Chapter 11
Asthma Epigenetics

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Abstract Asthma is the most common chronic disease of childhood, and a growing body of evidence indicates that epigenetic variations may mediate the effects of environmental exposures on the development and natural history of asthma. Epigenetics is the study of mitotically or meiotically heritable changes in gene expression that occur without directly altering the DNA sequence. DNA methylation, histone modifications and miroRNAs are major epigenetic variations in humans that are currently being investigated for asthma etiology and natural history. DNA methylation results from addition of a methyl group to the 5 position of a cytosine ring and occurs almost exclusively on a cytosine in a CpG dinucleotide. Histone modifications involve posttranslational modifications such as acetylation, methylation, phosphorylation and ubiquitination on the tails of core histones. MicroRNAs are short ~22 nucleotide long, non-coding, single-stranded RNAs that binds to complementary sequences in the target mRNAs, usually resulting in gene silencing. While many studies have documented relationships of environmental exposures that have been implicated in asthma etiology with epigenetic alterations, to date, few studies have directly linked epigenetic variations with asthma development. There are several methodological challenges in studying the epigenetics of asthma. In this chapter, the influence of epigenetic variations on asthma pathophysiology, methodological concerns in conducting epigenetic research and future direction of asthma epigenetics research are discussed.

Keywords Epigenetics • DNA methylation • CpG island • Histone acetylation • Histone acetyltransferase (HAT) • Histone deacetylase (HDAC) • Sirtuin1 (SIRT1) • microRNA (miRNA) • Single nucleotide polymorphism (SNP) • Telomerase • CpG pyrosequencing • Chromatin immunoprecipitation-next generation sequencing (ChIP-Seq)

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11.1 Introduction

Epigenetics is the "structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird 2007). In simpler terms, it is the study of mitotically or meiotically heritable states of gene expression potential that occur without directly altering the DNA sequence. DNA methylation, histone modifications, and microRNAs are major epigenetic variations in humans that are currently being investigated for asthma etiology and natural history. Furthermore, researchers in the field of environmental epigenetics are interested in evaluating the role of epigenetics as mediating factors that link environmental exposures (e.g., tobacco smoke, air pollution, and dietary factors) to asthma occurrence(Cortessis et al. 2012). In this chapter, our current understanding of the role of epigenetic variation on asthma pathophysiology is described and future direction of asthma epigenetics research is discussed.

11.2 DNA CpG Methylation

DNA methylation refers to the covalent addition of a methyl group to cytosine nucleotides (5-methylcytosine or 5mC) adjacent to guanine residues in the DNA sequence—so-called CpG sites (Fig. 11.1). CpG sites clustered in high frequency near gene promoters are referred to as CpG islands (CGI). In contrast, comparatively low CpG density areas located within 2000bp of traditional CGI are called CpG islands shores (Irizarry et al. 2009). DNA methylation is a nonrandom biological process, mediated by members of the DNA methyltransferases family (DNMTs). While gene promoter DNA hypermethylation is often associated with gene silencing, the effect of hypermethylation of intragenic regions (exons and introns) on gene transcription is complex and less clear.

Although there is a growing interest among researchers in exploring the role of epigenetic variation in asthma etiology (Miller and Ho 2008), only one study has documented the relationship between gene-specific DNA methylation and asthma (Perera et al. 2009). In that study, Perera and colleagues first used umbilical cord blood (UCB) white blood cells and conducted methylation-specific PCR (MS-PCR) to bisulfite-converted DNA. They found that maternal exposure to polyaromatic hydrocarbon (PAH; a constituent found in diet, tobacco smoke and traffic-related pollution) was associated with DNA methylation level in a CpG site in *ACSL3* in UCB white cells. Subsequently, these authors found that DNA methylation level in *ACSL3* was associated with increased asthma risk in children (odds ratio [OR] = 3.9; 95% confidence interval (CI): 1.14–14.3). Using this same study sample, these authors recently reported that subjects with higher benzo[a]pyrene concentration in UCB had higher DNA methylation in IFNγ promoter in UCB white cells and lower expression of IFNγ; however, the authors did not report whether variation in IFNγ promoter was associated with asthma (Tang et al. 2012).

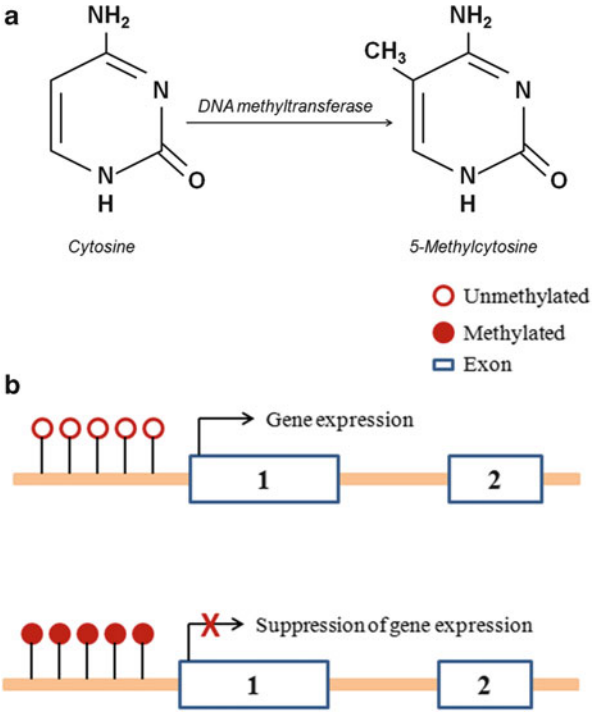

Fig. 11.1 Schematic diagram DNA methylation (**a**) and unmethylated and methylated CpG island in the promoter region of a gene. DNA methyltransferases mediate the conversion of cytosine to 5-methylcytosine by adding addition of a methyl group. Unmethylated CpG (*open red circles*) in the promoter region allows gene to be expressed, whereas methylation of promoter CpGs (*closed red circles*) suppress gene expression

Prenatal exposure to dichlorodiphenyldichloroethylene (DDE) has been associated with increased asthma in children (Sunyer et al. 2005). Morales et al. (2012) examined the role of DNA methylation in mediating this relationship in two cohorts of children. The discovery sample had 122 subjects (17 with persistent wheeze), whereas the replication sample included 236 children (37 with persistent wheeze). Using Illumina GoldenGate Methylation Cancer Panel I array, the authors found 54 CpGs that were differentially methylated by wheeze status; at 4 CpG islands (*ZNF264*, *ALOX12*, *EPO*, and *PDGFB*) the difference was more than 12 % between children with and without persistent wheeze. The methylation levels was lower in children with persistent wheeze at *ZNF264*, *ALOX12*, and higher in *EPO* and *PDGFB* compared to children who never wheezed. *ALOX12* (encoding arachidonate 12-lipoxygenase) belongs to the arachidonic acid metabolic pathway, prompting the investigators to attempt validation and replication of the findings. Technical validation in the discovery sample by Pyrosequencing showed that the Illumina panel overestimated *ALOX15* methylation levels; moreover, the associations with persistent wheeze in the discovery cohort were no longer significant upon repeat testing using the Pyrosequencing-based *ALOX12* methylation level. In the replication sample, however, lower cord-blood DNA methylation levels at two of the four investigated *ALOX12* CpGs was significantly associated with increased risk of persistent wheeze. In the discovery sample, levels of DDE in cord blood (but not

maternal blood samples) were inversely correlated with DNA methylation in one of the *ALOX12* CpGs (labeled as E85). This chapter provides the first epigenetic link between exposure to pesticides and asthma and suggests that *ALOX12* DNA methylation could be an epigenetic biomarker of susceptibility to asthma.

11.3 Histone Modification

DNA is tightly packaged by histone proteins to form a highly organized chromatin structure. The core histones are H2A, H2B, H3, and H4. Two H2A–H2B dimers and one H3–H4 tetramer form the nucleosome (Fig. 11.2a). Histone H1 is not part of the nucleosome but binds the latter to the linker DNA and seals off the entry and exit points of the DNA that wraps the nucleosome. Posttranslational histone modifications, such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation on the tails of core histones, are important epigenetic modifications for gene transcription. Acetylation of histone tails, for example, often results in a relatively loose structure of chromatin, which increases the accessibility of binding sites of transcription factors and thus the activation of gene expression. The status of histone acetylation is reversibly regulated by two distinct enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC). Increased histone acetylation by HATs leads to the unwinding of chromatin structure and transcriptional activation, whereas removal of acetyl groups by HDACs causes chromatin condensation and transcriptional silencing (Fig. 11.2b).

Among histone modifications, HDACs are implicated in development and regulation of T lymphocytes, the latter implicated in asthma etiology. In a mouse model, conditional deletion of HDAC1in T-cell lineage resulted in increases eosinophil recruitment into the lung, mucus hypersecretion, parenchymal lung inflammation, and increased airway resistance with parallel increase in Th2 cytokine (IL-4 and IL-13) in HDAC-deficient Th2 cells (Grausenburger et al. 2010), suggesting that HDAC inhibition may result in allergic airway inflammation. Although the role of histone modifications on development of asthma in children has not been published to date, few studies have documented the effect of HDAC inhibitors (trichostatin A and sirtinol) on airway inflammation in humans and in animal models with conflicting results. An in vivo study in a murine model showed that trichostatin A (TSA, a reversible HDAC inhibitor) attenuated allergen-induced airway inflammation and airway hyperresponsiveness (AHR) and reduced IL-4, IL-5, and IgE in bronchoalveolar lavage fluid (BALF) (Choi et al. 2005). Mammalian sirtuin1 (SIRT1) is a class III HDAC that deacetylases several transcription factors (i.e., PPARγ, p53, and NF-κB) and play a role in inflammation and aging (Kim et al. 2010; Yang et al. 2007). In another murine model, ovalbumin (OVA) inhalation resulted in increased SIRT1, hypoxia-inducible factor 1α (HIF-1α), and vascular endothelial growth factor (VEGF) protein levels in lung tissues and increased AHR (Kim et al. 2010). These effects were significantly attenuated by administration of sirtinol (inhibitor of SIRT1).

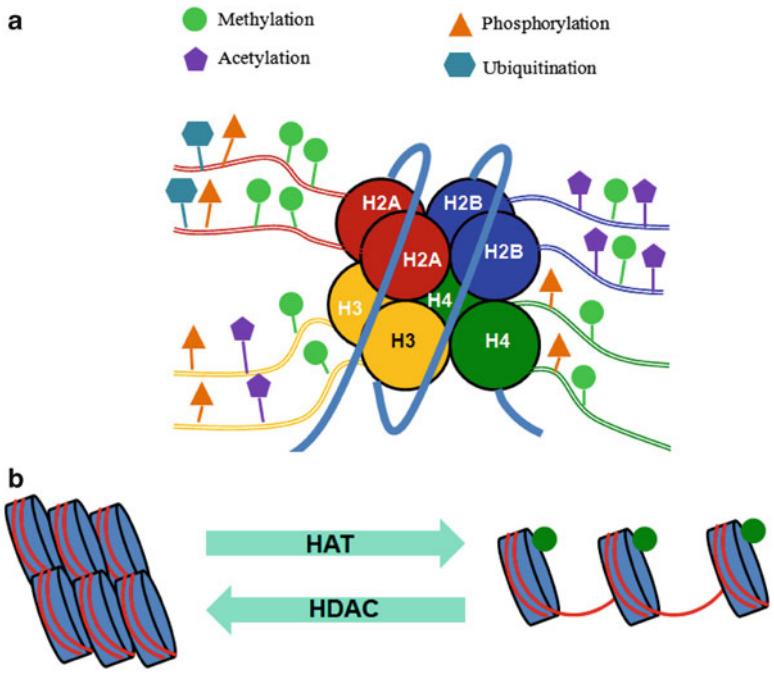

Fig. 11.2 Schematic illustrations of (**a**) a nucleosome with common histone modifications on histone tails and (**b**) function of histone acetyltransferases (HATs) and histone deacetylases (HDACs) o chromatin structure

Contrary to these potential benefits of HDAC inhibitors, other studies showed effects that could be detrimental in subjects with asthma. For example, in a study where human alveolar epithelial cell line (A549 cell) was used, IL-1β induced transcription of transforming growth factor β1 (TGF-β1) and resulted in increased histone H4 and H3 acetylation in distinct promoter regions of TGF-β1 (Lee et al. 2006). Prevention of histone deacetylation by administration of HDAC inhibitor TSA further increased TGF-β1 expression in this study, further supporting the role of histone acetylation in activation of TGF-β1. However, HDAC inhibitor-mediated increase in TGF-β1 concentrations in airway could have detrimental effects in asthma. A large body of evidence indicates that in inflammatory milieu (i.e., in the presence of IL-6 or IL-1β), TGF-β1plays a critical role in the development of pro-inflammatory Th17 cells (Gutcher et al. 2011; Qin et al. 2009). Earlier studies have also shown that the variant -509T allele in a promoter SNP is associated with increase TGF-β1 gene transcription, increased plasma TGF-β1 concentrations, and increased risk of asthma (Salam et al. 2007; Silverman et al. 2004).

Finally, there are conflicting reports on the role of HDACs on steroid-resistant asthma. Earlier studies documented that downregulation in HDAC2 expression is one reason for steroid resistance in asthma (Ito et al. 2006; Li et al. 2010). However,

data from a recent study suggest that downregulation of HDAC1 and HDAC2 expression does not occur in severe asthma and the earlier finding may have resulted due to clathrin cross-reactivity with commercial antibodies (Butler et al. 2012). Based on these contradictory findings, the therapeutic potential of HDAC inhibitors in asthma management remains questionable. Additional studies are needed to fully appreciate the effects of HDAC inhibition in different pathophysiological processes that are involved in asthma.

11.4 MicroRNAs

MicroRNAs (miRNAs) are small (~22 nucleotides), noncoding, single-stranded RNAs that bind to target mRNA through complementary sequences and negatively regulate gene expression at posttranscriptional level. The majority of miRNAs repress gene expression by mRNA destabilization and translational inhibition. The miRNAs are generated from much longer primary miRNA by a multistep process, which is regulated by RNase III endonuclease (Drosha and Dicer). The target sequence of a given miRNA is typically seen in many 3′ UTR sequences, such that one miRNA sequence can regulate the expression of multiple genes in a coordinated fashion (Fig. 11.3).

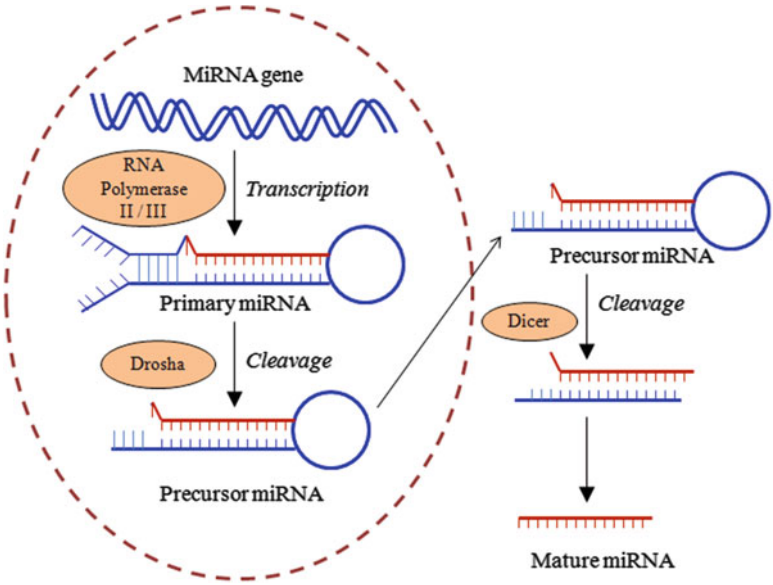

Fig. 11.3 Schematic diagram showing steps of microRNA (miRNA) synthesis

Many miRNAs have been implicated in key pathophysiological asthma processes including immune development and differentiation, AHR, and airway inflammation. While the role of some candidate miRNAs are reviewed here, other candidates implicated in immune development but not yet linked to asthma directly [i.e., miR-9 (Bazzoni et al. 2009), miR-155 (Louafi et al. 2010; Martinez-Nunez et al. 2009; O'Connell et al. 2007, 2010), miR-127 (Xie et al. 2012), miR-147 (Liu et al. 2009), and miR-221 (Mayoral et al. 2011)] are not.

DNA sequence variation in miRNA genes (including the pri- and pre-miRNAs) has been found to influence miRNA function (Duan et al. 2007). Few studies have evaluated the impact of polymorphisms in miRNA target sites on asthma. Following up on the earlier finding that HLA-G as an asthma-susceptibility gene (Nicolae et al. 2005), Tan et al. (2007) found that the +3142C/G SNP (*rs1063320*) in the HLA-G 3′ untranslated region affects binding of miR-148a, miR-148b, and miR-152 to the gene and the SNP interacted with maternal asthma to influence asthma risk in the offspring. The GG genotype was associated with reduced risk of asthma in children born to asthmatic mother, whereas the GG genotype was associated with increased risk of asthma in children born to nonasthmatic mothers. In another study, SNPs in pre-miRNA sequence (miR-146a rs2910164 and miR-149 rs2292832) were associated with a reduced risk of asthma in a Chinese population (Su et al. 2011).

In an experimental study, stretch stimulation of human airway smooth muscle cells (HASMC) induced transcription of miR-26a and resulted in hypertrophic responses in HASMCs (Mohamed et al. 2010). The miR-26a targeted the mRNA 3′ untranslated repeats of glycogen synthase kinase-3β and suppressed GSK-3β protein expression. However, using tissues collected by airway biopsy, no significant difference was detected for miR-26 family and more than 200 other miRNAs (including let-7, miR-125, and miR-30 families) between normal and mild asthmatic subjects (Williams et al. 2009). The investigators also compared miRNAs profiles between airway biopsy samples before and after budesonide treatment; however, no differential expression pattern was revealed.

One of the pathologic hallmarks of asthma is AHR. In animal models of asthma, upregulation of RhoA (a monomeric GTP-binding protein) has been associated with increased contraction of bronchial smooth muscle. Using human bronchial smooth muscle cells (hBSMC), Chiba et al. (2009) found that miR-131a negatively regulated expression of RhoA in BSMs. Downregulation of miR-131a and upregulation of RhoA occurred when hBSMCs were treated with IL-13 and in airway tissues of OVA-challenged mice. Inhibition of miR-131a by treatment with antagomir-133a also resulted in upregulation of RhoA in this study.

Animal models of asthma have implicated several miRNAs. In one study, upregulation of miR-21 was observed in three asthma models (OVA, *A. fumigatus*, and induced IL-13 transgenic mice) compared to control mice (Lu et al. 2009). The authors also found that miR-21 was predominantly expressed in the cytoplasm of cells in monocyte/macrophage lineage with highest expression in dendritic cells in bone marrow. IL-12p35 mRNA was found as the potential target gene of miR-21 and IL-12p35 mRNA was decreased in all three asthma models. Recently,

miR-21 is implicated in allergic rhinitis (AR) in children. In a study where a panel of 157 miRNAs was interrogated using mononuclear leucocytes from human umbilical cord blood, miR-21, miR-96, and miR-126 were significantly downregulated in neonates with high cord blood immunoglobulin E (CBIgE), whereas the expression of miR-21 and miR-126 was significantly lower in children with AR (Chen et al. 2010). The authors found that miR-21 targeted transforming growth factor receptor 2 (TGFBR2) and low miR-21 expression in CB was associated with significantly higher TGFBR2 expression is cord blood leucocytes in children with high CBIgE and those with AR compared to children with low CBIgE and those without AR, respectively. Because rhinitis is a significant risk factor for asthma (Chawes et al. 2010), further studies could examine whether miR-21 expression is associated with asthma risk.

Therapeutic potential of miRNA inhibitors have also been investigated in animal models of asthma. In OVA-sensitized female BALB/c mice, exposure to low levels of OVA resulted in marked increase in miR-126 expression in exposed mice compared to control by 2 weeks; however, the expression was reduced to baseline level by 6 weeks (Collison et al. 2011a, b). Inhibition of miR-126 by administration of an antagomir suppressed eosinophil recruitment into the airways but had no effect on chronic airway inflammation and remodeling. Another study also documented change in miRNA profiles by lipopolysaccharide exposure in male BALB/c without any significant modulating effect of dexamethasone (Moschos et al. 2007). In contrast to these findings, another study documented that exposure to house dust mite (HDM) increased expression of miR-145, miR-21, and let-7b by fivefold in BALB/c mice (Collison et al. 2011a, b). Furthermore, inhibition of miR-145 by using an antagomir reduced eosinophilic inflammation, mucus hypersecretion, Th2 cytokine production, and AHR; however, inhibition of miR-21 or lel-7b had no significant influence on HDM-mediated airway inflammation. The authors concluded that the anti-inflammatory effects of miR-145 were comparable to steroid treatment. The emerging evidence suggests a complex role of miRNAs in mediating the effects of exposure on underlying pathophysiological mechanisms involved in asthma.

11.5 Telomere Length

Telomeres are specialized protein-bound, multiple short DNA repeats that are located at the end of chromosomes (Nandakumar and Cech 2013). They are critically important in chromosomal stability and in the regulation of somatic-cell replication (Allsopp et al. 1992). Telomere shortening may reflect the total number of divisions experienced by a somatic cell and is associated with replicative senescence. Telomere shortening, a marker of biological aging, may represent an additional cellular level biomarker of adversity. While telomerase, a cellular enzyme, extend telomeres in germ cells and stem cells, it is absent in the majority of somatic cells. Therefore, telomere length shortens with each successive cellular division in somatic cells, and increased oxidative stress due to environmental exposures

(e.g., tobacco smoke, and air pollution) can further accelerate telomere shortening (Hou et al. 2012; Hoxha et al. 2009; McGrath et al. 2007; Morla et al. 2006; Valdes et al. 2005; von Zglinicki 2002). In telomerase null mice with short telomeres, exposure to tobacco smoke has been associated with emphysematous changes in the lungs (Alder et al. 2011). In humans, telomere length was found to shorter in subjects with chronic obstructive pulmonary disease (COPD) compared to healthy control in few cross-sectional studies suggesting accelerated aging in these patients (Houben et al. 2009; Savale et al. 2009). However, there has been no published report describing association between telomere length and development or natural history of asthma.

11.6 Assay Methods

In the last few decades, multiple assay techniques have been developed and utilized for characterizing epigenetic variation across including quantitative measures of DNA methylation, microRNA expression, and histone modifications, at a gene or genome-wide level (Bhandare et al. 2010; Laird 2003, 2010; O'Geen et al. 2011; Wei et al. 2012). A review of all such methodologies is beyond the scope of this chapter. Here, we review the most commonly used contemporary methods for studying DNA methylation (i.e., pyrosequencing) and histone modification (i.e., ChIP-seq). The assays for microRNA expression utilize quantitative RT-PCR or commercially available miRNA microarray analysis (Jardim et al. 2012; Levanen et al. 2013; Solberg et al. 2012), protocols similar to the ones used for gene expression, which are reviewed elsewhere in this text.

11.6.1 DNA CpG Pyrosequencing

Pyrosequencing is a real-time DNA sequencing-by-synthesis method in which enzymatic reactions and bioluminescence are used to determine methylation status at one to few CpG sites (Nyren 2007). Initially, bisulfite treatment of the genomic DNA converts unmethylated cytosine to uracil without affecting methylated cytosines. During PCR of the bisulfite-treated DNA, the uracil base is converted to thymine, which results in reading the methylated cytosine sites as virtual C/T polymorphisms. The ratio of cytosine and thymine present at each CpG site is then quantified and reflects the methylation level of that site in genomic DNA.

There could be several major technical issues that might affect measurement of DNA methylation. Insufficient denaturation of DNA may lead to bisulfite conversion failure. In such instances, bisulfite treatment has to be repeated (Tost and Gut 2007). There might be no or very weak PCR products, which may necessitate reoptimizing the assay by reducing extension time or amount of polymerase. If problem persists, then pyrosequencing may not be a suitable method for estimating DNA

methylation. In addition, any problem with annealing and hybridization of the pyrosequencing primer may lead to weak pyrosequencing signal. A redesign of primer and reoptimization of the assay may be required to obtain relatively stronger pyrosequencing signal (Mikeska et al. 2011; Tost and Gut 2007). For some of this problem, this assay may be time consuming and labor intensive.

11.6.2 CpG Genotyping

Although pyrosequencing allows accurate measurement of DNA methylation, one of the major limitations of this method is that only few CpG sites could be analyzed in a single sequencing reaction. Because it is difficult to automate the method to interrogate multiple CpGs at one time, it becomes labor intensive and costly as the number of CpGs investigated in a study increases. Currently, there are many commercial array-based (e.g., Illumina GoldenGate and Infinium assays) and next-generation sequencing-based [e.g., bisulfite sequencing with padlock probes (BSPP) (Deng et al. 2009), reduced representation bisulfite sequencing (RRBS) (Meissner et al. 2005), whole-genome shotgun bisulfite sequencing (WGSBS) (Cokus et al. 2008)] technologies that are available that can measure hundreds of thousands of CpGs in genome-wide scale, albeit the number of CpGs each analysis techniques can measure vary by order of magnitude (Laird 2010). Because these high-throughput technologies have some biases, pyrosequencing of the CpGs that are most significantly associated with an exposure or health outcome of interest remains a standard practice to validate DNA methylation level observed in genome-wide arrays in those CpGs.

11.6.3 ChIP-seq

Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) allows investigating protein–DNA interactions such as histone modification, transcription factor binding. The principles, guidelines and recommendations for ChIp-seq assay has been recently published based on the work done in the ENCODE and the modENCODE consortia (Landt et al. 2012). The first step of ChIP-seq requires cross-linking proteins to DNA using formaldehyde to stabilize the interaction between protein factors and chromatin (Fig. 11.4). Sonication, cell disruption, or enzymatic digestion (by micrococcal nuclease or MNase) is then used for chromatin fragmentation to obtain 100–300 bp target size for enrichment. The target DNA with its histone mark is then precipitated with specific antibody or epitope tag to purify and enrich the protein (histone mark in this case) (Landt et al. 2012). Once target histone marks are enriched through immunoprecipitation, cross-links are reversed and the DNA sequenced using high-throughput sequencing technologies. Sequence reads are then aligned to reference genome, and "peak calling" within regions of high numbers of aligned reads are performed. There are

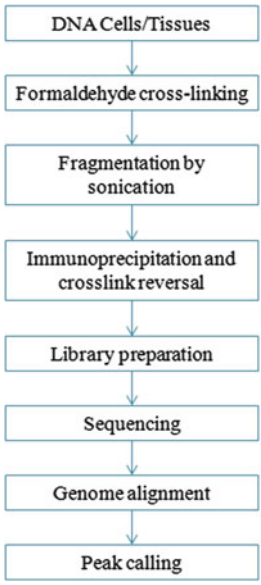

Fig. 11.4 A diagram showing ChIp-seq workflow

many software packages that are available for peak calling, which rely on different statistical models to calculate *p*-values and false discovery rates (Landt et al. 2012; Park 2009). Appropriate thresholds are necessary to detect enriched regions while limiting false discovery rate. Histone modifications may have sharp peaks at regulatory elements and broad peaks at transcribed or repressed regions (Park 2009; Wang et al. 2013).

Although ChIP-seq provides high coverage and high resolution within a reasonable cost per sample, however, there are some of the major constraints of this method. One problem is antibody deficiency that can either cause poor reactivity with the target or cross-reactivity with other DNA-associated proteins (Landt et al. 2012). As much as 25 % of the commercially available histone-modification antibodies were found to have significant problems of antibody specificity (Egelhofer et al. 2011). It is thus recommended to perform primary and secondary tests for antibody characterization and that antibody characterization data should be reported to evaluate the assay method (Landt et al. 2012). For antibody characterization, immunoblot analysis can be used as primary test, while mass spectrometry, peptide binding test, or immunoreactivity analysis in cell lines that contains knockout of specific histone modification could be used as secondary test (Landt et al. 2012).

Potential biases introduced during the sequencing protocol include issues related to DNA amplification (Landt et al. 2012) and sequence alignment. Amplification-related biases can be identified and adjusted for by creating appropriate control libraries using non-ChIP genomic DNA. Mapping of target to genomic region (i.e., sequence alignment) can be challenging, particularly with short tags or for targets in genomic regions with high homology or repetitive sequencing (Park 2009).

As the ChIP-seq technology (including pre- and postprocessing steps) has been evolving very rapidly, the method is poised to be utilized in clinical and epidemiological studies to further our understanding of asthma etiology and natural history.

11.7 Future Directions

Some of the major questions that remain to be addressed in the field of asthma are (1) Do environmental exposures have relatively stable effects on some epigenetic profiles and dynamic effects on other? (2) Do epigenetic changes on individual cell types that have been found to be involved in asthma predicts asthma risk and modulates natural history of asthma? (3) Are there critical developmental windows which modulate exposure-mediated asthma susceptibility? (4) Are there synergistic effects of environmental exposures, and genetic and epigenetic variations on asthma risk and natural history of asthma? (5) Can intervention strategies modulate epigenetic profiles to affect asthma risk and exacerbations in children? and (6) Are there transgenerational effects of exposures that are epigenetically mediated that affect asthma risk? These research questions need to be critically evaluated in future studies to advance our understanding of the role of epigenetics on asthma and to find strategies to reduce the burden of asthma worldwide.

To address these research questions, appropriate study design, data collection, and analytic strategies are needed. First, epigenetic profiles need to be assessed in specific cell types, preferably more than one cell types that are implicated in asthma. With the backdrop of this methodological limitation, studying the role of environment on epigenetic variation and how that relates to future disease risk becomes even more challenging, as a sample collected during susceptible windows (i.e., before disease onset) would be required for etiologic research.

Repeated measure of epigenetic marks from birth to disease onset together with longitudinal evaluation of the effects of environmental exposures on those epigenetic marks could identify whether there are subsets of relatively stable and dynamic epigenetic marks and whether different windows of exposure has differential impact on epigenetic profiles. While the less-modifiable epigenetic marks could serve as biomarker of susceptibility, the modifiable ones could be target for intervention to alter risk profile and/or improve natural history of disease.

There are other methodological concerns that must be addressed. Multiple assay methods are available for measuring DNA methylation that relies on enzymatic digestion, affinity enrichment, and/or bisulfite conversion, discussed in more detail elsewhere (Harris et al. 2010; Laird 2010). Some of the genome-wide approaches for methylation assays are not sensitive to detect DNA methylation with low frequency; however, with next-generation sequencing approaches (albeit more expensive) could be used to detect rare CpGs (Taylor et al. 2007). In terms of assays using common DNA methylation array platforms, genetic variations (SNPs, repeats, deletions, copy number variations, etc.) need to be excluded and background correction and normalization of the data are needed (Adriaens et al. 2012; Johnson et al. 2007; Sabbah et al. 2011).

Once epigenetic biomarkers are identified, technical validation of the most significant loci by another assay (i.e., Pyrosequencing) and replication of the findings in independent samples are needed to assure data quality and to show consistency of findings, respectively. Although the bisulfite-based assay methods are quite accurate, incomplete bisulfite conversion could introduce measurement error in estimation of DNA methylation (Laird 2010). Monitoring of completion of bisulfite conversion is therefore needed (Campan et al. 2009). Linking variation in epigenetic marks to difference in gene expression could provide functional relevance of the epigenetic marks.

Although some animal studies showed transgenerational effects of exposures (diet and obesity) on DNA methylation (Hollingsworth et al. 2008; Waterland et al. 2008), true transgenerational effects of exposure on epigenetic profiles and on asthma have not been reported in humans to date. Although the area is of great interest, some major methodological problems exist. Evaluation of transgenerational effects of exposure on the epigenome requires at least four generations because any exposure to an F_0 female during pregnancy exposes not only the F_1 embryo but also affects germline of the F_2 generation (Perera and Herbstman 2011). In addition, as opposed to controlled animal studies, the exposures in humans could happen postnatally in all subsequent generations and effects of the exposure to paternal sources of chromosomes could further complicate the matter in distinguishing true transgenerational effects from prenatal and postnatal exposure mediated effects on the epigenome.

11.8 Conclusion

Investigation of the etiology and natural history of a complex trait such as asthma in children is difficult. Studying epigenetics as a mediating factor for the associations between exposures and asthma development holds promise to unravel novel biologic pathways. While a good *dose* of optimism will advance the field of asthma epigenetics, too much hype must be avoided as even the robust effect sizes could be small, similar to what has been observed in GWAS. The potentially modifiable nature of the epigenetic profiles may allow us identify intervention approaches to reduce the risk of asthma in children and improve natural history of the disease.

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