EPIGENETICS AND DERMATOLOGY

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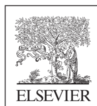

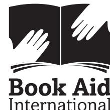

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Dedication

To our patients who suffer from skin diseases. May this book be a seed for future research and development of novel treatments to help alleviate dermatological illness of all forms, from allergic diseases to autoimmune skin diseases and cancer. We hope that epigenetics will provide potential cures and personalized approaches for many of these diseases.

QL, CC, and BR

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Preface

Epigenetics—the word epigenetics has been used since the 1940s, when Dr. Charles Waddington used the term to describe how gene regulation impacts development. In those days, before we even knew the structure of DNA, Dr. Waddington also coined the term *chreode*, to describe the cellular developmental process which leads to the paths that cells take toward development, a sort of cellular destiny. Now, some 70 plus years later, the term *epigenetics* has taken on a different meaning, though not necessarily a discordant philosophy, and is used to describe the study of how genes are regulated without a change in DNA sequence.

The concept of epigenetics embodies a broad range of cellular and biological phenomena, but the premise is based on the fact that gene expression may be altered in the absence of mutations or deletions, or other changes in DNA sequence, leading to different states of health and disease. How this is achieved is through the mechanisms of epigenetics, which includes DNA methylation and alterations in histone structure. MicroRNAs, which are short sequences of noncoding RNA that bind to promoter regions of genes to affect translation, have also been classified by some as an epigenetic phenomenon, but this is not without controversy.

The skin is the largest organ in the body. It is a dynamic, living, immunologic structure that possesses many functions, serving as a protective barrier to the outside world and a homeostatic system to support life. It is also an immune organ, and while it protects us from the dangers of microbes, pollutants, and toxins, it also participates in how we identify safety from hazardous exposures, thus acting as a medium for the development of tolerance. The systems in the skin are complex, involving numerous cell types and signaling molecules, and the pathways that govern the regulation of skin function add an additional layer of complexity. Thus, much can go wrong. Therefore, diseases of the skin range from neoplasms to infections to autoimmune diseases and allergic conditions. Solving the mysteries of skin function will help us find new ways to restore skin "health" or "normalcy." Epigenetics will no doubt play a significant role in these endeavors.

The first application of epigenetics was in cancer diagnosis and treatment. Interestingly, research scientists, pharmacologists, and physicians

have been using products that act by impacting epigenetics for many years without knowing it. For example, many herbal products were found to be efficacious in the treatment of some diseases, and were therefore widely used, and though we did not know it at the time, some of these herbal products actually act through epigenetic mechanisms. We are gradually recognizing that epigenetics is involved in many aspects of diseases, and the acquisition of data on how these processes work will help guide us in the development of novel, epigenetic treatment modalities that promise to help diagnose, treat, or even cure diseases in the coming future.

This book is divided into three sections. The first includes chapters addressing the basic science of epigenetics in various skin cell types. The second describes the role of epigenetics in dermatological conditions, and the third touches upon more general epigenetic diagnostic and therapeutic concepts and discusses the future of epigenetics and skin diseases.

It is the hope of us, the editors, that this book on epigenetics in dermatology will benefit readers from many disciplines, including but not limited to dermatologists, rheumatologists, biologists, allergists, immunologists, and oncologists. We hope that the reader will enjoy the discussions on all the various aspects by which epigenetics can impact skin function and diseases.

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CHAPTER

1

Introduction to Epigenetics

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The human genome project has been one of the most important scientific achievements in modern history. It has ushered in a new era in the field of life science research. However, among the project's many great discoveries, surprising findings such as only particular subsets of genes being able to be expressed at a particular location and time, led to the realization that knowledge of DNA sequences is insufficient to understand phenotypic manifestations. The mechanism by which DNA, or the genetic code, is translated into protein sequences is not merely dependent on the sequence itself but also on a sophisticated regulatory system that interplays between genetic and environmental factors. These mechanisms comprise the science of epigenetics, and the control of genes through various chemical interactions for the basis of at least part of the regulatory system overseeing the expression of the genetic code [1].

Epigenetics is defined as heritable changes in gene expression without changes in the DNA sequence. The prefix epi- is derived from the Greek preposition ἐπί, meaning above, on, or over. The term was first coined in 1942 by C.H. Waddington to denote a phenomenon that conventional genetics could not explain [2]. Since then, epigenetics has evolved into a branch of science that studies biological pathways and systems with well-understood molecular mechanisms. Simplistically, epigenetic mechanisms may involve modifications to DNA and surrounding structures such as DNA methylation, chromatin modification, and noncoding RNA (ncRNA).

DNA methylation is a stable and inheritable epigenetic mark. This genetically programmed modification is almost exclusively found on the 5′ position of the pyrimidine ring of cytosines (5mC) adjacent to a guanine. These sites are referred to as CpG sites, and the modification is

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mediated by specific enzymes called DNA methyltransferases (DNMTs). Transcription is generally repressed by hypermethylation of active promoters associated with CpG-rich sequences [3]. DNA methylation-based imprinting disorders play an important role in skin diseases such as systemic lupus erythematosus (SLE) [4], psoriasis vulgaris [5], primary Sjögren's syndrome [6], and other diseases. In addition, aberrations in the function of DNMTs and methyl-CpG-binding proteins (MBDs) can also contribute to skin diseases [7]. Recently, another modified form of cytosine, 5-hydroxymethylcytosine (5hmC), has been identified and is now recognized as the "sixth base" in the mammalian genome, following 5mC (the "fifth base") [8]. 5mC can be converted to 5hmC by the ten−eleven translocation (Tet) family proteins, which can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) to achieve active DNA demethylation [9]. Emerging evidence has indicated that 5hmC-mediated DNA demethylation and Tet family proteins may play essential roles in diverse biological processes including development and diseases, as illustrated by the critical function of 5hmC in the development of melanoma [10].

The other main mechanism in epigenetics involves changes to non-DNA gene components. DNA is tightly compacted by histone proteins. Posttranslational modifications on the tails of core histones, including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination, and sumoylation are important epigenetic modifications that regulate gene transcription. Abnormalities in these modifications, especially acetylation and deacetylation, can alter the structure of chromatin and perturb gene transcription, which can then contribute to disease development and progression. Histone acetylation status is reversibly regulated by two distinct competing families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Until now, four classes of HDACs have been identified (including Class I, Class II, and Class IV). HDACs are zinc-dependent proteases consisting of HDAC1−11, and Class III, also known as sirtuins (SIRT1−7), which require the cofactor NAD+ for their deacetylase function [11].

Another widely studied histone modification is methylation. Methylation of lysine or arginine in histone proteins alters the compaction or relaxation of chromatin depending on the position of amino acid and the number of methyl groups; for example, histone 3 tri-methylated at lysine 4 promotes gene transcription, while histone 3 tri-methylated at lysine 9 inhibits gene transcription [3]. Increasing evidence indicates the critical role of histone modifications in skin diseases including immune-mediated skin diseases, infectious diseases, and cancer [12−14].

It is debatable whether or not the role of ncRNAs constitutes an epigenetic phenomenon. There are some who will claim that ncRNAs such as microRNAs (miRNAs) are a fundamental part of nature and do not

satisfy the definition of epigenetics. However, others feel that since miRNAs do affect regulation of genes, they are a bona fide mechanism of epigenetic change.

The family of ncRNAs is diverse and complex. It can be divided into eight groups: ribosomal RNAs, transfer RNAs, miRNAs, long noncoding RNAs (lncRNAs), small nucleolar RNAs, small interfering RNAs, small nuclear RNAs, and piwi-interacting RNAs. ncRNAs are important epigenetic regulators in development and disease, especially miRNAs and lncRNAs. miRNAs are short ncRNA sequences (19−25 nucleotides) that regulate gene expression by binding to complementary sequences in the 3′ UTR of multiple target mRNAs, leading to translational repression (imperfect sequence match) or mRNA cleavage (perfect match) [15]. Since the first miRNA *lin-4* was characterized in 1993, an increasing number of miRNAs have been identified. Altered expression profiles of miRNAs in patients revealed a crucial role of miRNAs in cellular events and the development of diseases [16].

lncRNAs are functional ncRNAs, each exceeding 200 nucleotides in length and lacking functionally open reading frames. lncRNAs regulate gene expression through different molecular mechanisms. They can mediate the activity of proteins involved in chromatin remodeling and histone modification, or act as an RNA decoy or sponge for miRNAs. They can also bind to specific protein partners to modulate the activity of that particular protein [17]. Recent advancements in technology to identify ncRNAs using microarrays provide a great bulk of novel data from genomewide studies, and have revealed potential use of ncRNAs as diagnostic and prognostic biomarkers in various human disorders including skin diseases [18].

The role of genetics in disease is indisputable. But environmental exposures have also been demonstrated to play an essential role in the pathogenesis of skin diseases. Many diseases are now believed to occur as a result of a combination of genetic and environmental factors, but how do these two opposing forces interact? Epigenetic mechanisms may play a role in linking genetic and environmental factors, adding an additional element to the mechanism of disease.

Epigenetic regulation is generally accepted to play a key role in cellular processes. Aberrations of epigenetic modifications contribute to the pathogenesis of human diseases. With a growing knowledge of epigenetic mechanisms, we are confident that epigenetic markers can be applied as sensitive and specific biomarkers in disease diagnosis, evaluation, and prognosis. Moreover, epigenetic interventions may become an important supplement to traditional therapeutic approaches in the near future. The specific role of epigenetics in the pathogenesis, clinical phenotypes, and treatment of skin diseases is rapidly expanding as we continually increase our understanding of the mechanisms of epigenetics.

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CHAPTER

2

Laboratory Methods in Epigenetics

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2.1 INTRODUCTION

Epigenetic changes occur during cell differentiation, and serve to activate or suppress genes once the cells have reached terminal differentiation. Thus, epigenetics builds a bridge between genetics and environmental stimuli. Gene expression is up- or downregulated through epigenetic mechanisms in response to environmental changes. Abnormalities of epigenetic marks, such as DNA methylation, histone modifications, and aberrant expression of microRNAs (miRNAs), lead to the development of diseases. Mapping of the human epigenome is one of the most exciting and promising endeavors in terms of increasing our understanding of the etiology of diseases, and of developing new treatment strategies. Recent advances in technology have made it possible to interpret parts of the "epigenetic code." In this chapter, we summarize the classical strategies used in epigenetic studies and give a description of technological advancement in detection methodology.

2.2 DNA METHYLATION ANALYSIS

DNA methylation is an important epigenetic mark and a widely studied epigenetic change. The developments of DNA methylation studies keep pace with the advancements of detection technology. Over the past three decades, a large number of different methods have been

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applied in DNA methylation analysis. From the initial Southern blot analysis using methylation-sensitive restriction endonucleases to the current availability of microarray-based epigenomics, the technology used for DNA methylation analysis has been revolutionized [1]. Here, we discuss methods to distinguish 5-methylcytosine (5mC) from cytosine as well as methods that can distinguish 5-hydroxymethylcytosine (5hmC) from 5mC. Different methodologies available for analyzing DNA methylation are discussed, with a comparison of their relative strengths and limitations.

2.2.1 Methods to Distinguish 5-Methylcytosine from Cytosine

There are four major methods to distinguish 5-methylcytosine from cytosine. Many additional DNA methylation analysis techniques have been developed based on these primary methods (Figure 2.1).

2.2.1.1 Restriction Endonuclease-Based Analysis

2.2.1.1.1 Southern Blot

Southern blot analysis using methylation-sensitive restriction endonucleases is one of the classical and initial methods utilized in the measurement of DNA methylation in particular sequences. The two most commonly used pairs of isoschizomers are *Hpa*II-*Msp*I, which recognize CCGG, and *Sma*I-*Xma*I, which recognize CCCGGG. Neither *Hpa*II nor *Sma*I can digest methylated cytosine [2]. Although this method is relatively inexpensive and the interpretation of results is straightforward, it is limited by the availability of restriction enzyme sites in the target DNA. Other

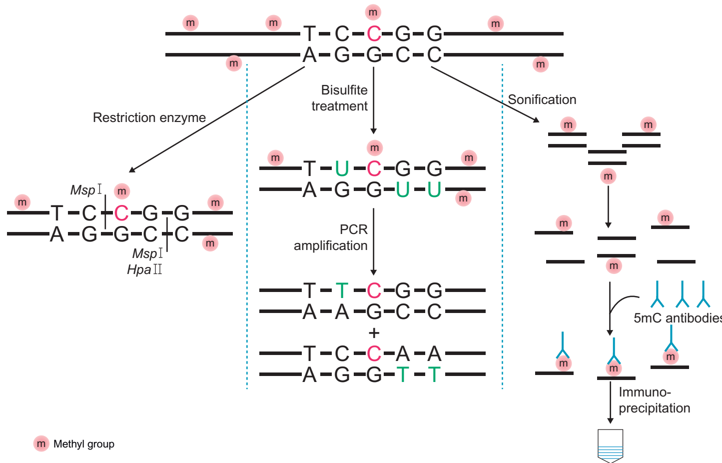

FIGURE 2.1 Principles to distinguish 5-methylcytosine from cytosine.

limitations include large amounts of high-quality DNA and problems with incomplete digestions. These disadvantages render this method time-consuming with relatively low resolution. Thus, it is not widely applicable.

2.2.1.1.2 Methylation-Sensitive Amplified Polymorphism

The methylation-sensitive amplified polymorphism (MSAP) method is based on digestion with methylation-sensitive restriction endonucleases followed by amplification of restriction fragments [3]. MSAP is a simple and relatively inexpensive genome-wide method for the identification of putative changes in DNA methylation. Unlike methods based on bisulfite modification or immunoprecipitation, MSAP is independent on the availability of genome sequence information, but the choice of the particular restriction enzymes may lead to ambiguous interpretation of MSAP data [4].

2.2.1.2 Bisulfite Conversion Technique and Derivatives

The bisulfite conversion technique is a revolutionary mark that has accelerated the study of DNA methylation. Treatment of the DNA with sodium bisulfite can convert unmethylated cytosine into uracil, while methylated cytosine remains unchanged. During the following polymerase chain reaction (PCR) process, uracil is then converted to thymidine. This chemical modification in the DNA sequence can be detected by using a variety of methods [5].

2.2.1.2.1 Bisulfite Sequencing PCR

Bisulfite sequencing PCR (BSP), which is regarded as the "gold standard" of DNA methylation analysis, is an unbiased and sensitive alternative to the use of restriction enzymes. This method combines the bisulfite treatment of genomic DNA with PCR amplification and sequencing analyses [6]. PCR products can be sequenced directly or as single clones. The latter is much more popular as it enables mapping of methylated sites at single-base-pair resolution. To acquire this high-quality data, the bisulfite-treated amplified DNA is usually cloned into bacterial cells with subsequent isolation of plasmids from numerous bacterial clones to be sequenced to determine the extent of methylation within the DNA sequence of interest; this is a process which is quite time-consuming and labor-intensive [7].

2.2.1.2.2 Pyrosequencing

Pyrosequencing is an attractive alternative to the conventional BSP. Pyrosequencing detects luminescence from the release of pyrophosphate on nucleotide incorporation into the complementary strand. Pyrosequencing studies also require the coupling of bisulfite treatment of genomic DNA with PCR amplification of the target sequence, but the advantage of

pyrosequencing is that quantitative DNA methylation data can be obtained from direct sequencing of PCR products without requiring cloning into bacterial expression vectors and sequencing a large number of clones [8]. On the other hand, the quality of the data decreases with the distance of the CpG from the 3′ end of the forward primer, thus the number of bases that can be analyzed in a single sequencing reaction is limited [9].

2.2.1.2.3 Combined Bisulfite and Restriction Analysis

Bisulfite treatment of DNA can lead to the creation of new methylation-dependent restriction sites or the maintenance of restriction sites in a methylation-dependent manner. Based on this property, a quantitative method termed "combined bisulfite restriction analysis" (COBRA) was developed which merged the bisulfite and restriction analysis protocols. The use of COBRA is again limited by the availability of restriction enzyme recognition sites in the target DNA. This method is relatively labor-intensive but is cost-effective [10].

2.2.1.2.4 Methylation-Sensitive Single-Nucleotide Primer Extension and SnuPE Ion Pair Reversed-Phase High Performance Liquid Chromatography

Methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) assay analyzes methylation status at individual CpG sites in a quantitative way and with the capability of multiple analyses. This method couples bisulfite treatment with strand-specific PCR which is performed to generate a DNA template. Subsequently, an internal primer that terminates immediately 5′ of the single nucleotide to be assayed is extended with a DNA polymerase that uses ^{32}P-labeled dCTP or dTTP [11]. This protocol can be carried out using multiple internal primers in a single primer-extension reaction; thus a relatively high throughput is possible. However, Ms-SNuPE assay is usually labor-intensive and requires radioactive substrates. To overcome this restriction, several variants which omitted radioactive labeling were developed, such as SNaPshot technology from Applied Biosystems (ABI) [12], SNuPE ion pair reversed-phase HPLC (SIRPH) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [13].

2.2.1.2.5 Methylation-Sensitive Melting Curve Analysis

Based on the principle that the higher GC (base pair of guanine and cytosine) content of DNA sequence makes it more resistant to melting, a new approach to DNA methylation analysis, methylation-sensitive melting curve analysis (MS-MCA), was developed. This method detects sequence difference between methylated and unmethylated DNA obtained after sodium bisulfite treatment by continuous monitoring of the change of fluorescence as a DNA duplex melts while the

temperature is increased. If equal proportions of fully methylated and fully unmethylated molecules are amplified, two distinct melting peaks are observed, and interpretation is easy. If the target sequence is heterogeneously methylated, a complex melting will result in a pattern which is difficult to interpret [14].

2.2.1.2.6 Methylation-Sensitive High-Resolution Melting

The principle behind methylation-sensitive high-resolution melting (MS-HRM) is the same as for MS-MCA, but MS-HRM possesses some methodological advantages. First, the HRM approach acquires more data points so that it is more sensitive to detecting subtle differences within the amplicons. Second, the temperature variations produced with HRM instrumentation are generally extremely small. Third, the data obtained in HRM are more stable and reliable because most of the software provided with the instruments allows normalization for end-level fluorescence, temperature shifting, and use of internal oligonucleotide calibrators [14]. This technique requires the use of double-stranded DNA-binding dyes that can be used at saturating concentrations without inhibiting PCR amplification. Both MCA and HRM are semiquantitative measurements that cannot offer detailed information about the methylation of single cytosines within the sequence of interest, but they can distinguish fully and partially methylated samples, which may enable early detection of diseases [15].

2.2.1.2.7 MethyLight

MethyLight technology is a sensitive, sodium-bisulfite-dependent, fluorescence-based real-time PCR technique that quantitatively analyzes DNA methylation. Execution of MethyLight requires the designation of methylation-specific primers and fluorogenic probes [16]. The MethyLight method has major advantages. First, it is a relatively simple assay procedure, without the need to open the PCR tubes after the reaction has ended, thereby reducing the risk of contamination and the handling errors associated with manual manipulation. Second, only small amounts and modest quality of DNA template are required, making the method compatible with plasma samples and small biopsies. Third, it has the potential ability to be used as a rapid screen tool and is uniquely well suited for detection of low-frequency DNA methylation biomarkers as evidence of disease. However, the drawback of MethyLight technology is that it is not designed to offer high-resolution methylation information [17,18].

2.2.1.3 Immunoprecipitation-Based Methods

Immunoprecipitation-based methods utilize methylation-binding proteins such as MeCP2 and MBD2, or 5mC-specific antibodies to enrich

the methylated fraction of the genome. Different strategies using this approach have been successfully applied for the analysis of DNA methylation information. The two most commonly used methods are methylated DNA immunoprecipitation (MeDIP) and methyl-CpG immunoprecipitation (MCIp). MeDIP is an adaptation of the chromatin immunoprecipitation (ChIP) technique and uses 5mC-specific antibodies to immunoprecipitate methylated DNA. MCIp uses a recombinant protein that contains the methyl-CpG-binding domain and the Fc fraction of the human IgG1 to directly bind and enrich methylated DNA. These methods are relatively straightforward without either digestion of genomic DNA or bisulfite treatment and the results are relatively easier to analyze and interpret. However, immunoprecipitation-based methods do not provide DNA methylation information at single-nucleotide resolution [19].

2.2.1.3.1 Methylated-CpG Island Recovery Assay

The methyl-CpG island recovery assay (MIRA) is based on the fact that methyl-CpG-binding domain protein-2 (MBD2) has the capacity to bind specifically to methylated DNA sequences and this interaction is enhanced by the methyl-CpG-binding domain protein 3-like-1 (MBD3L1) protein. DNA isolated from cells or tissue is sonicated and incubated with a matrix containing glutathione-*S*-transferase-MBD2b and MBD3L1. Then, specifically bound DNA is eluted from the matrix and gene-specific PCR reactions are performed to detect CpG island methylation. The MIRA procedure can detect DNA methylation using 1 ng of DNA or 3000 cells. It is quite specific, sensitive, and labor-saving [20].

2.2.1.3.2 Methyl-Binding-PCR

Methyl-binding (MB)-PCR relies on a recombinant, bivalent polypeptide with high affinity for CpG-methylated DNA. This polypeptide is coated onto the walls of a PCR vessel and can selectively capture methylated DNA fragments from a mixture of genomic DNA. Then, the degree of methylation of a specific DNA fragment is detected in the same tube by gene-specific PCR. MB-PCR is particularly useful to screen for methylation levels of candidate genes. Given the enormous amplification capability and specificity of PCR, MB-PCR provides a quick, simple, and extremely sensitive technique that can reliably detect the methylation degree of a specific genomic DNA fragment from <30 cells [21].

2.2.1.4 Mass Spectrometry-Based Methods

Mass spectrometry is recognized as an extremely useful and reliable measurement for acquiring molecular information. The principle of mass spectrometry is that a charged particle passing through a magnetic field is deflected along a circular path on a radius that varies with the mass-to-charge ratio (m/z). One adapted mass spectrometry platform

for DNA methylation analysis is MassARRAY EpiTYPER, which uses base-specific enzymatic cleavage coupled to MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) mass spectrometry analysis. Although the limited throughput and high cost restrict this approach in becoming a genome-wide technology, it is an excellent tool to analyze DNA methylation for its fast and accurate analysis power and its multichannel analysis capability [22].

2.2.1.4.1 MALDI-TOF Mass Spectrometry with Base-Specific Cleavage

The base-specific cleavage strategy involves amplification of bisulfite-treated DNA followed by *in vitro* transcription, and subsequent base-specific RNA cleavage by an endoribonuclease to produce different cleavage patterns. Bisulfite treatment of genomic DNA converts unmethylated cytosine into uracil and it appears as a thymidine (T) in the PCR products while the methylated cytosine remains unchanged. These C/T appear as G/A variations in the reverse strand. In the subsequent base-specific RNA cleavage reaction, methylated regions are cleaved at every C to create fragments containing at least one CpG site each. But both methylated and unmethylated regions are cleaved at every T to produce fragments in the T-cleavage reaction. G/A variations in the cleaved products generated from the reverse strand show a mass difference of 16 Da per CpG site. In MALDI-TOF analysis, the relative amount of methylated sequence can be calculated by comparing the signal intensity between the mass signals of methylated and unmethylated templates to generate quantitative results. This approach is recommended for purposes requiring the analysis of larger regions of unknown methylation content [23].

2.2.1.4.2 MALDI-TOF Mass Spectrometry with Primer Extension

The primer-extension strategy requires the designation of a primer that anneals immediately adjacent to the CpG site under investigation in a post-PCR primer-extension reaction. The primer is then extended with a mixture of four different terminators and the extension reaction will terminate on different nucleotides depending on the methylation status of the CpG site. Therefore, distinct signals are generated for MALDI-TOF mass spectrometry analysis. This approach should be used in routine analyses of a relatively small number of well-characterized informative CpG sites [14].

2.2.2 Genome-Scale DNA Methylation Analysis

Given the importance of DNA methylation, it is not surprising that many researchers have taken advantage of array- and sequencing-based

technologies that have become available in recent years to perform genome-scale association studies which will provide valuable new information with high throughput and lower cost.

2.2.2.1 Microarray-Based Analysis of DNA Methylation Changes

2.2.2.1.1 Sample Preparation

There are three basic techniques applied to sample preparation in microarray platforms: digesting the DNA with methylation-sensitive or methylation-insensitive restriction endonucleases, sodium bisulfite conversion of unmethylated cytosine into uracil, and affinity purification by applying antibodies binding to methylated cytosines. Coupled with these techniques, a wide range of microarray platforms have evolved to enable genome-scale DNA methylation analysis [22].

2.2.2.1.2 Microarray Used in DNA Methylation Profiling

The initially applied microarray platform was a CpG island microarray used to identify genomic loci that exhibited differential methylation. CGI microarrays used clones from libraries in which CpG-rich fragments had been enriched by MeCP2 columns [24]. However, these arrays have low resolution and limited methylome coverage. Therefore, microarrays made of short oligonucleotides are now commercially available to overcome the drawbacks of CGI microarrays [25]. These oligonucleotide arrays, such as a promoter array, can reach a high resolution, can be easily configured according to the user's need and often contain a high density of probes spanning each CGI [26]. The first "complete" high-resolution DNA methylome profile of a living organism (*Arabidopsis thaliana*) was generated using a tiling array platform [27]. This approach involves up to several million oligonucleotides and has greater methylome coverage than promoter and CGI microarrays. It has allowed researchers to study DNA methylation in noncoding areas in addition to regulatory regions of genes [28,29]. However, to cover the entire human genome, more array slides and a relatively larger amount of genomic DNA are required. The single-nucleotide polymorphism (SNP) arrays combine the use of methylation-specific endonucleases with an SNP-ChIP. This approach can provide an integrated genetic and epigenetic profiling and allows allele-specific methylation analysis at heterozygous loci [30,31]. Besides the methods cited above, microarrays based on methyl-sensitive restriction enzymes, methylation-dependent restriction enzymes, bisulfite conversion, or immunoprecipitation are widely used in epigenomic studies [32]. These microarray-based technologies show differences in terms of resolution, coverage, and sample preparation; therefore, it is necessary to determine the advantages and disadvantages of each specific technique (Table 2.1) [33–41].

TABLE 2.1 Comparison of Microarray Assays in DNA Methylation Detection

Microarray platform	Resolution (bp)	Coverage of CpGs	Principles	Advantages	Limitations	References
SNP arrays	1	10^4	Restriction endonuclease	Identify an integrated genetic and epigenetic profiling and allow allele-specific methylation analysis at heterozygous loci	Limit to restriction enzymes digested sites	[30,31]
HELP (dual-adapter approach)	50–200	10^6	Restriction endonuclease	Positive display of hypomethylated loci	Limit to restriction enzymes digested sites and relatively low resolution	[33,34]
CHARM	50–600	Better than HELP (lack exact data)	Restriction endonuclease	Detect hypermethylated CpG sites in CpG island core and CGI "shore" regions	Limit to restriction enzymes digested sites and relatively low resolution	[35,36]
Bead array (Infinium/ GoldenGate)	1	10^4	Bisulfite conversion	Low sample input and low cost		[37–39]
MeDIP	1000	10^6	Immunoprecipitation	Cost-effective and independent on a specific restriction site	Less sensitive to CpG-poor sites	[32,40]
MIRA	100	10^6	Immunoprecipitation	More sensitive to CpG-poor sites than MeDIP and do not require DNA to be denatured to single strands	Depending on MBD-binding ability	[32,41]

HELP, HpaII tiny fragment enrichment by ligation-mediated PCR; CHARM, comprehensive high-throughput arrays for relative methylation; MeDIP, methylated DNA immunoprecipitation; MIRA, methylated-CpG island recovery assay; CGI shores, stretches of ∼2 kb bordering CpG islands.

2.2.2.2 Next-Generation Sequencing Techniques

The evolution of sequencing technologies marked by the first massively parallel DNA sequencing platforms in 2005, has revolutionized research in biological science and ushered in a new era of next-generation sequencing (NGS). There are three major NGS platforms, namely Roche/454, Illumina/Solexa, and Life Technologies/SOLiD, and each of them has different features (Table 2.2) [42–47]. Compared with microarray-based methodologies, NGS offers higher resolution and a larger coverage, and is independent of knowledge of the reference genome or genomic features. Most importantly, NGS methods allow for assessment of DNA methylation in interspersed repetitive genomic regions that are inaccessible using microarrays [22]. However, sequencing-based methods would produce a dramatically large number of bioinformatics data, which leads to extreme difficulties in downstream data management. Thus, the selection of bioinformatics software tools is particularly critical for efficient and appropriate data processing [48]. NGS techniques also include methylation-sensitive restriction enzymes (MRE-seq), affinity-based methods (such as MeDIP-seq, MBD-seq), and bisulfite conversion approaches (e.g., reduced-representation bisulfite sequencing (RRBS)). MRE-seq methods evaluate relative rather than absolute methylation levels through incorporating parallel digestions with three to five restriction endonucleases. With single CpG resolution and the ability to assay a more significant portion of the methylome including most CGIs, MRE-seq becomes a relatively simple and accurate method to analyze DNA methylation [49]. Compared with MRE or bisulfite-based sequencing, MeDIP-seq shows lower resolution, but an important advantage of MeDIP over restriction enzyme methods is that it lacks bias for a specific nucleotide sequence, other than CpGs [49]. RRBS can assess absolute quantification of methylation of more than 1 million CpG sites at single base-pair resolution, which prevails over other sequencing methods [50]. However, all the methods cited above can generate largely comparable methylation calls, but differ in CpG coverage, resolution, quantitative accuracy, efficiency, and cost [51].

2.3 TECHNIQUES USED FOR 5hmC MARK DETECTION

5′-hmC is an oxidative metabolite of 5′mC catalyzed by ten–eleven translocation dioxygenases (TET). It is widely distributed among tissues, especially in embryonic stem cells and Purkinje neurons, but depleted in cancer cell lines, which indicates that 5hmC might serve biologically important roles [52]. Traditional methods for detecting 5mC cannot differentiate 5mC from 5hmC. Methylation-sensitive restriction enzymes