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An Overview of Epigenetic Assays

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Abstract

A significant portion of ongoing epigenetic research involves the investigation of DNA methylation and chromatin modification patterns seen throughout many biological processes. Over the last few years, epigenetic research has undergone a gradual shift and recent studies have been directed toward a genome-wide assessment. DNA methylation and chromatin modifications are essential components of the regulation of gene activity. DNA methylation effectively down-regulates gene activity by addition of a methyl group to the five-carbon of a cytosine base. Less specifically, modification of the chromatin structure can be carried out by multiple mechanisms leading to either the upregulation or down-regulation of the associated gene. Of the many assays used to assess the effects of epigenetic modifications, chromatin immunoprecipitation (ChIP), which serves to monitor changes in chromatin structure, and bisulfite modification, which tracks changes in DNA methylation, are the two most commonly used techniques.

Keywords

Epigenetics; DNA methylation; DNMTs; CpG islands; HDACs; HMTs; Chromatin immunoprecipitation; Bisulfite modification

Introduction

The field of epigenetics has rapidly developed into one of the most influential areas of scientific research and has been shown to regulate essential biological processes such as aging, development, and memory formation [1-3]. The number of pathologies linked to the dysregulation of epigenetic systems continues to grow and with it, the list of potential targets for epigenetic-based therapeutics. In recent years, epigenetic research has seen a shift from site-specific studies aimed at determining how epigenetic processes, such as DNA methylation and histone acetylation, regulate specific genes to a genome-wide assessment of when and why epigenetic alterations occur. The assays which are used to monitor changes in the epigenetic environment have also undergone a similar progression, marked by both the appearance of new

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and innovative assays as well as the modification and coupling of more traditional epigenetic assays such as bisulfite modification of DNA or chromatin immunoprecipitation (ChIP).

Epigenetics is widely defined as any heritable change between DNA and its surrounding chromatin that does not alter the sequence of the DNA. Epigenetic regulation of gene expression has been linked to discrete mechanisms that affect the stability, folding, positioning, and organization of DNA [1]. The most studied of these mechanisms includes DNA methylation and chromatin remodeling, which work synergistically to organize the genome into transcriptionally active and inactive zones. Patterns of DNA methylation are established during development, and once established remain fairly stable through adult life [1]. On the other hand, the modification of histones is more of a reversible process, allowing genes to be switched between transcriptionally active and inactive states. Events such as tumorigenesis can lead to a drastic alteration in established methylation patterns or to dysregulation of chromatin remodeling processes, both of which can result in significant and consequential changes in gene expression [4].

In order to better understand these gene regulatory mechanisms, several techniques have evolved that enable investigators to physically map chromatin modifications and changes in DNA methylation patterns. Of these techniques, chromatin immunoprecipitation and bisulfite modification of DNA form the foundation for tracking chromatin changes as well as changes in DNA methylation, respectively. These techniques, coupled with an ever-growing interest in the field of epigenetics, have proven to be the fundamental driving forces responsible for the proliferation of epigenetic research.

DNA Methylation

Methylation at the five-carbon of cytosine is a reversible process that can directly influence gene activity. Essential for normal embryonic development, DNA methylation plays an important role in the regulation of gene expression and affects a wide range of essential biological processes. Patterns of hemimethylation are established throughout the stages of embryogenesis, and alterations among established methylation patterns have a wide range of effects. Aberrant DNA methylation has received the most attention for its role in tumorigenesis but has also been shown to play a role in a number of other genetic disorders.

DNA methylation is a result of the enzymatic activity of the DNA methyltransferase (DNMT) family of enzymes. DNMTs catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to cytosines in CpG dinucleotides [5]. The vast majority DNA methylation is carried out by DNMT1, DNMT3a, and DNMT3b. DNMT1 was the initial methyltransferase to be identified and is largely responsible for maintaining pre-existing methylation patterns. DNMT1 is known as the maintenance methyltransferase, due to a preference for hemimethylated DNA over unmethylated DNA. The very low level of de novo methylation by DNMT1 has led to the designation of DNMT3a and DNMT3b as the de novo methyltransferases, even though they show no preference for nonmethylated over hemimethylated DNA. Inactivation of genes coding for the DNMT3a and DNMT3b family of methyltransferases via gene targeting disrupts de novo methylation in developing stem cells, while displaying no significant loss of maintenance methylation in imprinted methylation patterns [6].

Evolutionarily conserved nonmethylated regions of DNA abundant in cytosine and guanine bases known as CpG islands are the most common sites of DNA hyper-methylation. Promoter regions of nearly 40% of mammalian genes have been shown to either contain or to be in close proximity to one or more CpG islands [5]. Hypermethylation of CpG islands usually leads to a loss in gene expression or in a small number of cases to upregulation of gene expression [7, 8]. Methylation profiling of specific CpG islands before, during, and after events such as the

formation of a tumor may 1 day provide physicians with the ability to accurately diagnose diseases based on the epigenetic state of the patient. The onset of CpG island profiling was one of the first steps in the shift to a global study of epigenetic modifications and has led to the discovery of novel tumor suppressor genes regulated via DNA methylation.

The generally accepted principal surrounding DNA methylation is that hypermethylation leads to a loss of gene expression. The physical extension of the methylated cytosine into the major groove of the DNA can result in a steric hindrance thus rendering the DNA inaccessible to the active site of a transcription factor. Binding of methylcytosine binding domain proteins (MBDs) to methylated DNA has also been shown to prevent transcription. In addition to the direct silencing effect of MBDs, it has now become clear that they provide the link that allows DNA methylation and chromatin remodeling to cooperatively prevent gene transcription. Association of MBDs with histone modifying enzymes, such as histone deacetylases (HDACs) and certain histone methyltransferases (HMTs) allows a gene silenced via DNA methylation to remain in a transcriptionally inactive state [9,10].

Chromatin Remodeling and the Connection to DNA Methylation

Basic chromatin structure within the nucleus consists of 146 kb sections of DNA wound tightly around a series of histone proteins forming the nucleosome. Histones can be modified by a number of chemical processes including acetylation, methylation, and phosphorylation, all of which can display profound effects on the regulation of gene expression [11]. Levels of histone acetylation are a function of the delicate balance that exists between acetyltransferases (HATs) and HDACs. In general, histone acetylation leads to an increase in gene activity, while histone deacetylation leads to transcriptional repression. Acetylation of the histone tails by HATs exposes the underlying DNA to a variety of cellular factors that directly affect gene expression. HDACs catalyze the removal of acetyl groups from histone tails, which leads to the local condensation of chromatin and the rendering of the associated DNA inaccessible [12,13]. Histone acetylation/deacetylation has been the most studied histone modifications; however, recently it has been shown that methylation of specific histone residues by HMTs can also play an important role in gene regulation. HMTs substitute an acetyl group with a methyl group at specific arginine and lysine residues on histone tails. Methylation of lysine residue K9 on histone H3 results in decreased transcription of the gene [14].

It is now believed that most genes silenced as a result of epigenetic changes undergo alterations in both chromatin structure and methylation status. Many of the epigenetic modifying enzymes have been shown to interact with and recruit one another. DNA methylation has been shown to influence chromatin structure due to the association of a type of MBD, methyl CpG binding proteins (MeCPs), with HDACs and HMTs. Localization of histone modifying enzymes to a gene targeted for silencing helps to maintain the gene in a transcriptionally inactive state [15]. MeCPs have also been shown to interact with the DNMTs, leading to a greater likelihood that methylation patterns will be efficiently maintained [16]. The complex and cyclic interactions of the various epigenetic modulators make it very difficult to attribute a change in gene expression to one single factor. As a result, investigators are now trying to understand in what order epigenetic changes occur. Does DNA methylation lead to histone deacetylation or vice versa?

In addition to attempting to understand the timing of epigenetic modifications, researchers are now trying to determine exactly how the cellular environment contributes to and regulates DNA methylation and chromatin structure. One of the most important mysteries facing epigenetists today is the elucidation of the chain of events that leads to the appearance of aberrant methylation patterns during tumorigenesis. A much more detailed picture of the shifts in methylation patterns and alterations in chromatin structure occurring across the entire

genome during abnormal processes is direly needed. The answer may lie in methylation profiling of CpG islands and other similar “epigenomic” studies that will provide insight into the timing of epigenetic alterations.

Analysis of Changes in DNA Methylation

There are many techniques to analyze changes in DNA methylation, with the best method depending on factors including but not limited to the availability of the DNA, total number of targets being analyzed or the desired specificity. Bisulfite modification of DNA is the foundation for the majority of assays geared toward analyzing changes in methylation patterns. The differences in bisulfite-based methylation assays arise from the manner in which bisulfite-modified DNA is analyzed. Bisulfite modification converts nonmethylated cytosines to uracils, which are then converted to thymines during DNA amplification by PCR, whereas methylated cytosines are protected from bisulfite modification.

DNA sequencing and the use of methylation-sensitive primers (MSPs) are the two most commonly used techniques to analyze bisulfite-treated DNA. Sequencing analysis of bisulfite-modified DNA can be used to reveal the methylation status of specific cytosines, whereas MSPs can be used to quickly assess a larger number of CpG islands. Recent studies have shown that a combination of bisulfite techniques and chromatin immunoprecipitation assays can allow assessment of methylation status and chromatin structure from one sample. Zinn et al. have shown that DNA collected from ChIP can be analyzed for methylation status using MSPs [17].

Single nucleotide primer extension (SNUPE) is another means to analyze bisulfite-modified DNA. The extension of an oligonucleotide to the 5' end of a CpG site using dideoxycytidines (ddCTP) or dideoxythymidine (ddTTP) followed by real-time PCR, allows for a quantitative assessment of methylation patterns and can be applied to multiple sites simultaneously [18]. A semi-quantitative method known as methylation sensitive-single strand conformation analysis (MS-SSCA) can be used to obtain an overall picture of DNA methylation. MS-SSCA can be applied across a broader range of samples and can be used to assess the ratio of methylated to nonmethylated DNA [19].

One of the oldest methods of mapping methylation alterations on a genomic scale does not use bisulfite treatment of DNA. Digestion of genomic DNA with endonucleases that differ in their methylation sensitivities is still an ideal method for obtaining a rough estimate of the totality of methylation. One of the best assays to assess a large number of CpG islands is restriction landmark genomic scanning (RLGS). This method involves the radioactive labeling of nonmethylated sequences that are targets of methylation sensitive restriction enzymes [20].

Methods of Assessing Chromatin Modifications

Assays aimed at assessing epigenetic changes have evolved remarkably over the last half-decade. The chromatin immunoprecipitation (ChIP) assay, which assesses changes in chromatin structure, comprises one of the most utilized assays in epigenetic research. ChIP assays monitor DNA–protein interactions and allow the chromatin structure surrounding a specific DNA sequence to be analyzed. A conventional ChIP (xChIP) uses formaldehyde to crosslink DNA and protein, followed by immunoprecipitation of DNA–protein complexes. Once the crosslinks are reversed, recovered DNA can then be analyzed using PCR. Another commonly used form of the ChIP assay is the native ChIP (nChIP). nChIP uses micrococcal nuclease digestion to prepare the chromatin for analysis. nChIP allows for modifications of histones, such as methylation or acetylation, to be assessed more accurately than with formaldehyde fixation; however, nChIP does not usually allow for assessment of proteins with a weak binding affinity for DNA [21]. Most ChIP assays are semi-quantitative although

combining either ChIP assay with real-time PCR (Q-ChIP) can achieve a quantitative measurement of the amount of DNA bound to a specific protein.

ChIP assays can also be combined with other epigenetic assays such as DNA bisulfite modification. DNA harvested from a ChIP assay can be treated with bisulfite and methylation-specific primers (MSP) can be used to assess changes in DNA methylation in a ChIP-MSP [17]. New uses for the ChIP assay are continuously appearing as epigenetic research comes of age. With the recent surge in epigenetic research, multiple modifications of ChIP assays have begun to appear, which allow assessment of global changes in chromatin structure. One of the most useful techniques to assess genome-wide epigenetic changes is the ChIP on Chip assay that utilizes traditional ChIP protocols combined with microarray analysis [22].

In addition to ChIP, many other assays exist that can be used to assess chromatin structure. For example, DNaseI hypersensitivity assays can be used if a more general determination of the changes chromatin has undergone is desired. DNaseI hypersensitivity sites are usually located in or around promoter regions thereby allowing for mapping of transcriptionally active versus inactive chromatin [23]. One of the most useful techniques to assess changes in chromatin structure is the use of the deacetylating agent trichostatin A (TSA), which has also been shown to be capable of treating diseases caused by dysregulation of histone acetylation [24]. At low concentrations, TSA inhibits HDACs and allows determination of exactly what role acetylation/deacetylation plays in regulation of a specific gene.

Analysis of Epigenetic Modulating Enzymes

Comparison of results obtained from methylation or chromatin structure analysis with results obtained from quantification of either the expression or analysis of epi-genetic players like DNMTs, HDACs, or MeCPs can provide valuable insight into the causes of epigenetic alterations. Analysis of the differences in mRNA and protein levels can be quantitatively preformed using real-time PCR and Western blot protocols, respectively. Immunoprecipitation assays such as ChIP or co-immunoprecipitation (co-IP) can be highly effective means of assessing the functions of epigenetic modulators. For example, ChIP analysis using antibodies specific to MeCPs has been used to identify new sites of DNA hypermethylation during oncogenesis [25]. If an analysis of the interactions between epigenetic modulators is needed, a co-IP can be preformed. Immunoprecipitation using antibodies specific to one enzyme, followed by detection with antibodies specific to others, can help to determine recruitment capabilities. Most of the assays that are used to assess enzymatic activity involve addition of synthetic substrates specific to the enzyme being analyzed (i.e., DNA rich in CpG dinucleotides for DNMT activity assays) with cellular protein extracts. The differences in the various activity assays stem from the type of substrate used as well as from the method used to quantify enzymatic activity.

Conclusion

Over the last decade, it has become very clear that epigenetic mechanisms play important roles in gene regulation. In epigenetic research two major questions are yet to be fully answered: (1) What events lead to the dysregulation of epigenetic systems? and (2) How do certain epigenetic changes regulate the appearance of other aberrant epigenetic alterations? To answer these questions, epigenetists now have begun a genome-wide assessment of epigenetic alterations during certain biological processes. Elucidation of both the timing and the interactions of the epigenetic modulating enzymes may hold the key to unlocking what could be one of the 21st century's greatest scientific discoveries. As epigenetics truly comes of age, so too do the assays that have led to the realization of just how important of a role epigenetic mechanisms play in gene regulation.

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