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Advances in Epigenetic Technology

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Abstract

Epigenetics refers to the collective heritable changes in phenotype that arise independent of genotype. Two broad areas of epigenetics are DNA methylation and histone modifications and numerous techniques have been invented to analyze epigenetic processes not only at the level of specific genes, but also to analyze epigenetic changes that occur in defined regions of the genome as well as genome-wide. Advances have also been made in techniques devised to assess the enzymes that mediate epigenetic processes. These methods that are currently driving the field of epigenetics will greatly facilitate continued expansion of this exponentially growing discipline of genetics.

Keywords

Epigenetics; DNA methylation; chromatin; methods; histone; technique

1. Introduction

Although the term “epigenetics” has several variants of its definition, taken together they can be summarized as the collective heritable changes in phenotype due to processes that arise independent of primary DNA sequence. DNA methylation is the most studied of the subfields of epigenetics and in most eukaryotes is characterized by the enzymatic addition of a methyl group to the cytosine-5 position. This usually occurs in CpG dinucleotides and the DNA methyltransferases (DNMTs), of which there are three major types in mammals, are responsible for most of the methylation that occurs in the genome. DNMT1 is the enzyme primarily involved in the maintenance of methylation patterns with each cell replication and it preferentially methylates hemimethylated DNA (1). Also very important in shaping the methylome are the enzymes DNMT3a and DNMT3b, which have relatively high *de novo* methylating activity (2). The most significant aspect of DNA methylation is its role in gene expression and the more methylated a gene regulatory region, the more likely it is that the gene activity will become down-regulated and *vice versa* although there are exceptions to this general rule (3).

Like DNA methylation, chromatin modification is another central epigenetic process that impacts gene expression as well as many other biological processes. For example, histone

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acetylation has been associated with an increase in gene activity whereas histone deacetylation often silences transcription (4). However, methylation of histones has variable effects on gene activity where lysine 4 (K4) methylation of histone H3 is frequently associated with increasing gene activity while methylation of lysine 9 (K9) of histone H3 may lead to transcriptional repression. Even more significant perhaps is the fact that there is often cooperation between DNA methylation and histone modifications (5) such that cytosine methylation may contribute to the methylation of H3-K9. Likewise, H3-K9 methylation may promote cytosine methylation to lead to gene down-regulation. Therefore, it is often the case that both DNA methylation and histone modifications act interdependently to contribute to the overall chromatin state and its epigenetic control of numerous cellular processes.

2. DNA Methylation Gene- or Region-specific Techniques

A major breakthrough in the analysis of DNA methylation occurred with the development of bisulfite methylation sequencing. This method (Chapter 2) is a gold-standard for detection of DNA methylation largely because it allows identification of 5-methylcytosine (5mC) at single base-pair resolution. In this technique, bisulfite treatment of denatured DNA converts cytosines to uracil while 5mC is not converted. After PCR amplification and DNA sequencing, nonmethylated cytosines are recognized as thymines and methylated cytosines (largely in CpG dinucleotides) remain as cytosines. This leading method of DNA methylation analysis has lead to numerous subsequent methods such as Methylation Specific PCR (MSP), Combined Bisulfite Restriction Analysis (COBRA) and Methylation-sensitive Single Nucleotide Primer Extension (MS-SNuPE) which are also covered in this volume.

The real power of fundamental bisulfite methylation sequencing is in its application to a variety of methods and notable among these techniques is MSP (Chapter 3) that allows assessment of methylation patterns in CpG islands. Advantages of MSP are that it requires no cloning or methylation-sensitive restriction enzymes and it can be performed on very small quantities of DNA as well as DNA from paraffin-embedded samples. For discrimination of methylated or nonmethylated DNA, two primers are synthesized with one pair specific for methylated DNA and the other specific for nonmethylated DNA. Amplification using PCR of a methylated primer pair indicates methylated DNA and amplification from a nonmethylated primer pair occurs if the DNA does not contain methylation within the site covered by the primer. MSP is especially useful for screening DNA samples and has found numerous applications in clinical medicine.

A strong feature of bisulfite pyrosequencing (Chapter 4) is its quantitative nature. This biotin-based method also amplifies PCR products from bisulfite-treated DNA and incorporated nucleotides allow for quantification based on emission of light. Although methylation patters at a single allele are not derived with this method, it does allow the identification of heterogeneous DNA methylation patterns. A drawback of pyrosequencing can be its more limited covered regions of DNA (typically about a hundred base pairs) but serial bisulfite pyrosequencing allows longer PCR products to be assessed. This method can also be applied to formalin-fixed paraffin-embedded tissue which greatly extends its useful applications.

In certain locus-specific DNA methylation analyses, the need for removing PCR products can be eliminated in that amplification and analysis of bisulfite-modified DNA are performed in one tube. This is referred to in Chapter 5 as a closed-tube method and some examples of this convenient approach are real-time analysis—methylation specific PCR (SMART-MSP), methylation-sensitive high resolution melting (MS-HRM) and MethyLight. The MethyLight approach utilizes a fluorescent hydrolysis probe while the SMART-MSP

method uses a double-stranded DNA binding fluorescent dye and melting curve analysis. If the methylation level or patterns of the DNA of interest is not known, MS-HRM can be used as a pre-screening technique. Detection and quantification of methylation is better achieved for DNA that has less than 5% methylation using the MethyLight and SMART-MSP approaches (Chapter 5).

COBRA is commonly used for analyzing specific regions of DNA for methylation and is also dependent on bisulfite treatment of DNA and subsequent restriction enzyme treatments. Chapter 6 introduces a novel visualization software referred to as Methyl-Typing. In this new approach comprehensive restriction enzymes for sequences containing 5mC after bisulfite conversion are provided. This method, which makes COBRA analysis much easier, provides computation and visualization of the essential information for performing COBRA and provides all possible methylation sites of restriction enzymes.

A very promising and useful region-specific DNA methylation analysis method is the SIRPH technique (Chapter 7). SIRPH refers to Single nucleotide primer extension (SNuPE) that is combined with Ion paired Reverse Phase HPLC. This method utilizes one universal HPLC gradient and one SNuPE annealing temperature for all primers. This quantitative approach to analyzing methylation in specific regions of DNA requires only small quantities of PCR product. Another advantage is that it is highly accurate and reproducible.

Thus, there are numerous approaches for determining the DNA methylation status of specific genes or regions of DNA of interest. Many of these methods have bisulfite conversion of DNA as their fundamental basis. Any of the techniques covered in Chapters 2–7 can be incorporated into most laboratories depending on the specific needs of each laboratory and the desired resolution and applications that are needed. Most notably, each of these methods are reliable in achieving accurate assessments of DNA methylation in defined DNA sequences.

3. Methods of Epigenomic Analysis

In some cases, one may wish to achieve a broader view of DNA methylation status within the genome and a number of exciting techniques have been developed that allow analysis of DNA methylation of very large segments of DNA if not the entire genome. One of the early developments in this regard is referred to as restriction landmark genome scanning (RLGS). This approach described in Chapter 8 allows methylation status determination of the whole genome based on 2-dimensional electrophoresis. An improvement of this method is described in Chapter 8 in that the original method utilized *NotI* to detect methylated sites and this has now been refined to the use of the methylation-sensitive isoschizomers *MspI* and *HpaII*. A widely used method for genome-wide methylation analysis is the Methylated DNA Immunoprecipitation (MeDIP) technique. This method is based on the uses of antibody specific for 5mC and allows quantification of enriched methylated DNA fragments. However, analysis of MeDIP results can be complex. In Chapter 9, Pelizzola and Molinaro describe newly-developed analyses for MeDIP results referred to as MEDME (Modeling Enrichment Derived from MeDIP Experiments) and BATMAN (Bayesian Tool for Methylation Analysis) which are designed to assist with evaluation of MeDIP enrichment measures to estimate the absolute or relative number of 5mCs in a genomic sample.

Two additional approaches to assessing the methylome are the Methylated-CpG Island Recovery Assay (MIRA) originally developed by Rauch and Pfeifer (6) and the Luminometric Methylation Assay (LUMA). An advantage of the MIRA method (Chapter 10) is that it is not dependent upon bisulfite conversion of DNA and, unlike MeDIP, does

not require specific antibody recognition. The fundamental basis of MIRA is that the high affinity of methylated DNA binding proteins, MBD2b and MBD3L1, are able to recover methylated DNA at single nucleotide resolution and can also be used with microarray analysis or next-generation sequencing for methylome determinations. In Chapter 11 another exciting genome-wide approach to assessing DNA methylation status is described that is dependent upon methylation-specific restriction enzymes followed by pyrosequencing. This LUMA technique allows for quantification of the number of restriction enzyme cuts in a genomic sample based on methylation sensitivity relative to an internal standard. Notable advantages of the LUMA approach are its quantitative abilities and the high-throughput potential of this method.

The advent of techniques to analyze the methylome has been one of the most remarkable advances in epigenetics and these methods described in Chapters 8–11 represent some of the promising developments in this area. However, advances in epigenomics are occurring at a remarkable pace and it is likely that this area will herald the most breakthroughs over the next decade.

4. Techniques to Inhibit DNA Methylation and Assess DNA Methylation Activity

Since analyses of DNA methylation as a central component of epigenetic processes, and DNA methyltransferases (DNMTs) are the mediating enzymes of DNA methylation, methods to inhibit the DNMTs as well as to measure their activity are important components of epigenetics. Inhibition of DNA methylation in somatic cells often allows determination of the role of DNA methylation in gene expression as well as other processes such as cellular differentiation and nuclear reprogramming (Chapter 12). The most commonly used approach for inhibiting DNA methylation is the employment of pharmacological agents such as 5-azacytidine, 5-aza-2’deoxycytidine and non-nucleoside inhibitors such as procainamide which inhibit the activity of DNMTs. DNMT heterozygous mouse models have also been used although mice null in DNMT1 result in embryonic lethality. The employment of small interfering RNA (siRNA) has also been applied to reducing DNA methylation in cells and this approach has the advantage of specificity in terms of which of the three major mammalian DNMTs (1, 3a or 3b) are selectively knocked down.

Another basic method of DNA methylation analysis involves assessment of the enzymatic activity of the DNMTs. Techniques to accurately determine the activity of the DNMTs have many uses such as monitoring the effectiveness of knockdown of the DNMTs as well as measuring changes in DNMT enzymatic activity during various biological processes such as cellular differentiation and tumorigenesis. In Chapter 13 three reliable approaches to determining DNMT activity are described. These assays consist of use of radioactively labelled S-adenosyl-L-methionine, use of bisulfite conversion of in vitro methylated DNA and a novel fluorescence-coupled array using restriction enzymes that has high throughput applications for screening of DNMT inhibitors. Therefore, inhibition of DNA methylation as well as methods to determine the activity of the DNMT mediators of DNA methylation are fundamental approaches to understanding the role of DNA in biological processes.

5. Chromatin Immunoprecipitation (ChIP)-based Protocols

ChIP assays are widely used for a number of different assessments such as analysis of DNA-protein interactions and determining posttranslational modifications of histones. The most common use of ChIP assays in epigenetic analyses involve the determination of histone modifications which, along with DNA methylation, shares the role as a mainstay of epigenetic processes. However, a drawback to ChIP assays is they it often require a large

number of cells for reliable measurements. Recent advances have overcome many of these limitations. For example, in Chapter 14 a micro-ChIP protocol is detailed that can be used for multiple parallel ChIPs using only a thousand cells which is remarkably less than the typical number of about 10^6–10^7 cells for conventional ChIP analyses. This development should greatly enhance the utility of ChIP-based measurements and expand its practical applications. However, conventional ChIP requires cross-linking of DNA and protein while the development of native ChIP (nChIP) bypasses the cross-linking process (Chapter 15). Some advantages of foregoing cross-linking and using nChIP are that the native form of the proteins are preserved, more sensitivity is generally possible and special equipment (e.g., sonicator) is not required.

Two very useful modifications of the ChIP method are Q-PCR in combination with ChIP and sequential ChIP or SeqChIP. Q-PCR is a highly sensitive and reproducible technique and when combined with conventional ChIP, these characteristics of Q-PCR are maintained which greatly enhances traditional ChIP assays. For example, Q-PCR can be combined with ChIP to assess the presence of acetylated histones H3 and H4 on different regions of a target locus as described in Chapter 16. Often it is desirable to assess the binding of more than one protein to a particular DNA sequence. For instance, epigenetic proteins such as the DNMTs can have many interactions with other proteins and it is often useful to assess how these proteins are interacting at a particular site on the DNA. SeqChIP is a relatively newer technique that provides this advantage. In SeqChIP one can assess the interactions of two or more proteins or various histone modifications at a specific site in the genome (Chapter 17). The sequential nature of this technique refers to the use of different antibodies to specific proteins that are provided sequentially during ChIP reactions. The significant advantage of SeqChIP is that many epigenetic modifications do not occur in a single-protein manner but rather, often involve interactions. Likewise, more than one histone modification often is involved in many epigenetic processes. Therefore, SeqChIP allows a more accurate determination of the key interdependent processes that occur in vivo. Both Q-PCR in combination with ChIP and SeqChIP are extremely useful techniques that are greatly expanding the potential of epigenetic analyses.

6. Combined Chromatin Immunoprecipitation and DNA Methylation Analysis

Most of the chapters describe techniques that are valuable in assessing either DNA methylation or chromatin modifications. However, these two major mediators of epigenetic changes often cross-talk and can modulate each other. We therefore developed a novel technique that is a combination of conventional ChIP with bisulfite methylation sequencing which we refer to as ChIP-BMS (Chapter 18). This method renders DNA methylation information at the single-nucleotide level of defined DNA fragments precipitated by specific antibodies to histones or transcription factors of interest. This allows investigation of the interaction patterns between histone modification and DNA methylation as well as transcription factor binding and methylation of recognition sites. It can also serve to provide valuable information about multiple interactions between genetic and epigenetic factors Additional applications of this new technique besides analyses of interactions between histone modifications and DNA methylation are identification of methylation-sensitive transcription factors as well as simultaneous assessment of epigenetic regulation of gene expression by the two major epigenetic mediators, DNA methylation and the vast array of chromatin modifications. It also seems possible that ChIP-BMS could be combined with Seq-ChIP to render more meaningful results of the myriad of epigenetic and genetic factors that can contribute to transcriptional regulation.

7. In Vivo RNA-protein Interaction Assessment

RNA also has important roles in epigenetics and many proteins interact with RNA to modulate RNA-based epigenetic processes. It is therefore important to have the tools available to detect direct and indirect interactions between specific proteins and RNA in vivo. This is best achieved through the RNA immunoprecipitation technique (RIP) that is not too unlike ChIP assays. The key difference, however, between ChIP and RIP is that DNA is enzymatically removed and RNA that is bound to the proteins captured by the antibody are immunoprecipitated (Chapter 19). The uses of the RIP technique are vast and may be applied to epigenetics to help unravel the increasingly appreciated role of RNA in epigenetic processes.

8. Epigenomic Chromatin Methods

Genome-wide assessments of epigenetic modifications have definitely come of age and many investigators have a strong interest in methods that can achieve global information about chromatin modifications such as histone acetylation or histone methylation. ChIP-chip, or the application of purified ChIP products to a microarray (chip), was for many years the leading method of choice for epigenomic changes in histone modifications. However, multiple DNA microarrays are required for global analyses using the ChIP-chip approach and in part due to this reason ChIP-seq (Chapter 20) is rapidly becoming more popular than ChIP-chip. ChIP-seq can interrogate the whole genome with only one lane of sequencing, it renders high resolution and it requires only very small amounts of chromatin for analyses.

One of the major roles of epigenetic processes is the control of gene expression and it is often advantageous to determine regions of open chromatin associated with gene activity on a genome-wide scale. Chapter 21 describes the mapping of open chromatin using Formaldehyde-assisted Isolation of Regulatory Elements (FAIRE). This method is also based on cross-linkages and in this case it identifies open regulatory regions due to their relatively lower levels of association with nucleosomes. FAIRE has also been combined with high-throughput sequencing (FAIRE-seq) as described in Chapter 21 and is relatively easy to perform. FIARE can be used for small amounts of tissue and has numerous applications such as facilitating understanding of how sequence variation affects open chromatin structures. When applied to a global analysis, FAIRE can be a very powerful tool in epigenetics.

9. Knockdown of Histone Deacetylases

Similar to the case with DNA methylation where inhibitors can render information about the role of DNA methylation in various contexts, knockdown of histone deacetylases (HDACs) can also be a very useful tool in epigenetic studies. Also reminiscent of DNA methylation inhibition, HDAC inhibition has a number of applications perhaps most notably in the anticancer field since HDAC inhibitors have been found to have utility in a number of different cancer treatments. A major mechanism through which HDAC inhibitors affect cancer is through re-activation of aberrantly silenced tumor suppressor genes and Chapter 22 details protocols for HDAC inhibition in human breast cancer cells as one important application of this approach. HDAC inhibitors can be subdivided into hydroxamic acids, short-chain fatty acids, benzamindes and cyclic tetrapeptides groups and assessment of their effectiveness can be determined through processes such as Q-PCR for gene-specific expression changes or microarray expression analyses to determine global gene expression changes after HDAC inhibition. Given the increasing clinical significance of HDAC inhibition, advances in technology development in this field could have a major impact.

10. Bioinformatics Applied to Epigenomics

The development of sophisticated methods to determine changes in the epigenome under various conditions has been a major advancement in the field of epigenetics. For example, massively parallel sequencing allows mapping of epigenetic variants but also renders vast amounts of data that can be daunting to understand and analyze. Computational methods for epigenetic analysis have therefore developed rapidly to answer the call for efficient techniques that can manage the volumes of data that are derived using epigenomic methods. For instance, a protocol has been developed consisting of computational analysis for modified methylation-specific digital karyotyping (MMSDK) based on massively parallel sequencing (Chapter 23). A protocol is described for a mapping process based on the in silico simulation of combined enzyme cutting and tag extraction of the reference genome. The tags are mapped to the simulated library using Mapping and Assembly with Qualities (MAQ) which is suitable for any tag profiling-based method. It can also be applied to other epigenetic analyses based on massively parallel sequencing. The endonuclease digestion-based MMSDK method allows for mapping of DNA methylation in the human genome and is an accurate and fast computational tool that is being developed for epigenomic studies.

11. Conclusion

Numerous methods have been developed that provide means to accurately assess many epigenetic processes, most notably, DNA methylation and chromatin modifications as the most prevalent epigenetic mediators. Two of these methods, bisulfite sequencing for DNA methylation and ChIP for chromatin modifications and DNA-protein interactions, have given rise to numerous additional techniques that improve the speed, accuracy and applications of these fundamental methods. New protocols have also been developed, such as ChIP-BMS, that combine these two basic techniques to not only render information about the interdependence of DNA methylation and chromatin modifications, but also to provide a more comprehensive understanding of the overall dynamics of epigenetic processes in modulating the phenotypic expression of the genome. Along with significant advances in methods to unravel the epigenome have come new challenges in managing massive amounts of information that is derived with these advanced techniques. Further developments in computational epigenetics will be needed for deciphering the many data that are accumulating due to the exciting advances in experimental epigenomics.

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