Epigenetics in humans: an overview

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Purpose of review

The purpose of review is to describe the recent advances in the field of human epigenetics.

Recent findings

With the completion of the genome project in 2003, high expectations existed for the DNA sequence information to provide answers about the causative mutations for common diseases. However, this was not completely the case. Another interesting finding that resulted from the genome project was that the perceived level of complexity of humans was not accompanied with a relative increase in the number of genes when compared to 'lower species'. Epigenetics is able to provide answers to previously unanswered health-related questions and can explain differences in level of complexity between organisms. Epigenetic studies accomplished in the last few years have exposed a very complex multilayered regulatory mechanism that is able to answer previously puzzling questions in biology.

Summary

Understanding and interpretation of the role for epigenetic modifications in the human genome has progressed rapidly over the past decade with the advancement of microarray-based and sequence-based technologies. The complex interaction between DNA methylation, histone modifications, protein complexes and microRNAs has become better appreciated in the context of both local and long range epigenetic control of transcription in both normal cellular differentiation and tumorigenesis.

Keywords

DNA methylation, histone modifications, microRNA

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Introduction

Various definitions of epigenetics have emerged since Waddington [1] first coined the term in the 1940s. Generally, epigenetic refers to chemical alterations to DNA or associated histone proteins that change the structure of chromatin and modulate the readability of genomic regions, but do not involve alterations in the DNA sequence. Importantly, these modifications are heritable and can be stably transmitted through many cell divisions, but can also be reset. The classic epigenetic modifications include DNA methylation, post-translational modifications of histone proteins, silencing of the extra copy of the X chromosome in women, and genomic imprinting. In addition, proteins (e.g., DNA methyltransferases, histone deacetylases and methyltransferases, heterochromatin-associated proteins) and protein complexes [e.g., polycomb group (PcG) proteins] with epigenetic modifying capabilities have been included under the umbrella definition of epigenetics. More recently, with the identification of the RNA interference machinery in the late 1990s and several classes of functional noncoding RNAs [e.g., microRNA (miRNA), small-interfering RNA, long noncoding RNA], a new layer of gene regulation has been added to the definition. In this opinion, we touch upon the basic mechanisms of several epigenetic modifications as well as highlight some of the current literature.

DNA methylation

DNA methylation (DNAm) is a highly stable heritable covalent modification that alters DNA without changing its sequence. It involves the addition of a methyl (CH_3-) group to the fifth carbon of a cytosine nucleotide predominantly in the context of a CpG dinucleotide. Generally in normal cells, regions of repetitive DNA are methylated which is proposed to be important for genomic stability. DNAm is responsible for silencing parasitic DNA sequences and the inactive X chromosome, genomic imprinting and tissue-specific, and developmental specific silencing/activation of gene transcription. CpG-rich regions known as CpG islands (CGIs) found at the 5-prime regulatory regions of more than 50% of human genes are generally unmethylated in normal cells, with some germ-line and tissue-specific

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genes being notable exceptions. Methylation in those regions is generally repressive by directly preventing binding of transcription factors or facilitating the binding of methyl-CpG-binding proteins, which also prevent transcription factor binding. DNAm patterns are cell lineage-specific, established during embryonic development and then maintained throughout adulthood.

A new epigenetic discovery is the hydroxymethylcytosine mark, the genomic distribution and function of which remains to be determined [2,3]. Hydroxymethylcytosine cannot be distinguished from methylcytosine using enzyme-based techniques to study DNAm or procedures that use bisulfite treatment of DNA. However, affinity-based methods such as MeDIP can distinguish between the two marks depending on the antibody used.

DNA methyltransferases

DNAm is mediated by DNA methyltransferases (DNMT1, DNMT3A and DNMT3B, DNMT3L) in eukaryotes. DNMT1 is responsible for maintaining DNAm patterns during replication. DNMT3A and DNMT3B invoke de-novo methylation, particularly during embryogenesis. DNMTs are overexpressed in many tumor types and may be at least partly responsible for hypermethylation observed in tumor suppressor genes. However, it is becoming increasingly recognized that upregulation of DNMTs is only observed in subsets of patients. For example, in a recent study of 765 colorectal carcinomas, DNMT3B protein was increased in only 15% of cases [4] and, therefore, other mechanisms modulating DNMT activity must exist, such as by splice variants, trans-acting factors that target DNMT mRNA or miRNAs.

Methodological advances for investigating the DNA methylomes

A rapidly evolving field is genome-wide profiling to study the human methylome, described in detail in recent reviews [5–7]. Microarray-based methylation profiling has been widely used and the most popular methodologies involve either restriction enzyme-based enrichment of methylated or unmethylated DNA, affinity-based enrichment of methylated DNA or bisulfite conversion-based microarray. More recently, next generation sequence-based profiling (NGS) provides single base resolution of methylation. The three major techniques to generate bisulfite sequencing libraries are BS-seq, methylC-seq, and reduced representation bisulfite sequencing (RRBS), which are described in detailed reviews by Lister and Ecker [8] and Ansorge [9]. MethylC-seq and RRBS have advantages over BS-seq in that they reduce the complexity of the genome prior to sequencing, which facilitates less sequencing and lower costs. However, those methods involve enzymatic cleavage and so are biased toward CpG-rich regions and may, therefore, miss less CpG dense biologically relevant regulatory regions. Targeted approaches for reduced representation such as 'padlock' probes do not yet facilitate genome-wide analysis. The most widely used platforms for NGS are the 454 GenomeSequencer FLX instrument (Roche Applied Science, Indianapolis, Indiana, USA), the Illumina (Solexa, Walnut, California, USA) Genome Analyzer, the Applied Biosystems ABI SOLiD system, and the HeliScope Single Molecule Sequencer (Helicos BioSciences, Cambridge, Massachusetts, USA). Emerging technologies not yet commercially available include real-time sequencing using SMRT technology (Pacific Biosciences, San Francisco, California, USA) and nanopore-based methods that bypass the requirement for bisulfite treatment [10].

Rapid evolution of NGS has facilitated the first single-base-resolution human methylome, published in a landmark paper in 2009 using MethyC-seq [11••]. Widespread differences in the distribution of cytosine methylation were observed in two human cell lines: H1 human embryonic stem cells and IMR90 fetal lung fibroblasts. Abundant non-CG DNAm was observed in the embryonic stem cell line, but not in differentiated cells and this article emphasizes the highly dynamic nature of DNAm, previously thought to be much more static. It was suggested that non-CG methylation in stem cells may play a role in the maintenance of pluripotency. Higher throughput and lower cost sequencing technologies and improved bioinformatic tools will open up the field for further comparative analyses of normal and disease methylomes.

DNA methylation and disease

Disruption of DNAm patterns has been observed in a growing number of disease processes, cancer being the most rigorously investigated. The dogma was that gene-specific hypermethylation leads to transcriptional repression, which is generally the case for hypermethylation occurring in promoters. Recently, however, it is recognized that hypermethylation occurring in the body of genes can lead to transcriptional activation.

Cancer cells are characterized by global hypomethylation accompanied by de-novo hypermethylation in CGI associated with genes, which can increase during progression from preneoplastic lesions to metastatic tumors, often leading to silencing of tumor suppressor genes or miRNA genes. The list of tumor suppressor genes silenced by DNAm in neoplasia is ever-expanding and these are too numerous to mention in this review, but an important unanswered question is why particular subsets of CGI become hypermethylated in cancer. One simple explanation could be that there are particular sequences in the genome that are more 'susceptible' to becoming methylated. Long-range epigenetic silencing (LRES)

mechanisms (discussed in more detail below) also provide a feasible explanation for concordant methylation of groups of loci.

A role for DNAm in many other diseases such as autoimmunity, developmental and neurological disorders, and diseases related to imprinting or X-chromosome inactivation has been less intensively studied, but nevertheless is becoming increasingly accepted. A recent article reported hypomethylation at multiple maternally methylated imprinted regions of imprinted genes in Beckwith–Wiedemann syndrome [12]. DNAm also controls gene dosage reductions during X-chromosome inactivation in women and when disrupted can lead to developmental disorders such as fragile X syndrome [13].

Environmental effects

During embryogenesis widespread, almost complete CpG demethylation occurs and must then be re-established during early development, which necessitates the availability of nutritionally derived methyl donors like methionine and co-factors like folic acid. Diseases such as coronary artery disease, schizophrenia, and other congenital abnormalities have been associated with inadequate establishment of DNAm due to nutritional deficiency prenatally. Numerous other environmental factors, including stress or exposure to chemicals such as fungicides and pesticides can alter epigenetic components of the genome.

One of the largest groups of environmental factors that humans are exposed to daily is endocrine disrupters that alter hormone production and/or signaling, promoting conditions such as reproductive failure, infertility, or cancer (reviewed in [14]). The distribution of DNAm in the developing embryo is tightly controlled, and disruption of normal methylation patterns by exposure to environmental factors such as endocrine disruptors during that time can result in developmental or transgenerational abnormalities, or adult-onset diseases. Interplay between genetics, the environment, and epigenetics may play a critical role in the pathobiology of diabetes [15]. Recent studies suggest a role for DNAm in the regulation of insulin production in mice and humans, as the insulin promoter is methylated in embryonic murine cells, but demethylated in both mouse and human insulin-producing cells [16•].

Long-range epigenetic silencing

The drive to identify genes that are methylated in cancer has mainly focused on discrete CGI-associated genes, but it is becoming increasingly recognized that epigenetic mechanisms can act over large megabase regions containing multiple genes that are coordinately suppressed. Concordant DNAm of adjacent CGIs encompassing a large genomic region was first reported in colorectal cancer and since in other cancers and is speculated to be a common phenomenon in malignancies.

Earlier this year, Coolen *et al.* [17••] reported that LRES is common in prostate cancer cells and that regions of LRES and loss of heterozygosity (LOH) overlap significantly, although the mechanistic link between the two phenomena is unclear. Global deacetylation was accompanied by combinations of repressive marks, including DNAm. LRES is much more abundant in cancer than in normal cells and leads to a major reduction in the accessible genome potentially available for normal transcriptional regulation. Another recent report identified an 800 kb region spanning more than 50 transcripts, encompassing three clusters of protocadherin genes, on chromosome 5q31.3 that is hypermethylated in Wilms' tumors and was associated with transcriptional silencing [18].

One popular hypothesis for LRES is the 'silencing and seeding' theory, based on the hypothesis that active transcription protects CGI-associated genes from de-novo DNAm, loss of which allows methylation to spread from adjacent loci. This introduces the concept of CGI 'shores': low CpG density regions located close (within 2 kb) to CGI, which exhibit high tissue-specific DNAm. These regions have been called tissue or cancer differentially methylated regions (T-DMRs and C-DMRs) in normal cells and cancer cells, respectively. Hypermethylation involving C-DMRs in colon cancer extended into a nearby CGI in 24% of cases, which may partly explain CGI hypermethylation in cancer [19•]. This group used the same approach to discover CGI 'shores' that are differentially methylated between human-induced pluripotent cells, embryonic stem cells, and fibroblasts.

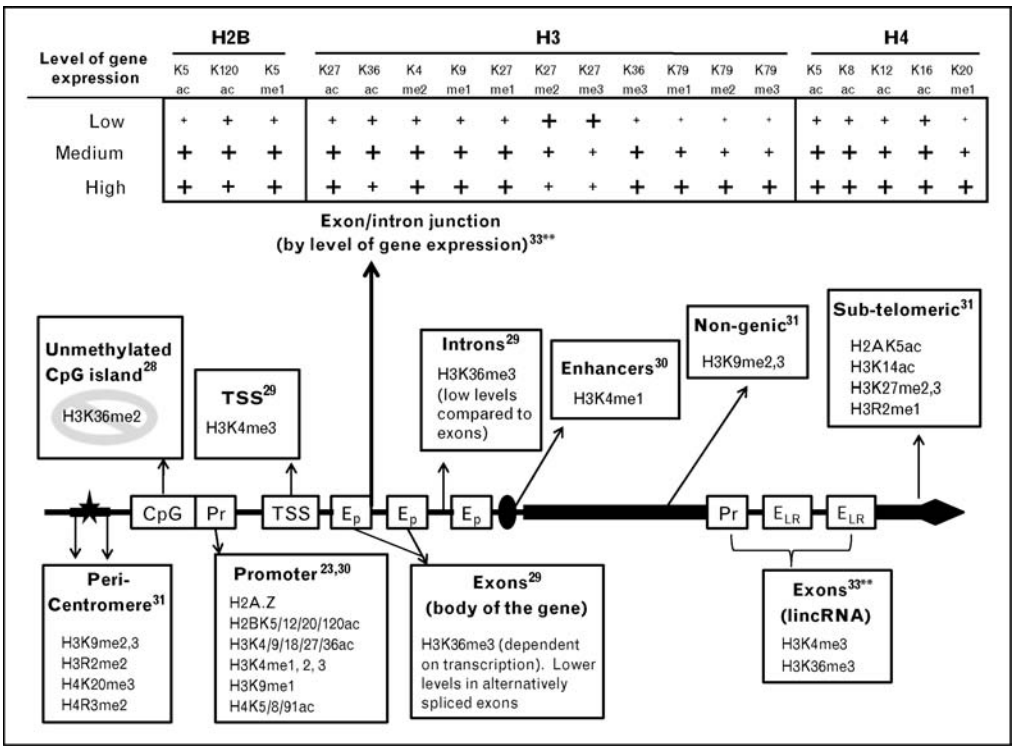
Epigenetic control at the local level and across large genomic regions has mostly been studied at a one-dimensional level. However, there is a paradigm shift toward three-dimensional genome regulation. Recently, Hsu *et al.* [20] reported epigenetic repression of large chromosomal regions through DNA looping in response to estrogen stimulation. Fourteen distinct loci were coordinately repressed by recruitment of H3K27me3 and DNAm.

Integration of linear and three-dimensional genomic and epigenomic studies using methods such as chromosome conformation capture (3C) and recently developed Hi-C [21••,22] has great potential to provide a much more comprehensive understanding of epigenetic control in normal tissues and reprogramming in carcinogenesis and other diseases.

Histone modifications

The functional unit of chromatin in eukaryotes, the nucleosome, is composed of 147 bp of DNA wrapped

Figure 1 Histone covalent modification signature



This figure summarizes recent findings in the prevalence of histone modifications throughout the chromosomes in humans. **Only examples of the most dramatic changes in modifications at exon/intron junctions in relation to level of gene expression are shown; for a complete list of 38 modifications in H2A, H2B, H3, and H4, please refer to Andersson *et al.* [32]. Size of the plus sign represents the relative level of modification for that particular signal between high, medium, and low expression genes. ac, acetylation; E_{LR}, exon (lincRNA; large intergenic noncoding RNA); E_p, exon (protein-coding gene); me, methylation; PR, promoter; TSS, transcript start site. Numbers next to each heading refer to the reference [28,29,30••,31,33••].

around an octamer of core histone proteins [two units each of histone 2A (H2A), H2B, H3, and H4]. Histones are small proteins (11–17 kDda) with an overall positive charge that have affinity for the negatively charged DNA. A linker histone (H1 or H5) associates with the entry and exit of the DNA from the nucleosome. Covalent post-translational modifications of the histone tails (e.g., acetylation, methylation, ubiquitylation, and phosphorylation) change the structure and function of chromatin by modifying the interactions between these proteins and DNA. Whereas histone acetylation and phosphorylation are always associated with a transcriptionally active state, histone methylation and ubiquitylation may convey a repressive or activating signal depending on the residue modified. Recent informatic studies suggest that histone modifications act in a combinatorial and cooperative fashion to dictate gene activity [23]. An example of this cross-talk among modifications has been observed by Zippo *et al.* [24]. They show that H3S10p at the FOSL1 enhancer induces H4K16ac within the enhancer and this initiates several protein interactions that ultimately results in the release of the promoter-stalled RNA polymerase II (Pol II) as well as increases its processivity. Further, a relationship exists between histone modifications and DNAm and it has become apparent that they may be dependent on each other. For example, recognition of an unmethylated lysine on H3 (H3K4) by DNMT3L is necessary prior to Dnmt3a recruitment and de-novo methylation [25,26]. Additionally, cross-talk among epigenetic modifications is necessary for proper chromatin structure and nuclear organization [27]. Figure 1 summarizes recent findings on chromatin signature [28,29,30••,31,32,33••].

Polycomb group proteins

First described in *Drosophila melanogaster* 50 years ago, PcG proteins are essential for normal development in multicellular organisms and target hundreds of developmentally important genes, impacting the epigenetic landscape. These most recent reviews describe polycomb repressive complexes (PRC1 and PRC2) and their roles in chromatin remodeling and transcriptional regulation [34,35].

Disruption of the epigenome caused by deregulated expression and/or binding of PcG proteins is involved in cellular transformation in cancer. PcG proteins were

also shown to regulate expression of *Ink4a/Arf*, which may play a role in regeneration of pancreatic islet β-cells [36,37], which has implications for diabetes pathogenesis, although those studies were performed in mice.

Deciphering the mechanisms for recruitment of PRCs to their target genes has been somewhat elusive in humans, even though polycomb response elements (PREs) had been identified in *Drosophila*. Earlier this year, a putative PRE was identified between *HOXD11* and *HOXD12* in embryonic stem cells to which PRC1 and PRC2 were recruited [38]. Alternative mechanisms for recruiting PRCs may involve noncoding RNAs and PcG-associated proteins. Recently, a new class of approximately 3300 RNAs called large intergenic noncoding (linc) RNAs was described, 20% of which are associated with PRC2 [39•]. Furthermore, PRC2 target genes were reactivated by knockdown of their associated lincRNAs. There are a number of proteins that have been reported to associate with PcGs, but four very recent articles describe the association of PRC2 with the Jumonji C-containing protein JARID2 (listed in [34]). Evidence from those studies implicates a role for JARID2 in PcG recruitment, but that other factors such as ncRNAs and/or additional proteins are also required.

miRNA

Organization, biogenesis, and function

miRNA is a class of noncoding RNAs that are transcribed primarily by Pol II. The long transcript is 3-prime polyadenylated and 5-prime capped. This primary transcript forms one or several stable hairpins due to base complementarity within the sequence. Upon transcription, the primary transcript is processed in the nucleus by the RNAse III enzyme Drosha, which associates with the hairpin together with DGCR8 in a complex referred to as the microprocessor complex. DGCR8 binds to double-stranded RNA and directs Drosha to cleave the hairpin 11 bp away from the double-stranded/single-stranded junction. Cleavage of the primary miRNA generates the precursor (pre) miRNA, which is approximately 70 nucleotides in length with a 3′ OH overhang. The 3′ overhang is recognized by a complex formed by exportin-5 and RanGTP, which shields the double-stranded RNA from degradation and catalyzes the export of the pre-miRNA from the nucleus [40]. Once in the cytoplasm, another RNAse III enzyme, Dicer, associates with the premiRNA and cleaves it to generate the mature approximately 22 nt miRNA duplex. The miRNA then associates with the protein Argonaute 2 to form the RNA-induced silencing complex (RISC). One strand of the duplex is retained and used by the RISC complex to target one or several mRNAs, most often at their 3′ UTRs, leading to downregulation of target mRNAs by translational repression (imperfect complementarity of miRNA and target mRNA) or degradation (perfect complementarity) [41]; the other strand is targeted for degradation. The current miRNA count is nearing a thousand in humans (miRbase; http://www.mirbase.org/cgi-bin/browse.pl). This estimate comes mostly from computational predictions and only a few have known functions. Approximately, 50% of miRNAs in humans are found in clusters [42] containing two to eight members and encompassed within no more than 50 kb (typically deriving from a single transcript) [43]. A functional relationship of clustered miRNAs has been recently documented [44•]. In that study, the Human Protein Reference Dataset was used to retrieve information on interacting proteins and by using a protein-centered perspective observed that proteins within a network tended to be regulated by miRNAs in the same cluster. Furthermore, miRNA also seems to regulate cellular pathways by co-targeting several members of a network. To that effect, Tsang *et al.* [45•] assessed the incidence of pathway-specific targeting by specific miRNAs with the use of computational predictions. They found many instances in which several members of a signaling pathway all shared complementarity to a specific miRNA: several of these predicted interactions were corroborated with published experimental results.

Epigenetic regulation by miRNAs

Controversy exists as to whether or not miRNAs should be considered part of the epigenetic program given that the best characterized function of these small RNAs is posttranscriptional gene regulation (e.g., translational repression). However, recent data place these ncRNAs as pivotal regulators of DNAm and histone modifications by directly or indirectly targeting epigenetic modifiers such as histone deacetylases and methyltransferases as well as DNA methyltransferases. The knowledge gained thus far in this area comes from studies using mainly nonhuman models and cellular systems, although some work with human cell lines and primary malignancies has supported findings in other species. It has long been recognized that the RNAi machinery is involved in regulating several epigenetic modifications in lower organisms. In mammals, Dicer deficiency results in decreased DNAm [46]. This was shown to be the result of reduced expression of the DNA methyltransferases. Upon further investigation, this group found increased levels of a negative regulator of the DNMTs, namely, retinoblastoma-like 2 protein (RBL2). Increased levels of RBL2 were due to a deficiency of miR290. Interestingly, RBL2 is also targeted by nonendogenous miRNAs as is the case with Kaposi's sarcoma-associated herpes virus (KSHV). During latency, a miRNA that targets RBL2 is transcribed from the KSHV's genome creating an environment with high DNMT expression and hypermethylation of a transcriptional activator involved in viral reactivation [47].

miRNA and malignancies

Global DNA hypomethylation together with localized hypermethylation of CGIs (particularly that of tumor suppressor genes) is a hallmark of tumorigenesis. Recently, Garzon *et al.* [48] linked global hypomethylation to a direct action of miR29a on the transcripts of DNMT3A and 3B, whereas DNMT1 levels were downregulated indirectly by way of downregulation of the transactivator SP1. Beyond being hypermethylated in their promoter regions, the aberrantly silenced tumor-suppressor genes are often characterized by the presence of high levels of silencing histone modifications. The histone methyltransferase and PcG protein EZH2 is overexpressed in several types of cancer. EZH2 catalyzes the trimethylation of H3K27 and its mRNA is targeted by miR101. Two recent studies showed an inverse correlation between EZH2 and miR101 in human prostate tumors [49,50]. Yet another characteristic of some cancers is histone deacetylase (HDAC) overexpression that promotes cell proliferation and survival. Noonan *et al.* [51] found that miR449a is downregulated in prostate cancer tissues. Using a 3′-UTR luciferase assay, they established that HDAC-1 is targeted and regulated by miR449a and they propose this miRNA deficiency in cancer cells to be a mechanism by which HDAC becomes overexpressed in human cancers.

Conclusion

The field of epigenetics is rapidly changing and ever-expanding. It has become evident that chromatin is not a static entity, but rather very dynamic. We now know that the different epigenetic modifications, the proteins and ncRNAs involved in orchestrating the acquisition and/or removal of these signals are tissue and cell-specific, genomic region-specific, spatial and temporally regulated, dependent on each other, labile to the environment, affected by health status, and able to be hijacked by exogenous agents making it quite difficult to draw an accurate picture of the epigenome. Due to this, studies in which single epigenetic modifications are determined are becoming less informative. This has led the way for the advent of high throughput technologies, data depositories, and bioinformatic approaches, which are providing the tools necessary to make testable genome-wide predictions.

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