CHAPTER 1

AN INTRODUCTION TO EPIGENETICS

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Abstract: Eukaryotic genomic information is modulated by a variety of epigenetic modifications that play both a direct role in establishing transcription profiles, modulation of DNA replication and repair processes and also indirect effects on the aforementioned processes through the organization of DNA architecture within the cell nucleus. Nowadays, the role of epigenetic modifications in regulating tissue-specific expression, genomic imprinting or X-chromosome inactivation is widely recognized. In addition, the key role of epigenetic modifications during cell differentiation and development has been highlighted by the identification of a variety of epigenetic alterations in human disease. Particular attention has been focused on the study of epigenetic alterations in cancer, which is the subject of intense multidisciplinary efforts and has an impact not only in understanding the mechanisms of epigenetic regulation but also in guiding the development of novel therapies for cancer treatment. In addition, a number of genetic disorders such as Immunodeficiency-Centromere Instability-Facial anomalies (ICF) or Rett syndromes are directly associated with defects in elements of the epigenetic machinery. More recently, epigenetic changes in cardiovascular, neurological and autoimmune disorders as well as in other genetically complex diseases have also started to emerge. All these examples illustrate the widespread association of epigenetic alterations with disease and highlight the need of characterizing the range and extension of epigenetic changes to understand their contribution to fundamental human biological processes.

INTRODUCTION

The size and complexity of eukaryotic genomes has evolved in association with the generation of mechanisms that are able to manage the genetic information that is needed at different specific physiological situations. Considering that a 10 μm-diameter

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eukaryotic nucleus has to accommodate an enormous length of DNA (in the order of magnitude of centimeter/meter), cells have to face two major challenges: firstly, how to optimize folding of DNA in such a tiny compartment and secondly, how to make accessible DNA sequences that will be needed at specific situations throughout their lifetime. Folding of DNA within the cell nucleus is achieved through its interaction with histones and other types of proteins, characteristic of chromatin. Regarding the second aspect, i.e., how DNA sequences retain the ability to stay functional despite their folding within chromatin, it is now obvious that the mere availability of transcription factors or DNA repair machinery cannot simply overcome the repressive effect of chromatin without additional help. Epigenetic marks provide cells with a dynamic signaling system that compartimentalizes the genome and allows the specific regulation of gene expression and accurate tuning of DNA replication and repair processes, as well as the appropriate nuclear organization in a manner adapted to particular physiological situations. These epigenetic marks are established and maintained by different groups of enzymes which are specifically targeted to their correct genomic locations through different nuclear factors, some of which directly respond to environmental signals or constitute the final step of a cell signaling cascade.

EPIGENETIC MODIFICATIONS ARE ESSENTIAL FOR CELL IDENTITY AND FUNCTION

Basically, cells encode their epigenetic information through the modification of two different groups of macromolecules: DNA and histones. DNA methylation is a major epigenetic modification with a direct contribution in the establishment of expression patterns in multicellular organisms.[1] In mammals, DNA methylation is restricted to cytosines in the context of CpG dinucleotide sequences, whose presence in the genome is much lower than expected based on GC content and exhibit a non uniform distribution. CpG sites can be present in the genome at high density in regions known as CpG islands, that are 0.4-3 kb in length, are relatively rich in G + C (>55%) and are enriched in the CpG dinucleotide relative to the remainder of the genome regions.[2] In the human genome, CpG islands are in and near approximately 76% of promoters of genes.[3,4] It has been proposed that methylation occurring at CpGs located in repetitive and parasitic elements plays a role in their stabilization.[5] Also, methylation of repetitive elements appears to contribute in the maintenance of nuclear architecture and organization of heterochromatic domains.[6] On the other hand, the majority of promoter CpG islands remain unmethylated under physiological conditions.[7] Methylation and subsequent transcriptional repression are confined to a relatively small set of genes, including some tissue-specific[8,9] and imprinted genes,[10] as well as those in the inactive X-chromosome of females.[11] The fact that promoter CpG islands can be unmethylated in both expressing and nonexpressing tissues indicates that the activity of the associated genes is not controlled by methylation. It is nonetheless widely believed that cytosine methylation regulates development. Many of the expression-methylation studies involve non-CpG island genes, which tend to have light and variable cytosine methylation that may be less in cells that express the gene. A standard method to map the methylation status of CpGs is based on the treatment of DNA with sodium bisulfite, that results in conversion of unmethylated cytosines into uracil whereas methylated cytosines remains unmodified, followed by genomic sequencing (BS).[12]

DNA methylation is a postreplicative process. The methyl group is transferred from S-adenosyl methionine to cytosines in DNA by DNA methyltransferases (DNMT) in a reaction that involves base flipping, whereby a cytosine base is swung completely out of the DNA helix into an extrahelical position so that the enzyme can access and methylate the cytosine.[13] The mammalian family of DNMTs is composed of five members [see for a review Goll and Bestor].[14] DNMT1 has been considered to be devoted to the maintenance of methylation patterns across DNA replication cycles, however whether faithful maintenance methylation is enforced by other factors that inhibit the de novo activity of DNMT1 in vivo (no such factors have been described) or the protein has de novo activity in vivo remains an unresolved issue. DNMT3A and DNMT3B have been proposed to be implicated in de novo methylation. They have a close homologue, DNMT3L, that lacks a conserved PWWP domain present in DNMT3a/b, is essential for establishment of maternal genomic imprints in the growing oocyte and at dispersed repeated sequences in the prospermatogonia. Finally, DNMT2 is an enigmatic DNMT that, despite its conservation across species and its expression in all sort of tissues, appears to lack catalytic activity.

In general, DNA methylation is considered to be a stable epigenetic mark, although mechanisms of active DNA demethylation have been reported to occur in specific physiological contexts, including development and cell differentiation.[15,16] In contrast with the precise identification of the machinery involved in promoting or maintaining DNA methylation, there is some controversy with respect to the enzymatic activities involved in active demethylation. Genetic and biochemical studies in *Arabidopsis* demonstrated that a subfamily of DNA glycosylases function to promote DNA demethylation through a base excision-repair pathway. These specialized bifunctional DNA glycosylases remove the 5-methylcytosine base and then cleave the DNA backbone at the abasic site, resulting in a gap that is then filled with an unmethylated cytosine nucleotide by as yet unknown DNA polymerase and ligase enzymes. Evidence suggests that active DNA demethylation in mammalian cells is also mediated at least in part by a base excision repair pathway where activation-induced cytosine deaminases (AID) convert 5-methylcytosine to thymine followed by G/T mismatch repair by the DNA glycosylase MBD4 or TDG.[17-19] However other possible mechanisms of active DNA demethylation have also been proposed.

In parallel with DNA methylation, histone modifications constitute a fundamental source of epigenetic information. Core histones are the building elements of the octameric complex around which the DNA is wrapped within chromatin. Histones can be modified at different amino acid residues. There are over sixty different sites on histones where modifications have been detected either by mass spectrometry or by specific antibodies, but not all these modifications will be on the same histone at the same time. However, this represents a huge underestimate of the number of modifications that could potentially take place on histones. Additional complexity comes also from the fact that methylation at lysines or arginines may be one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. This vast array of modifications confers enormous potential for functional responses. The timing of the appearance of a modification will depend on the signaling conditions within the cell. The use of modification-specific antibodies in chromatin immunoprecipitation assays (ChIP) coupled to gene array technology (ChIP-on-chip) has revolutionized our ability to monitor the global incidence of histone modifications. Such global analysis has only been done on a subset of modifications (acetylation and lysine methylation), but the results clearly show that modifications are not uniformly distributed. Combinations or sequential addition of

different histone modifications have different functional consequences for gene activity and chromatin organization.[20] For instance, reversible acetylation of histone lysines at their N-terminal tails is generally associated with transcriptional activation,[21] although there are some particularities on the functional consequences that depend on the specific lysine that it is acetylated (see for example refs. 22 and 23). On the other hand, methylation of histones can occur in lysine and arginine residues; the functional consequences depend on the type of residue and specific site that is modified.[24-26] For instance, methylation of H3 at K4[27] and R17[28] is closely linked to transcriptional competence, whereas methylation of H3 at K9, or H4 at K20, is associated with transcriptional repression.[29,30] The identification of the enzymes that direct modification has been the focus of intense activity over the last 10 years. Enzymes have been identified for acetylation,[31] methylation,[32] phosphorylation,[33] ubiquitination,[34] ADP-ribosylation,[35] sumoylation,[36] deimination[37,38] and proline isomerization.[39]

Histone modifications have been implicated in a number of epigenetic phenomena. The classic definition of epigenetics is the study of heritable phenotype changes that do not involve alterations in DNA sequence. The use of the term "heritable" has been eliminated in more recent definitions, allowing the term epigenetic to mean the information carried by the genome (e.g., on chromatin) that is not coded by DNA. However the classic term, that includes heritability, is important to maintain as it defines a nongenetic memory of function that is transmitted from generation to generation. A number of cellular phenotypes are transmitted in this way, including imprinting (discussed in Chapter 8), X chromosome inactivation, aging, heterochromatin formation, reprogramming and gene silencing. In addition there are environmentally induced changes, which are passed on from generation to generation, without the need for the original stimulus.

Several mechanisms link DNA methylation with specific histone modifications. However, not every posttranslational modification at the core histone tails depends on the methylation status of the DNA. The mechanisms by which promoter CpG island methylation leads to gene silencing involve changes in the modification of profile of histones. This process has been reported to occur place through the direct recruitment of histone modification enzymes by DNMTs[40] and other nuclear factors such as methyl-CpG binding domain (MBD) proteins.[41,42] Additional mechanistic connections between elements of the histone modification and DNA methylation machineries exist. For instance, Polycomb group (PcG) protein EZH2, which catalyzes methylation of K27 of histone H3, associates and targets DNA methylation to specific sites.[43-45]

Therefore, histone modification patterns can be established through DNA methylation-associated mechanisms or, alternatively, in a DNA methylation-independent fashion. In the latter case, recruitment of histone modification enzymes at specific genomic loci depends on the availability of particular transcription factors or through the ligand-dependent response of nuclear receptors which in turn recruit histone modification enzymes.

In multicellular organisms, cells usually respond to environmental or intracellular signals in a manner that depends on the participation of transcription factors which often recruit epigenetic enzymes.[46] Within any particular organism, different cell lineages share a common genome although available sets of transcription factors and epigenetic marks are cell type specific and functionally interconnected. The balance between epigenetic modifications, transcription factors and their ability to respond to environmental stimuli is delicate and guarantees proper cell function. Epigenetic modifications and the required sets of transcription factors are also transmitted through successive rounds of DNA replication

and cell division. However, disruption of a variety of pathways results in different types of epigenetic alterations, some of which have already been associated with disease.

EPIGENETIC DEREGULATION MECHANISMS IN HUMAN DISEASE

The recognition of the widespread occurrence and significance of epigenetic alterations in cancer has been essential in attracting the attention of the Biomedicine field towards Epigenetics. Cells from most cancer types suffer dramatic changes in their DNA methylation and histone modification content and distribution. Tumor cells undergo a decrease in the global content of 5-methylcytosine, which can be mainly attributed to loss of methylation at repetitive sequences.[47,48] However, the promoter CpG islands of many tumor suppressor genes become hypermethylated and this process represents an important mechanism by which these genes are inactivated.[49] Early analysis of the profile of promoter CpG island hypermethylation of candidate genes encouraged the view that the profiles of promoter methylation are tumor-type specific.[50,51] This notion has been reinforced with more recent genome-wide analysis that evidence the specificity of the DNA hypermethylation profiles in different tumor types.[52,53]

Several lines of evidence indicate that hypermethylation of tumor-suppressor genes plays an active role in the development and progression of cancer. In the first place, hypermethylation occurs early in cancer. This is the case of p14ARF in colorectal adenomas[54] and hMLH1 in endometrial hyperplasias[55] and gastric adenomas.[56] A second observation highlighting the functional relevance of promoter CpG island hypermethylation in tumorigenesis is its occurrence in the absence of genetic mutations. Both events (genetic and epigenetic) abolish normal gene function and their coincidence in the same allele would be redundant from an evolutionary point of view. However, one of the main findings that support for a critical role for CpG island hypermethylation in the origin and progression of a tumor is the demonstration of the existence of relevant biological consequences associated with the epigenetic inactivation of a particular gene. A classical example of this statement is represented by the hypermethylation of the DNA repair gene O6-methylguanine DNA methyltransferase (MGMT).[57] The MGMT gene product removes the promutagenic O6-methylguanine, generated from the addition of a methyl group to the base guanine, which is then read as an an adenine by DNA polymerases and thus may generate G to A mutations. It has been shown that the DNA repair gene MGMT is transcriptionally silenced by promoter hypermethylation in primary human tumors.[58] These tumors might accumulate a considerable number of G to A transitions, some of them affecting key genes, in a similar way that loss of the hMLH1 mismatch repair gene by methylation targets other genes. This information has led to the finding that the hypermethylation- associated inactivation of MGMT gives rise to the appearance of G to A transition mutations in the oncogene K-ras[59] and the universal tumor suppressor p53[60] in human colorectal tumorigenesis. These findings indicate that epigenetic lesions can cause genetic lesions in genes that are of key importance in the development of cancer.

As mentioned above, the comprehensive analysis of methylation in many different tumor types and gene promoters has provided evidence for the existence of the tumor-type-specific methylation profile indicated above. In theory, CpG islands should be the most ‘attractive’ substrate for DNA methylation, since, by definition, they contain a high concentration of CpG-rich sequences. However, under physiological circumstances most CpG island promoters remain unmethylated. It has been speculated on the existence

of mechanisms that would normally prevent unscheduled methylation at CpG islands and for some reasons those mechanisms would lose stringency in cancer cells. Many other questions then arise including the reason why certain CpG islands (while others never do) become methylated in cancer and whether this is a targeted process or a random one.

Genome-wide analysis of DNA methylation changes in cancer cells have shed some light on these questions by revealing that tumor-specific methylated genes belong to distinct functional categories, have common sequence motifs in their promoters and are found in clusters on chromosomes.[61] These results are consistent with the hypothesis that cancer-related de novo methylation may be specifically targeted through a trans-acting mechanism. Schlesinger and colleagues[44] showed that genes methylated in colon cancer cells are specifically packaged with nucleosomes containing histone H3 trimethylated on K27. The early establishment of this chromatin mark in unmethylated promoter CpG island-containing genes early in development and then maintained in differentiated cell types by the presence of an EZH2-containing Polycomb complex suggests that PcG proteins predefines genes that are methylated in cancer. In cancer cells, as opposed to normal cells, the presence of this complex brings about the recruitment of DNMTs, leading to de novo methylation. These results suggest that tumor-specific targeting of de novo methylation is preprogrammed by an established epigenetic system that normally has a role in marking embryonic genes for repression.[44]

DNA methylation and histone modification profiles are mechanistically coupled by multiple mechanisms. In addition to PcG mediated-connections between histone modifications and DNA methylation, other factors have been implicated in DNA methylation-dependent silencing. In this context, MBD proteins have been proposed to play a pivotal role.[62] MBD proteins have the ability to bind selectively to methylated CpGs and recruit different HDAC- and HMT-containing complexes.[42] In addition to the association of MBDs, promoter CpG island hypermethylation has been found to be associated with a decrease in the acetylation levels of histones H3 and H4 and loss of 3mK4 of histone H3.[62] In contrast, hypomethylation of repetitive sequences in cancer is associated with a loss of monoacecetyl K16 and trimethyl K20 of histone H4.[63,64] It has been suggested that this change could be associated with changes in the expression levels of specific histone modification enzymes, like K16 H4-specific HAT MOF[65] or K20 H4-specific HMT Suv4-20h2.[66]

The importance of epigenetic mechanisms involved in the pathogenesis of cancer is also revealed by a mechanism frequently found in hematopoietic malignancies. In leukemias and lymphomas, in contrast to most solid tumors, an additional mechanism for epigenetic dysregulation arises from the occurrence of nonrandom chromosomal translocations that disrupt genes residing in the translocation breakpoint region. In many cases, genes residing at these breakpoint regions are epigenetic enzymes or transcription factors that can themselves recruit epigenetic enzymes and are directly involved in hematopoietic cell differentiation, apoptosis, or proliferation. Therefore, generation of fusion proteins through this mechanism is commonly associated with epigenetic dysregulation at the target sites of the enzymes involved. These chromosomal translocations indicate how disruptions of the function of the enzymes that control chromatin structure can cause alterations of the histone modification profile in a target-specific fashion, resulting in an altered chromatin structure that affects gene expression at specific loci and ultimately causes cellular transformation. Typical examples of proteins include MLL (mixed-lineage leukemia), a histone H3 K4-specific methyltransferase, RUNX1 (also known as AML1)

which is associated with HATs or HDACs and PML, whose frequent fusion partner (RAR) has been described to interact with various epigenetic modifiers.[67]

Our knowledge on the importance of epigenetic alterations in cancer has greatly increased in the last few years. The contribution of DNA methylation-dependent epigenetic inactivation of tumor suppressor genes is widely recognized. More specifically in hematological malignancies, the epigenetic switch at many genomic sites is also commonly recognized. We need a better understanding on the causes that result in epigenetic deregulation in cancer. Mapping epigenomic changes, at the DNA methylation, histone modification and factor binding level, in cancer cells will surely provide a solid ground to address these issues.

Despite the enormous efforts invested in epigenetics studies, our knowledge on epigenetic alterations in other disease contexts is relatively poor. Epigenetic alterations occur in a wide range of biological scenarios, including the occurrence of genetic defects in the enzymes that regulate the epigenetic balance or epigenetic changes that result from a change in the environment. Although the best studied relationship between epigenetic alterations and disease are in the context of cancer, a number of diseases have proved to exhibit a fundamental epigenetic component. The first group of disorders for which an epigenetic component has been recognized includes diseases for which there is a genetic defect involving proteins implicated in the maintenance of epigenetic regulation. In this group are included a few rare syndromes such as Immunodeficiency-Centromere Instability-Facial anomalies (ICF) syndrome or Rett syndrome among others.

ICF syndrome, a rare autosomal recessive disorder characterized by the presence of variable immunodeficiency and a unique type of instability of pericentromeric heterochromatin, has been shown to be associated with mutations in DNMT3B. Epigenetic alterations associated with this defect include hypomethylation at various repetitive sequences[68] and chromosomal territory reorganization which may have an impact in alterations of gene expression of many genes.[69] In Rett syndrome, an X-linked dominant neurodevelopmental disorder affecting almost exclusively girls, mutations in MECP2, the archetypical member of the MBD family, have been found to be present in up to 80% of classical cases.[70] It has been proposed that loss of function of MECP2 results in the DNA methylation-dependent deregulation of genes,[71] although more recently it has been proposed that binding of MeCP2 outside gene boundaries may organize chromatin into functionally important domains or loops of imprinted regions, thereby modulating gene expression in either a positive or a negative manner.[72]

In other groups of disorders, genetic defects have been associated with clear distinctive epigenetic defects. This is for instance the cases of facioscapulohumeral muscular dystrophy (FSHD), where the deletion of a critical number of repetitive elements (D4Z4) is associated with hypomethylation,[73] or the imprinting disorders Beckwith-Wiedemann syndrome (BWS) and the Prader-Willi/Angelman syndromes (PWS/AS), where deletion of imprinting control regions results in biallelic expression of associated genes.[74]

The existence of an epigenetic component has been suggested for many other diseases for which a direct genetic defect is not obvious or complex genetic patterns have been suggested. This is for instance the case of autoimmune or neurological disorders and cardiovascular disease. The evidence for an epigenetic component in these diseases has been highlighted by the existence of discordance rates in sets of monozygotic twins.[75] Analysis of global and locus-specific differences of DNA methylation and histone modification in a cohort of identical twins has suggested the existence of an age-dependent epigenetic 'drift', which may result from the independent influence of environmental

factors.[76] More recent studies have addressed the relevance of epigenetic differences between twins discordant for autoimmune diseases.[77,78]

Our knowledge on the epigenetic contribution for these diseases requires additional research efforts: firstly, a detailed description of the type and extent of epigenetic alterations needs to be addressed. In addition, identification of the upstream mechanisms that lead to the generation of epigenetic changes should be investigated.

The availability of novel technologies for the genome-wide analysis of epigenetic alterations and systematic analysis of DNA methylation changes in specific diseases will surely lead to the discovery of specific markers with both basic research an clinical implications (discussed in Chapter 12). Initial high-throughput studies looking for epigenomic changes in atutoimmune disease, such as those carried out for systemic lupus erythematosus,[77] multiple sclerosis[78] or Type 1 diabetes[79] highlight the need of defining the range and extent of epigenetic alterations in this set of complex disorders.

CONCLUSION

In eukaryotic organisms, epigenetic modifications act as a signaling system that defines, in concert with transcription factors and other nuclear elements, the functionality of the genome in each particular cell type and in specific stages of the cell cycle during differentiation and development. Histone modifications and DNA methylation are major elements of the epigenetic signaling system and have a direct impact in the cell transcriptome, as well as in DNA repair and replication. The aberrant establishment or maintenance of epigenetic profiles is directly associated with cell misfunction and ultimately disease. This has been extensively studied for cancer and various genetic syndromes. The availability of novel strategies to characterize the profile of epigenetic modifications at the genome-wide level together with the possibility of pharmacological reversion of epigenetic alterations has attracted researchers to investigate the epigenetic component of genetically complex diseases, including autoimmune disorders.

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