marks, it is becoming evident that underlying genetic variation and epigenetic marks work together. The best example is allele-

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specific gene expression where allele-specific differences in gene expression may arise because of sequence variation that may be marked by differences in DNA methylation[66–68], histone modifications or chromatin structure.

Epigenetic marks (DNA methylation and histone marks) are a key component of cell-specific gene expression and, as such, are erased during germ cell development (meiosis) and re-established following fertilization. This process is referred to as epigenetic reprogramming[69] and constitutes of comprehensive erasure and re-establishment of DNA methylation and extensive remodeling of histone modifications in two steps. Epigenetic reprogramming is a key feature of inheritance of epigenetics marks. Genes that are expressed from only one parental allele, known as imprinted genes, are protected during the second reprogramming step by mechanisms that are being unraveled[70].

Epigenomic Study Design

The first step in epigenomic analysis is experimental design, including the choice of tissue/cells to be profiled and study design. Challenges in selection of the material to be used include limited availability of lung tissue for asthma studies and cell heterogeneity in available samples (such as DNA from whole blood). One way to address the first challenge will be to analyze paired lung-blood samples to identify epigenetic marks that carry over from the lung to peripheral blood and test whether surrogate tissue (peripheral blood mononuclear cells, nasal epithelia, sputum, etc) adequately reflect activity in the lung. The second challenge can be addressed by collecting white blood cell count data and including the constituent cell counts in the analysis. If this information is not available, established epigenomic profiles for constituent cell cells (such as data generated by the Roadmap Epigenomic project, http://www.roadmapepigenomics.org/) can be used to estimate relative abundances of different cell types[71].

Study design and power calculations based on previously collected data are important in designing studies that are able to identify significant epigenetic changes after adjustments for genome-wide comparisons. The most powerful study design for epigenomic analysis uses monozygotic twins that are essentially identical genetically so that all differences in phenotype can be contributed to environmental factors with paired-sample design allowing for better statistical power[72]. Another design with reasonably high power includes siblings (not necessarily twins) discordant for disease phenotypes with parental DNA also available for estimates of heritability. Case-control design with a large enough number of individuals included in the analysis is often used due to availability of samples. The final considerations in the study design are clinical and immune phenotypes of interest. Prior to sample selection from available specimens for epigenomic profiling, clinical/immune variables must be analyzed to identify those that have reliable measurements, normal distribution, and have a strong clinical or biological rationale to include in statistical models. Once epigenomic profiles are collected and data are normalized, principal components analysis (PCA) can be used to prioritize variables based on how much variance in the dataset they account for.

Epigenomic Profiling

Epigenetic marks can be studied using focused and genome-wide approaches (Table 2)[73]. Generally, studies begin with genome-wide approaches to identify targets followed by focused approaches to internally validate (confirmation in the same cohort) or externally validate (independent cohort) the initial findings. Microarrays have been the tool of choice for profiling epigenetic marks on a genomic scale, with several platforms and protocols available for DNA methylation (Table 2)[74]. Most commonly used array platforms for DNA methylation are Illumina 450k BeadChip, Comprehensive Analysis of Relative DNA Methylation (CHARM) platform[75], and MeDIP arrays (Agilent Technologies and Roche

Nimblegen). Array platforms have also been used to examine histone modifications by chromatin precipitation followed by hybridization on microarrays (CHIP-chip)[74] and for miRNAs[76].

However, the most substantial advance in the area of technologies for the assessment of epigenetic marks on the genome scale in recent years has been the introduction of next-generation sequencing technologies[77]. Application of next-generation sequencing to epigenomic research has been recently reviewed[78]. These technologies have been widely used for the study of histone marks (CHIP-seq) and microRNAs (miRNA-seq) as they provide superb quality data compared to array platforms. They have also been used to identify open chromatin areas of the genome (FAIRE-seq)[79] and spatial chromatin organization (3C-seq)[80]. The majority of methylation profiling is still done on array platforms as bisulfite-converted DNA sequencing (BS-seq) on the genomic scale is expensive. However, a number of techniques that examine only regions of the genome enriched for methylation marks have been developed and are being increasingly used[81]. Recent advances in the development of techniques for epigenomic profiling include attempts to define genome-wide patterns of DNA hydroxymethylation[82, 83] and to study DNA methylation and histone modifications in one experiment[84, 85]

Pyrosequencing[86] and Epityper assays on the Sequenom MassARRAY platform are commonly used techniques for interrogation of a small number of CpG sites while quantitative PCR (qPCR) methods are typically used for focused studies of histone modifications and miRNAs. In addition to site-specific methods for assessment of DNA methylation, some studies assess overall level of methylation in each sample (global methylation); this is often measured by assessing methylation in repeat regions of the genome (Alu, LINE-1, Sat2), mass spectrometry methods, or luminometric methylation assay (LUMA)[87].

Epigenomic Data Analysis

The first step in analysis of collected epigenomic data is to identify statistically significant differences between disease states. Statistical methods used for microarray analysis have generally been applicable to epigenomic profiles collected on arrays or sequence data after alignments and tag counts have been performed[78]. Strategies for analyzing tiling arrays have also been used in epigenomic analyses (such as CHARM or CHIP-chip)[88]. One of the problems with this type of analysis is that it is only associative and does not demonstrate causality. Methods used in epidemiology and genetical genomics are beginning to be applied to epigenomes to identify causal relationships[89].

The second and most complex step in the analysis of epigenomic data is understanding how different epigenetic mechanisms together influence gene expression. Each of the three epigenetic mechanisms is independently complex but when combined, the complexity of these interactions presents unique analytic challenges. We are just beginning to understand how one type of epigenetic mark affects gene expression[90]. However, the evidence for cross-talk among different types of epigenetics marks is accumulating. The complexity of epigenetic regulation of gene expression is high even when one is interested in examining only one gene or locus and there are considerable challenges associated with understanding these interactions and effect on gene regulation genome-wide. Analytical strategies for these types of integrative epigenomic analyses have not reached maturity but are starting to be applied to disease datasets[91]. Two types of integrative analysis will be important to apply to epigenomic data mapping strategies and network analysis. Expression quantitative trait loci (eQTL) mapping approaches[92] can be applied to identify genetic variants that underlie methylation status (methyl-QTL) or methylation marks that control expression changes

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(methyl-expression QTL). Similarly, co-expression network analysis strategies that have been applied to expression analysis can be applied to epigenomic analysis[93].

Epigenetic Marks and the Immune System

A substantial body of evidence suggests that epigenetic mechanisms affect the expression of cytokines and binding of transcription factors that control the lineage of Th_1, Th_2, and T_{reg} cells. In the context of Th_1/Th_2 differentiation, the most extensively studied are the Th_1 cytokine IFNγ, and Th_2 cytokines IL-4 and IL-13. It has been shown that *de novo* DNA methyltransferase DNMT3A methylates CpG -53 in the *Ifng* promoter[35] and cord blood $CD4^+$ cells enhance the development of Th_1 (but not Th_2) lineage through progressive demethylation of the *Ifng* promoter[36]. Methylation of the *Ifng* promoter was reduced in $CD8^+$ cells from atopic children in the age range in which hyperproduction of IFNγ occurs, suggesting that DNA methylation at this locus may be a contributing factor in the development of atopy in children. Differentiation of human $CD4^+$ cells into the Th_2 subtype is accompanied by the appearance of DNase I hypersensitive sites (DHS) and CpG demethylation around these DHS sites within IL-4 and IL-13 promoters[37–39]. Extensive studies of the Th_2 cytokine locus control region (LCR)[40] have shown that rad50 hypersensitive site 7 (RHS7) within the Th_2 cytokine LCR undergoes rapid demethylation during Th_2 differentiation[41].

In addition to DNA methylation, histone modifications are also important in guiding T cell differentiation. T-bet and GATA-3 transcription factors control lineage-specific histone acetylation of *Ifng* and *Il4* loci during Th_1/Th_2 differentiation[42]. Rapid methylation of H3K9 and H3K27 residues (repressive marks) at the *Ifng* locus are associated with differentiating Th_1 cells while demethylation of H3K9 and methylation of H3K27 was associated with Th_2 differentiation. In a study of human cord blood $CD4^+$ cells, histone acetylation marks at the proximal *Il13* promoter were selectively observed in Th_2 cells[39], suggesting that permissive histone marks together with DNA demethylation lead to expression of IL-13 in Th_2 cells. In aggregate, these studies suggest that DNA methylation and histone modifications are highly dynamic and represent important determinants of Th_1 and Th_2 cell lineages.

Although miRNAs were discovered relatively recently, there is already a substantial body of evidence for the role of miRNAs in the development and function of the immune system[94–96]. A number of differentially expressed miRNAs have been identified in response to innate and adaptive immune stimuli with many commonalities in miRNA expression (miR-21, -103, -155, and -204) [94]. miRNA-155 is the most often identified differentially regulated noncoding RNA in studies involving the immune system[94, 95]; a recent study revealed that miR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4[97].

Epigenetic mechanisms controlling regulatory T cells (T_{regs}) development are also beginning to be explored. T_{regs} are a unique T-cell lineage with an important role in immunological tolerance whose development is primarily regulated by the transcription factor FOXP3. Evidence for the role of DNA methylation[98] and histone modifications[99] in regulation of FOXP3 expression are summarized in two recent reviews[100, 101]. There is also clear evidence that miRNAs are involved in T_{reg} development and function[96].

The Role of the Environment and *In Utero* Exposures in Modulating the Epigenome

Unlike an individual's genetic make-up, epigenetic marks can be influenced much more easily by exposures, diet, and ageing. Randy Jirtle's seminal experiments showed that

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maternal diet supplemented with methyl donors (folic acid, vitamin B12, choline and betaine) shifts coat color distribution of progeny towards the brown pseudoagouti phenotype, and that this shift in coat color resulted from an increase in DNA methylation in a transposon adjacent to the agouti gene [14, 15]. These studies also revealed that mice with yellow coat color are obese and develop cancer, suggesting for the first time that changes in DNA methylation caused by diet *in utero* may be linked to disease development. Other studies have shown that pesticides and fungicides can alter the methylome resulting in changes in male fertility[102], and that ageing is also associated with changes in DNA methylation and gene expression[103]. The concepts associated with environmental epigenetics were reviewed recently elsewhere[44].

More recent evidence suggests that environmental exposures relevant to the development of asthma, such as air pollution and cigarette smoke, also affect the epigenome. Decreased DNA methylation in peripheral blood (as measured by LINE-1 repeats) was found to be associated with exposure to PM2.5 particles amongst 718 elderly individuals in the Boston area[104], and although this correlated with time-dependent variables such as day of the week and season, there was no association with air pollution-related health effects. Another study demonstrated that hypomethylation of *iNOS* (*Nos2*) promoter in buccal cells was associated with exhaled nitric oxide (NO) levels and PM2.5 exposure among 940 participants in the Children's Health Study[105].

Several epidemiological studies have examined the relationship between exposure to cigarette smoke and epigenetic marks. Among 384 children, a global reduction in DNA methylation, as measured by the extent of methylation of Alu repeats, and differential methylation of 8 specific CpG motifs was found to be associated with *in utero* smoke exposure[106]. 15 specific genomic loci were significantly associated with current smoking, two with cumulative smoke exposure, three with time since quitting cigarettes in 1085 individuals enrolled in the International COPD Genetics Network and validated in the Boston Early-Onset COPD study (n = 369)[107]. Cigarette smoke exposure has also been shown to have a significant influence on expression of miRNAs[108–110]. Comparing current to never smokers, 28 miRNAs were differentially expressed, mostly downregulated in human bronchial airway epithelium of smokers[108]. miR-218 was found to be one of the strongly associated miRNA with cigarette smoke exposure and it was further shown that a change in miR-218 expression in primary bronchial epithelial cells and H1299 cell line resulted in a corresponding anti-correlated change in expression of predicted mRNA targets for miR-218.

Other studies have examined the influence of cigarette smoke exposure on epigenetic marks *in vitro* or in animal models. Normal human airway epithelial cells and immortalized bronchial epithelial cells exposed to cigarette smoke condensate (CSC) identified time- and dose-dependent changes in histone modifications (decrease in H4K16Ac and H4K20Me3, and increase in H3K27Me3) accompanied by decreased DNMT1 and increased DNMT3b expression; these changes are characteristic of lung cancer progression[111]. Two other studies also demonstrated changes in miRNA expression in lungs of mice[110] and rats[109] exposed to cigarette smoke with substantial overlap between mice and rats and some overlap of rodent miRNA expression changes in the lung with those observed in human airway epithelium[108].

In addition to influencing epigenetic marks as a result of direct exposure, *in utero* exposure to components or air pollution or cigarette smoke results in changes in global and site-specific DNA methylation. Maternal exposure to benzo(a)pyrene (BaP), a representative airborne polycyclic aromatic hydrocarbon (PAH), was associated with hypermethylation of IFNγ in cord blood DNA from 53 participants in the Columbia Center for Children's Environmental Health cohort[112]. In another study, global hypomethylation has been

associated with maternal smoking and cotinine levels in the umbilical cord blood from 30 newborns[113]. In a birth cohort of 90 women born 1959-63 in New York City, prenatal tobacco exposure, measured at the time of pregnancy and not retrospectively reported, was associated with a decrease in Sat2 methylation but not LINE-1 or Alu methylation[114]. Examination of two differentially methylated regions (DMRs) regulating two imprinted loci (*H19* and *Igf2*) in infants born to 418 pregnant women demonstrated that infants born to smokers had higher methylation at the *Igf2* DMR than those born to never smokers or those who quit during pregnancy (no differences were seen in the *H19* DMR)[115]. Similarly, DNA methylation in *Axl*, a receptor tyrosine kinase relevant in cancer and immune function, was 2.3% higher in peripheral blood of children exposed to maternal smoking *in utero*[116]. Finally, one study has demonstrated association of maternal cigarette smoking during pregnancy with downregulation of several miRNAs in placenta; expression of one of the miRNAs (miR-146a) was downregulated in dose-dependent manner in immortalized placental cell lines exposed to nicotine and BaP[117].

Asthma Epigenetics – Animal Studies

Given the evidence for the strong influence of environmental exposures on epigenetic marks and the role of epigenetic regulation in T cell differentiation, it is becoming clear that epigenetic changes may be one of the factors to explain the increasing prevalence of asthma. Our group hypothesized that these dietary influences are, at least in part, mediated by the epigenome. To test this hypothesis, we conducted a study in which pregnant female mice were fed either a low or high methylation diet and progeny were sensitized and challenged with ovalbumin [17]. We observed an increase in airway inflammation, serum IgE, and airway hyperresponsivness (AHR) in pups of mothers who were fed high methylation diet compared to those of mothers on low methylation diet. Furthermore, we demonstrated hypermethylation of 82 gene-associated CpG islands throughout the genome, including extensive hypermethylation of the promoter and decreased expression of *Runx3*, a gene known to regulate allergic airway disease in mice. Importantly, we reversed the immune phenotype by treatment with a demethylating agent (5-aza-deoxycytidine). Epidemiological evidence for association of folic acid with the development of asthma in children has been mixed[118–122] but it may be that folate together with other methyl donors in the diet plays a role in this disease.

Importantly, a direct link between epigenetic control of the Th_2 cytokine locus and development of allergic airway diseases was further demonstrated in mice with deficiency in the Th_2 LCR[123]. A more recent study also identified a DNase I-hypersensitive site 2 (HS2) element in the second intron of the *Il4* gene as the strongest of all known *Il4* enhancers and showed that this enhancer is strictly controlled by GATA-3 binding[124]. Moreover, Tanaka *et al.* propose a new model in which independent recruitment of GATA-3 to locus-specific regulatory elements controls the status of the expression of genes encoding Th2 cytokines[125].

A number of other animal studies have since examined DNA methylation in the context of allergic airway disease. Fedulov *et al* demonstrated DNA methylation changes in splenic $CD11c^+$ dendritic cells (DCs) from neonate mice born to allergic mothers (mothers sensitized and challenged with ovalbumin)[126]. Brand and colleagues observed increased methylation of the *Ifng* promoter (and increased IFNγ cytokine production) in $CD4^+$ T lymphocytes after ovalbumin sensitization challenge and demonstrated that methylation of the *Ifng* promoter is required for development of allergic airway disease by using 5-aza-deoxycytidine (demethylating agent) and adoptive transfer experiments of $CD4^+$ T-cells from sensitized/challenged to naïve animals and reverse[127]. Although both demethylation and adoptive transfer experiments clearly demonstrate the importance of methylation marks

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in $CD4^+$ cells in development of allergic airway disease, loci other than *Ifng* may be important in this process and should be examined. Finally, DNMT3A, but not DNMT3B, deficiency is $CD4^+$ lymphocytes (conditional mutant mice) was shown to result in increased expression of IL-13 (and other Th2 cytokines), decreased DNA methylation and changes in H3K27 acetylation/methylation in the IL-13 promoter, increased airway inflammation and AHR in the ovalbumin model of allergic airway disease[127]. This study clearly demonstrates the role of DNA methylation in controlling expression of Th2 cytokines and development of allergic airway disease in mice.

Several recent studies have also begun to shed light on the role several miRNAs play in the development of allergic airway disease in animal models[128]. Selective blockade in miR-126 resulted in diminished Th_2 response, inflammation, and AHR in the house dust mite (HDM) model; these effects were shown to be mediated by activation of the MyD88 innate immune signaling pathway. Using the same HDM model, this group also demonstrated that inhibition of miR-145 inhibited eosinophilic inflammation, mucus hypersecretion, Th2 cytokine production, and airway hyperresponsiveness, and that the anti-inflammatory effects of miR-145 antagonism were comparable to glucocorticoid treatment[129]. Two studies identified a controversial role for the let-7 family of miRs in the ovalbumin model of allergic airway disease[130, 131]. The first study showed that multiple members of the highly conserved let-7 miRNA family are the most increased lung miRNAs in response to allergen[130]. The authors confirmed that IL-13 is regulated by let-7a *in vitro* and demonstrated that inhibition of let-7 miRNAs *in vivo* using a locked nucleic acid profoundly inhibited allergic inflammation and AHR, suggesting a proinflammatory role for let-7d. The second independent study demonstrated that let-7 miRNAs regulate IL-13 production in A549 cells and primary cultured T cells, and that intranasal administration of mature let-7 mimic to lungs of mice with allergic inflammation resulted in decreased IL-13, AHR and mucus metaplasia, implying an anti-inflammatory role for let-7[131]. More studies are needed to understand the discrepancy in these findings but this illustrates the complexity of miRNA regulation of gene expression.

Finally, three recent studies have demonstrated how miRNAs play a crucial role in regulation of IFNγ and therefore T-cell polarization. Targeted ablation of miR-21 led to reduced lung eosinophilia after ovalbumin sensitization and challenge, with a broadly reprogrammed immunoactivation transcriptome and significantly increased levels of the Th1 cytokine IFNγ[132]. Consistent with the miR-21 binding site in *IL-12p35*, DCs from miR-21-deficient mice produced more IL-12 after LPS stimulation and OVA-challenged $CD4^+$ T-cells from the same mice produced more IFNγ and less IL-4. Two studies showed that miR-29 suppresses IFNγ production[133, 134]. Steiner *et al.* performed gene expression profiling of cells that do not produce miRNAs (DGCR8-deficient cells[135]) transfected with a synthetic miR-29 and wild-type cells with antisense inhibitors of miR-29, respectively[134]. In this elegant experiment, they found reduced expression of two transcription factors that regulate IFN-γ production (*Tbx21*/T-bet and *Eomes*) under gain-of-function conditions and elevated expression of these two transcription factors under loss-of-function conditions. They further proved the role of miR-29 regulation of expression of these transcription factors in $CD4^+$ lymphocytes *in vitro* and in both $CD4^+$ and $CD8^+$ T-cells in an *in vivo* virus infection model. Ma *et al.* demonstrated an inverse correlation between IFN-γ production and levels of miR-29 in natural killer (NK) cells and T cells from mice infected with *Listeria monocytogenes* or *Mycobacterium bovis*[133]. Mice lacking miR-29 infected with *M. bovis* showed less inflammation, lower bacterial burden and increased numbers of IFNγ-producing $CD4^+$ T cells in their lungs compared with control mice.

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Asthma Epigenetics – Human Studies

While animal studies have begun to decipher the role of epigenetic regulation of gene expression associated with the development of allergic airway disease in the lung, several recent publications in human cohorts have examined DNA methylation in cells outside of the lung - peripheral blood[136], buccal cells[137, 138] and nasal cells[139]. These early studies have only demonstrated statistical association of DNA methylation and specific exposure or asthma phenotype but have not elucidated the role of DNA methylation in the control of gene expression in human asthma. Breton *et al.* demonstrated that DNA methylation in promoters of two arginase genes (Arg1 and Arg2) is associated with exhaled nitric oxide in children with asthma from the Children's Health Study and indicates a role for epigenetic regulation of nitric oxide production[137]. In a pilot study in the Columbia Center for Children's Environmental Health cohort, Kuriakose and coworkers found that iNOS methylation was not significantly associated with fraction exhaled NO (Fe_{NO}) but was associated inversely with J_{NO} (bronchial NO flux)[138]. This latter study emphasizes the importance of careful selection of clinical parameters used in the association study. A more recent study of DNA methylation in nasal cells from 35 asthmatic children 8–11 years old identified inverse association of Fe_{NO} and promoter methylation of both *Il6* and *iNOS*[139]. Finally, data from two independent pregnancy cohorts in Spain (discovery and validation)[136] showed that DNA hypomethylation in *Alox12* in peripheral blood of children was associated with a higher risk of persistent wheezing at age 4. In aggregate, these studies suggest that DNA methylation in easily obtained samples (buccal, nasal or peripheral blood cells) may be a useful biomarker for airway inflammation in pediatric research.

A recent study has also examined DNA methylation in *Foxp3* and T*reg* function in peripheral blood from children with and without asthma and with high and low exposures to air pollution[140]. T*reg*-cell suppression was impaired and T*reg*-cell chemotaxis was reduced as a result of exposure to air pollution. Changes in DNA methylation have also been associated with the development of asthma among older smokers in the Lovelace Smokers Cohort. Comparison of 184 smokers with asthma to 511 smoker controls with a similar smoking history (COPD cases excluded) identified an association of DNA methylation in the protocadherin-20 gene in sputum DNA with asthma as well as a significant synergistic interaction between methylation of protocadherin-20 and paired box protein transcription factor-5α on the odds for developing asthma[141].

A set of earlier studies suggested that acetylation of histones may also play a role in asthma. Increased acetylation of H4 has been demonstrated in individuals with asthma and is associated with an increase in expression of several inflammatory genes in the lung[142]. It has also been shown that increased acetylation of histones results in decreased HDAC activity which may be responsible for enhanced expression of inflammatory genes. In addition, glucocorticoids appear to suppress inflammation by altering acetylation of histones that regulate inflammatory and anti-inflammatory genes; these studies are described in detail in a recent review[143] and suggest that targeting histone acetylation (and possibly other epigenetic marks) may lead to novel anti-inflammatory therapies, especially in corticosteroid-resistant cases of asthma. A more recent study found that TGF-β2 suppresses expression of ADAM33, one of the most replicated asthma susceptibility genes, in normal or asthmatic fibroblasts and that this occurs by altering chromatin structure (deacetylation of histone H3, demethylation of lysine 4 on H3, and hypermethylation of lysine9 on H3) and not by gene silencing through DNA methylation as in epithelial cells[144].

The role of miRNAs in asthma and atopy in humans is also emerging. Although no detectable differences in expression of miRNAs from airway biopsies were observed between mild asthmatics and normal subjects in an early study[145], only mild asthmatics

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were included in this study and the number of miRNAs examined was limited. However, this study demonstrated cell-type specific expression of miRNAs in cells isolated from airways and lung tissue, suggesting a possible role for miRNAs in asthma. A more recent study has indeed identified miRNAs that play a role in specific cells in asthma. In a study of 8 controls, 4 non-severe and 12 severe asthma patients, widespread changes in mRNA and noncoding RNA expression in circulating $CD8^+$ but not $CD4^+$ T were associated with severe asthma[146]. miRNA expression profiles showed selective downregulation of miR-28-5p in $CD8^+$ lymphocytes and reduction of miR-146a and miR-146b in both $CD4^+$ and $CD8^+$ T cells. It is likely that some of the other miRNAs identified in animal models play a yet uncovered role in the development of asthma in humans.

Challenges in Understanding the Asthma Epigenome

Some of the key questions in regard to future studies in asthma epigenetics revolve around understanding how the epigenome contributes to inheritance of asthma, developmental vulnerability of the epigenome, effect of the environment/diet/ageing, and the influence of asthma (and other diseases) on the epigenome. While sorting out these factors will be challenging, it is absolutely essential that the proper tissue be chosen to study the effects of the epigenome on asthma. The more pure and relevant to disease state the cell population, the more likely will the epigenetic marks regulate expression of key genes involved in pathogenesis of asthma. In the absence of airway biopsies in pediatric asthma, nasal epithelial cells or sputum may be the closest surrogate for disease relevant cells. Specific cell populations, such as CD4+ and CD8+ T lymphocytes, isolated from peripheral blood may also be informative in identifying immune genes whose expression is mis-regulated by epigenetic marks in the disease state. Despite these concerns, epigenetic marks in the peripheral blood may provide biomarkers to identify those at risk, responses to different forms of environmental stress, or likelihood of responding to specific therapeutic agents.

Analogous to asthma genetics studies, the choice of the study population will be crucial to success of future epigenetic studies. Ancestry of study subjects will likely need to be taken into account given the early evidence for the role of genetic variation and DNA methylation at asthma-associated loci, such as *Ormdl3*[147]. Moreover, a recent study suggests that DNA methylation is highly divergent between populations of European and African descent, and that this divergence may be in large part due to a combination of differences in allele frequencies and complex epistasis or gene × environment interactions[148]. Based on this, population stratification may be a confounder in population-based genome-wide DNA methylation studies and may have to be accounted for by using principal components from the methylation profile, whole-genome association studies (GWAS) or ancestry-specific marker panels. Given the strong influence of the environment on epigenetic marks, environmental and dietary exposures as well as medication use must be measured/recorded in the study not only for exposures of interested but also for any confounding exposures that need to be adjusted for in the analysis. Despite the differences between disease phenotypes in human cohorts and animal models of allergic airway disease, animal models with fixed genetic background and controlled exposures are likely to remain a crucial component of future studies in the field.

One of the major hurdles to overcome in future asthma epigenetics research will be the validation component. Necessary components of the validation process include internal validation of epigenetic marks in the same samples by a different technique, association of epigenetic marks with changes in gene expression in the study population and external validation of epigenetic marks in an independent cohort (Figure 3). Some of the difficulties encountered in the validation process are platform differences in technologies; differences in DNA methylation measurements are encountered based on the approach used to capture

methylated marks (restriction digest, immunoprecipitation, bisulfite conversion) and probes to measure the extent of methylation (single CpG site vs a region covered by overlapping probes). Another major challenge in validations studies is interpretation of epigenetic marks in the context of changes in gene expression. Both *cis*- and *trans*-effects of methylation marks are likely to be important in gene regulation and this process is very complex. Depending on the site of methylation (promoter vs intron), epigenetic marks may play different roles in control of gene expression in *cis*. Mapping studies of methylation marks on gene expression (methyl-eQTL) will be essential in identification of *cis*- and *trans*-effects. The final major challenge will be identification of cohorts with comparable genetic background and environmental exposures to use as an independent validation step. It is likely that cohorts with similar exposures and phenotypes will be of most utility for broad validation of large number of epigenetics marks and identification of specific phenotype- and exposure-driven epigenetic changes while more divergent cohorts may still be useful in validation of a small number of epigenetic marks associated with disease regardless of other factors.

The Potential Impact of Epigenetics Research on Asthma

While we know that inheritance, parent-of-origin, environment, *in utero* exposures, and Th_2 immunity play important roles in the etiology of asthma, there is no well-developed unifying mechanism accounting for these etiologic events/triggers. Although the *Hygiene Hypothesis* is appealing conceptually[3] and ties a number of these basic etiologic events together, there are several competing hypotheses (T cell skewing, infection, diet, obesity, etc.), and none of them fully account for the complex interaction between host and environmental determinants of asthma. For example, the *Hygiene Hypothesis* suggests that a decrease of exposure to microbes would, through enhanced atopic immune responses, increase the incidence of allergies and allergic asthma[3]. However, the prevalence of atopy and asthma are not concordant, allergic mechanisms account for at most 50% of asthma cases, very high asthma rates are present in some countries where hygienic conditions are less than ideal, and although the prevalence and incidence of asthma continue to increase in inner cities in the U.S., housing conditions in these communities are becoming more hygienic.

While epigenetic mechanisms not only provide a unique cause of asthma, these basic transcriptional controls potentially serve to explain some of the prevailing hypotheses underlying the development of asthma. For example, the *Hygiene Hypothesis* is dependent on activation of innate immune genes, including genes activated by the Toll-like receptors; importantly, epigenetic mechanisms control the activation of these innate immune genes and, consequently, the extent of the inflammatory response[149, 150]. Moreover, a recent study demonstrated that microbes may also operate by means of epigenetic mechanisms[151]. In this animal study, prenatal administration of the farm-derived gram-negative bacterium *Acinetobacter lwoffii* F78A prevented the development of an asthmatic phenotype in the progeny, and this effect was IFN-γ dependent. Prenatal microbial exposure was also associated with a significant protection against loss of H4 acetylation in the promoter of *Ifng*, which was closely associated with IFN-γ expression in $CD4^+$ lymphocytes as well as a decrease in H4 acetylation at the *Il4* promoter. Pharmacologic inhibition of H4 acetylation in the offspring abolished the asthma-protective phenotype. So while epigenetic mechanisms have the potential of changing our basic concepts about asthma, these mechanisms may not only account for the etiologic events/triggers related to asthma but may also help to explain some of the prevailing hypotheses attributed to this disease.

Furthermore, identification of key epigenetic marks has the potential to transform asthma therapy from palliative to preventive, and may alter our recommendations for pregnancy throughout the world. Currently, other than avoidance of cigarette smoke, we are simply

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unable to prevent asthma. Most patients with asthma rely on chronic medications to reduce the severity of their symptoms. Understanding the importance of epigenetic mechanisms in the development of asthma and the periods of vulnerability in establishing epigenetic marks has the potential of preventing the development of this disease, not only in our offspring but in their children as well. Identification of critical epigenetic marks associated with the development of asthma and influenced by specific environmental factors at certain timepoints, *in utero* or postnatally, would allow us to advise on intake of dietary supplements and limiting harmful exposures during the critical windows when these dietary and environmental factors have the strongest influence on the development of disease. Understanding the complex interactions between *in utero* exposures and epigenetic vulnerability will provide insight into future interventions for individuals at risk for the development of allergic asthma and may lead to the prevention of this disease altogether.

However, asthma is a complex disease. And although epigenetic mechanisms may contribute to the etiology and pathogenesis of this disease, there are multiple pieces to the asthma puzzle. The challenge will be to understand how genetic variation, transcriptome, epigenetic marks, the environment, and the immune system interface with each other to result in the development of allergic and non-allergic forms of asthma.

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Abbreviations

CHIP	chromatic immunoprecipitation
CpG	cytosine followed by guanine in DNA sequence
DC	dendritic cell
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
GWAS	genomewide association
HAT	histone acetyltransferase
HDAC	histone deacetylase
QTL	quantitative trait locus
MBD	methyl-binding domain
miRNA	micro RNA
ncRNA	non-coding RNA
PCA	principal components analysis
qPCR	quantitative PCR
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
Th	T helper cells

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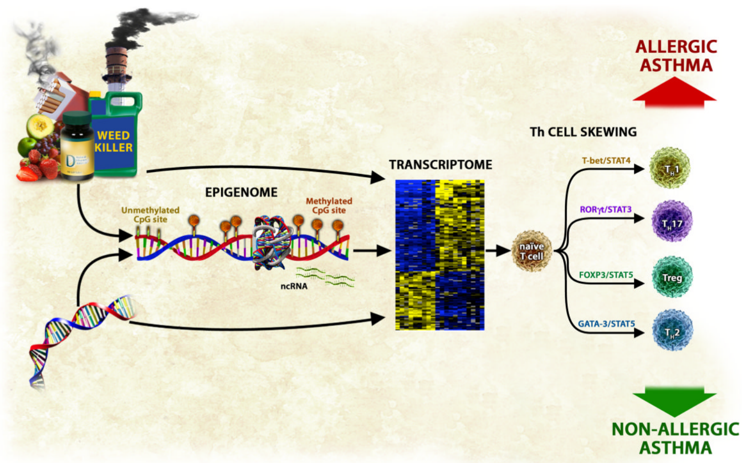

Figure 1.

An overview of epigenetic regulation of gene expression in asthma. Environmental exposures and dietary factors an individual is exposed to both in utero and postnatally influence epigenetic marks which in turns regulates genes expression. Underlying genetic variation can regulate gene expression by affecting epigenetic marks or by other mechanisms (alteration of transcription factor binding sites, for example). Alterations in epigenetic marks have consequences on expression of key immune genes that regulate Th subtype cell skewing, which in turn leads to development of disease. It is likely that distinct epigenomic profiles are associated with the development of allergic and non-allergic forms of asthma.

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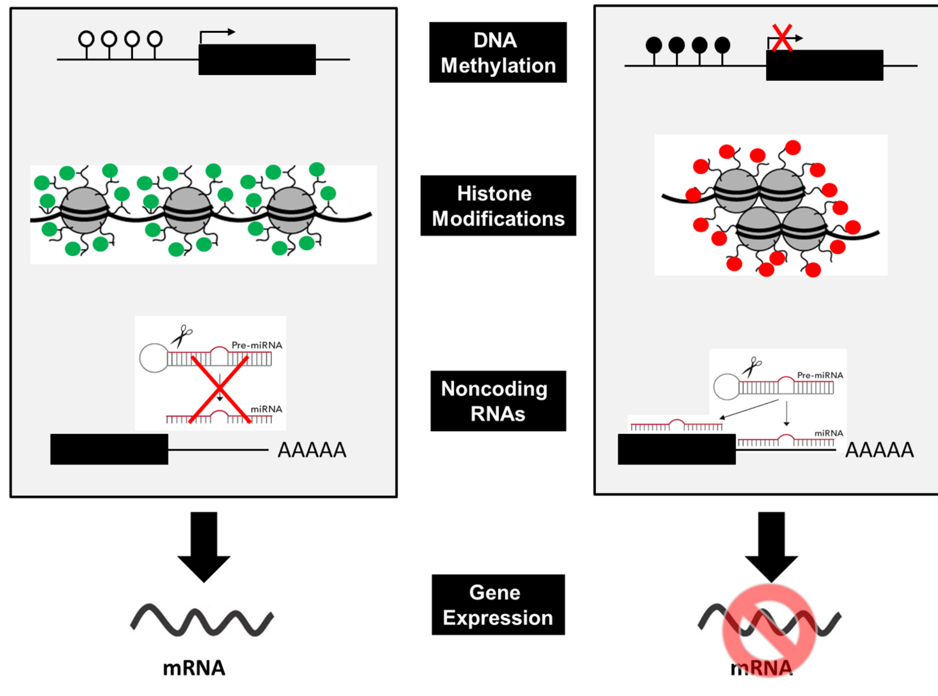

Figure 2.
Effect of epigenetic marks – DNA methylation, histone modifications, and miRNAs – on gene expression. White circles denote unmethylated CpGs while black circles denote methylated CpGs. Green circles refer to permissive histone modifications while red circles indicate repressive histone marks. miRNAs can affect gene expression by either RNA degradation (perfect complementarity and binding) or inhibition of protein translation (imperfect complementarity and partial binding).

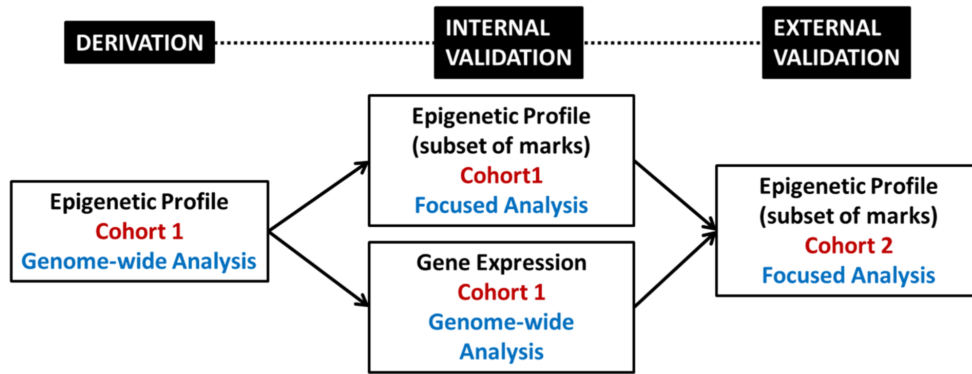

Figure 3.
An overview of the validation process for discovery of epigenetic marks associated with development of asthma. Internal validation refers to confirmation in the same cohort by a different technique and association of epigenetic marks with changes in gene expression while external validation refers to validation of epigenetic marks in an independent cohort. Genome-wide analysis of gene expression is preferable to focused approaches due to complexities in the relationship of epigenetic marks and gene expression alterations.

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Table 1

Key Components of the Epigenetic Regulation of Gene Expression

Epigenetics	The word epigenetics is derived from the Greek word epi for over or above, and genetics or the science of heredity. Two key components of epigenetics (DNA methylation and histone modifications) together define chromatin state beyond the information that is encoded in the genomic DNA sequence. Together they control degree of accessibility of the genomic DNA fraction of chromatin. Tightly bound 'closed' chromatin state is less accessible to transcription machinery and other regulatory proteins while more accessible 'open' chromatin state leads to active gene transcription. Epigenetic marks work in concert with other components of cellular regulatory machinery (transcription factors, enhancer, and repressors) to control spatial and temporal level of expressed genes.
DNA Methylation	The extent of methylation of cytosines in ~ 30 million CpG sites in the human genome. 60–90% of CpGs across the human genome are methylated while regulatory regions containing more dense areas of CpG motifs (CpG islands; stretches of DNA >200 bp in length with >50% GC content and observed/expected CpG>0.6[152]) are generally unmethylated. DNA methylation also correlates with spatial organization of the chromatin (proximity of chromosomal loci)[153].
Histone Modifications	In eukaryotes, DNA is packaged and ordered into nucleosomes, the basic structural unit of chromatin, by wrapping around the octamer consisting of 2 copies each of histone proteins (H2A, H2B, H3 and H4). Histone modifications include methylation, acetylation, phosphorylation and other modifications of specific amino acids in nucleosomal histones. Generally, histone marks are described as 'permissive' (active promoters), 'repressive' (inactive promoters) or 'poised' (accessible promoters).
Noncoding RNA	The term non-coding RNA (ncRNA) is commonly employed for RNA that does not encode a protein. ncRNAs include both small and large classes of RNA molecules that control gene expression by a variety of mechanisms.

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Table 2

Summary of Commonly Used Techniques for Epigenetic Profiling

Type of Epigenetic Mark	Type of Sample Preparation Approach	Type of Profiling Approach
DNA methylation	Bisulfite conversion	Microarray (Illumina), High-throughput sequencing (BS-seq, RRBS-seq), Epityper (mass spectrometry; focused) or pyrosequencing (focused)
	Methylated DNA immunoprecipitation (MeDIP)	Microarray or High-throughput sequencing (MeDIP-seq)
	Methyl-binding domain (MBD) precipitation	Microarray or high-throughput sequencing (MBD-seq)
	Restriction digest with methylation-sensitive restriction enzymes (McrBC, MspI, HpaII, MspJI etc)	Microarray (CHARM, HELP) or high-throughput sequencing (MRE-seq)
Histone modifications	Chromatin immunoprecipitation (CHIP)	Microarray (CHIP-chip), high-throughput sequencing (CHIP-seq) or quantitative PCR (focused)
DNA methylation associated with chromatin modifications	CHIP followed by bisulfite conversion	High-throughput sequencing (CHIP- BS-seq, Bis-CHIP-seq)
Noncoding RNAs	Size selection of appropriate RNA molecules	Microarray, high-throughput sequencing (miRNA-seq and RNA-seq) or quantitative RT-PCR (focused)
Chromatin accessibility	DNAse I cleavage	High-throughput sequencing (DNase sensitivity-seq)
Chromatin accessibility Chromatin spatial organization	Formaldehyde cross-linking	High-throughput sequencing (FAIRE-seq)
	Chromosome conformation capture	High-throughput sequencing (3C-seq, 4C-seq, 5C-seq)