2 Introduction to Epigenetics

Neelam Singla, Riya Thapa, Rashi Kulshrestha, Asif Ahmad Bhat, Saurabh Gupta, Manish Purohit, Santosh Kumar Singh, and Gaurav Gupta

Abstract

Epigenetics is the study of inherited variations in gene expression that do not involve changes to DNA sequences. Over 200 unique cell types in a human adult have a nearly similar genomic sequence. DNA and histones undergo reversible chemical alterations that contribute greatly to cellular variety via dynamic control of global gene expression, in addition to the genomic sequence. Epigenetics is based on chemical modifications of macromolecules rather than alterations to the chromosomal sequence. Epigenetic modifications, such as DNA methylation, chromatin modifications, nucleosome placement, and changes in noncoding RNA profiles, are reversible and fundamental processes in the regulation of gene expression. Alterations in gene activity and neoplastic transformation of cells may result from epigenetic disruptions. Epigenetic alterations occur at an early stage in neoplastic growth, before the corresponding genetic alterations have actually occurred. Researchers in this area will discover information on a wide variety of topics, all grouped together under the explanatory framework of chemical changes that affect gene expression regulation. In this chapter, we provide information on epigenetics, DNA methylation, and histone modifications.

Keywords

Epigenetics · Histone acetylation · HDAC inhibitors · DNA methylation

N. Singla · R. Thapa · R. Kulshrestha · A. A. Bhat · M. Purohit · S. K. Singh · G. Gupta (✉)
School of Pharmacy, Suresh Gyan Vihar University, Jagatpura, Jaipur, India

S. Gupta
Chameli Devi Institute of Pharmacy, Indore, Madhya Pradesh, India

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2.1 Introduction

Reproduction is crucial for the continuation of life and also aids in the transmission of characteristics from one generation to the next. Non-genetic variables, like epigenetics, also have a role in determining heritability and the genetic information stored in gametes. The reproductive processes most important for epigenetic landscape construction or maintenance are germ cell development and early embryo development. There is evidence that prenatal exposure to a certain lifestyle and environmental factors may alter the epigenetic blueprint of gametes, hence altering the phenotype of offspring. It's common knowledge that people's genes contribute to their susceptibility to a wide range of ailments. As time passes, more and more proof emerges that an organism's health state is determined by more than just hereditary elements; environmental influences may also play a role in shaping health via epigenetic modulations. Like genetic characteristics, epigenetic variables affect reproductive health and fertility [1, 2]. So, it's crucial to learn about epigenetic alterations, cell physiology, and the root of disease. In the last three decades, new developments in sequencing the human epigenome have helped scientists understand the origins of several diseases. This opens opportunities for understanding the epigenetic code and creating more effective therapies [3–5].

This section summarizes the most important epigenetic processes and introduces the many epigenetic analysis methods and cutting-edge epigenetic technologies that are now accessible.

2.2 Epigenetics

2.2.1 Definition

Although approximately 200 cell types in humans have the same DNA sequences, their gene expression patterns and phenotypes are very different. Developmental scientist Conrad Waddington first used the term "epigenetic landscape" to characterize the dynamic cellular phenotypic changes that occur throughout the evolution of a multicellular organism. Holliday later defined "epigenetics" as a branch of nuclear inheritance that does not rely on variations in DNA sequence. According to current molecular and mechanistic definitions, epigenetics is the combination of modifications to the chromatin template that generate and maintain separate gene expression patterns and silencing within a single genome. If genes are words, then epigenetics specifies how those words should be interpreted [6–9].

On the other hand, if we see sets of genes as computer hardware, then epigenetic regulation would be analogous to the operating system. That's why epigenetics is so important; it's a window into how cellular processes are orchestrated, functioning as an extra layer of regulation. Three primary processes cause epigenetic alterations to chromatin: DNA methylation, noncoding RNAs, and post-translational histone modifications. This chapter will try to give you a taste of these ideas (Fig. 2.1) [10, 11].

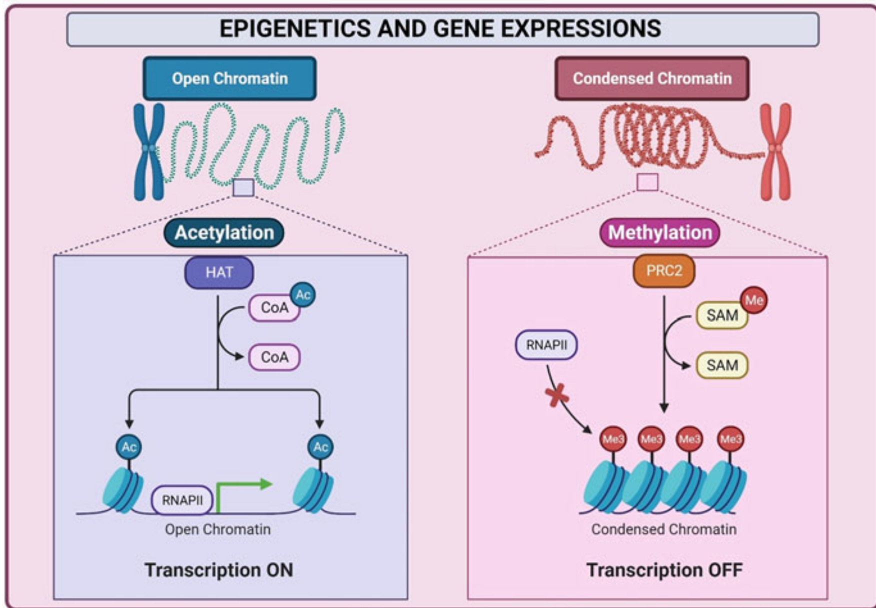

Fig. 2.1 Epigenetics and gene expressions

2.2.2 DNA Methylation

Over 35 years after its discovery, DNA methylation at cytosine residues is still widely considered a fundamental epigenetic mechanism controlling gene expression [6]. 5mC, or 5-methylcytosine, is converted to a cytosine residue in the DNA template by adding a methyl group. The most common location is the dinucleotide sequence 50CpG30 (CpG stands for cytosine and guanine, which a phosphate group separates in DNA). Both strands of DNA may carry it. Therefore, it can be passed down through generations of a family. As a kind of epigenetic marking for the genome that is conserved across cell division, DNA methylation patterns contribute to the formation of cellular memory. Clusters of CpGs, or CpG islands, are common. They tend to be abundant in interspersed repetitive elements and noncoding areas (like centromeric heterochromatin) (e.g., retrotransposons). In addition to their prevalence in gene enhancers, CpG islands are often located in the upstream area of gene promoters [12–15].

The methyl group of cytosine, found in the DNA helix’s major groove, is the primary site of interaction for many DNA-binding proteins. Then, different DNA-binding proteins are attracted to or repelled by methylation DNA. Methyl-CpG-binding domain (MBD) proteins attach to methylated CpGs, which in turn recruit repressor complexes (such as histone deacetylases, which remove activating histone acetylation marks) to methylated promoter areas, resulting in transcriptional suppression. CpG methylation, in contrast, prevents transcription by inhibiting the

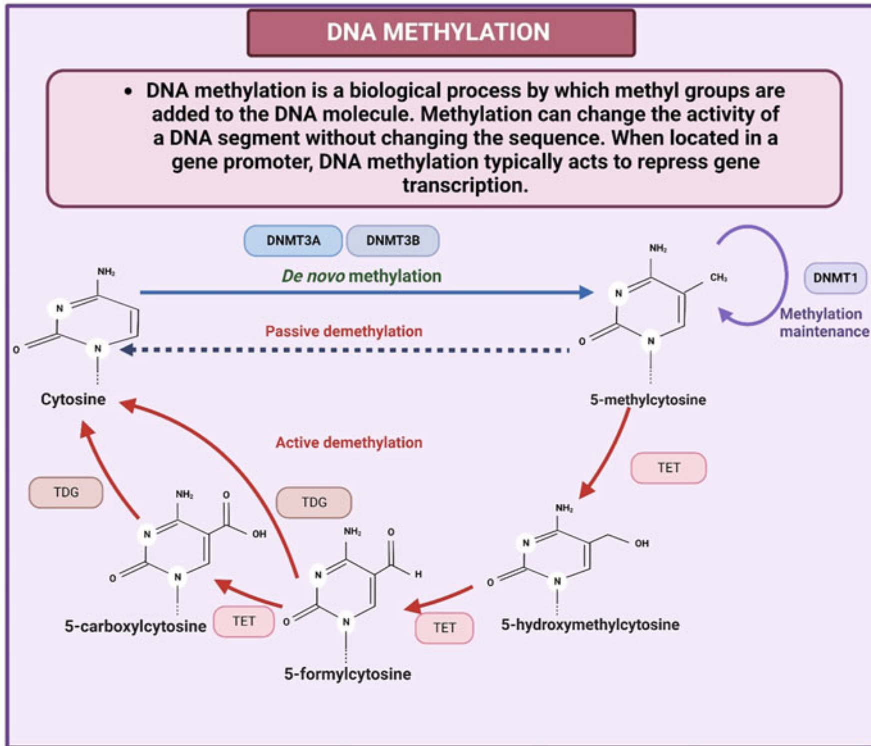

Fig. 2.2 DNA methylation

binding of certain transcriptional regulators like CTCF. Cellular differentiation, genomic imprinting, and inactivation of the X chromosome all rely on DNA methylation as a critical process [16, 17]. DNA methylation is essential for maintaining genomic stability and protecting against the spread of harmful transposable elements. Cell division results in the transmission of DNA methylation patterns from one cell to the next, but these patterns are not permanent. Researchers have discovered that DNA methylation patterns change throughout an individual's life. These alterations may be a healthy reaction to external stimuli or related to pathogenic processes such as neoplastic transformation or the natural aging process [18, 19] (Fig. 2.2).

2.2.3 DNA Methylation: Establishment and Erasure

DNA methylation in mammals is mediated by DNA methyltransferases (Dnmts), which consist of three proteins from two families with very different structures and functions. The first family of maintenance methyltransferases includes Dnmt1, an enzyme that preferentially methylates hemimethylated CpG dinucleotides (i.e., DNA

methylated at CpG in one of the two strands). This way, Dnmt1 is responsible for semiconservative DNA methylation patterns during replication. Dnmt3a and 3b belong to a different family of de novo methyltransferases that establish de novo DNA methylation patterns in the developing embryo. Even though it is inactive as a methyltransferase, Dnmt3l plays a crucial role in de novo methylation activities at certain DNA sequences and directs the activities of Dnmt3a and 3b. The ten-eleven translocation (Tet) protein family of DNA hydroxylases is involved in the active demethylation process [20, 21].

In contrast, the suppression of Dnmt1 during cell division is responsible for the passive demethylation process. Tet enzymes may convert the methyl group on CpG to 5-hydroxymethylcytosine (5hmC). This DNA alteration is abundant in the active chromatin areas of the genome and has a role in regulating gene expression. 5hmC may be oxidized further into 5-carboxyl cytosine (5caC) and 5-formyl cytosine (5fC) by the Tet family of proteins, which uses ATP in the process. This triggers the base excision repair process, which removes the mutated base and converts the 5mC back to an unmethylated cytosine, reactivating transcription [22–26].

2.2.4 Histone Modifications

Chromatin, found in multicellular organisms, is a nucleoprotein structure that houses DNA. DNA is wrapped around a ring of histones, which are uncomplicated proteins. Nucleosomes, the basic building blocks of chromatin, comprise 146 bp of DNA wrapped around an octamer of core histones. Two copies of the main histones H2A, H2B, H3, and H4 make up each nucleosome. The H1 linker protein connects each nucleosome's DNA and histone octamer core. The histone core is attached to the DNA through weak ionic contacts between positively charged residues on histone proteins and phosphate groups on the DNA [27–31].

2.2.5 Higher-Order Chromatin Organization

Nucleosomes are the fundamental units of chromatin structure. When the chromatin is fully unfolded, the structures may be seen as "beads on a string" made up of polymers with a size of 11 nm. According to the solenoid model of the chromatin fiber, nucleosomes are arranged in a helical array of around six to eight nucleosomes per turn, with the histone H1 on the inner of the fiber. Since the nucleosomes run in a spiral pattern, the linker DNA must be twisted to join them. This leads to a tighter chromatin shape that is transcriptionally inept at 30 nm. This allows the chromatin to be structured into bigger looping domains (300–700 nm). In the metaphase of mitosis or meiosis, chromosomes develop with the most compacted chromatin structure to ensure accurate genetic material distribution [32–35].

The chromatin structure is very flexible and may take on a wide variety of shapes. Traditionally, chromatin has been split into euchromatin and heterochromatin. Coding and regulatory (for example, promoters and enhancers) portions of the

genome are found in euchromatin, often known as “active” chromatin. It is in an accessible, decondensed confirmation that is “poised” for gene expression because it encourages active transcription. Heterochromatin describes the tight, highly compacted form of the “inactive” sections of the genome. It consists mostly of noncoding sequences and repetitive regions, yet it still makes up the vast bulk of the genome (e.g., retrotransposons, satellite repeats, and LINEs). Permanently suppressed “constitutive” heterochromatin is often located in pericentric and subtelomeric areas [36–39], whereas “facultative” heterochromatin allows for temporary derepression of genes within a certain cell cycle or developmental stage.

Histones in the nucleus are among the most conserved proteins in eukaryotes. The N-terminal portion is simple, whereas the C-terminal resembles a histone fold. The DNA is wrapped around the histone’s globular domain after heterodimerized with another histone (H3 with H4, H2A with H2B). The N-terminal “tail” domain is unstructured and exists outside the nucleosome. Although many post-translational modifications (PTMs) occur at specific residues in the tails of histones, notably histones H3 and H4, other modifications occur at specific residues in the more organized globular domains as well. These modifications foster nucleosomal and, by extension, chromatin variety. The first covalent modifications studied were histone H3 and H4 acetylation and methylation. Additional modifications to histones, including ubiquitination, phosphorylation, ADP-ribosylation, sumoylation, crotonylation, and biotinylation, have also been discovered [40–42].

2.2.6 Histone Acetylation

Lysine (K) residues at the N terminus of histone may be acetylated. Although acetylation of all core histones is possible in vivo, H3 and H4 acetylation have received the most attention. Lysines 9, 14, 18, and 23 in H3 and lysines 5, 8, 12, and 16 in H4 are available for acetylation. The affinity of histone tails for DNA is reduced when acetyl groups are added, which neutralizes the basic charge of the tails. Furthermore, it affects the contacts between histones inside nucleosomes and the interactions of histones with other regulatory proteins. The acetylation of histones is a hallmark of euchromatin and generates an “open” chromatin environment conducive to gene transcription [43, 44]. Histone acetyltransferases and histone deacetylases work in opposition to one another to regulate the dynamic process of histone acetylation (HDACs) [9, 31].

2.2.7 Histone Methylation

Lysine (K) and arginine (R) residues on histone may be methylated on their side chains. In contrast to acetylation, methylation of histones does not affect the protein’s charge. Lysines may be mono-, di-, or tri-methylated; arginines can be mono-, symmetrical, or asymmetric di-methylated. This improves the already high complexity of this update. Several lysine residues are at positions 4, 9, 27, and 36 on

H3 and at lysine 20 on H4 [45]. H3R2, H3R8, H3R17, H3R26, and H4R3 are all arginine methylation sites. However, histone methylation may signify either transcriptional activation or repression, depending on the methylation sites, in contrast to acetylation, which invariably leads to transcriptional activation. Methylation at H3K4 activates genes, whereas H3K27 methylation is known to silence them. The result is that most of these alterations occur in the promoter and enhancer elements of genes. Closed chromatin, caused by H3K9 methylation, is characteristic of heterochromatin areas in the genome. Heterochromatin-mediated transcriptional silence, X chromosome inactivation, and the DNA damage response are just a few of the many biological processes in which histone lysine methylation plays a role. Kinase methyltransferases (KMTs) and lysine demethylases (KDMs) coordinately control histone lysine methylation to preserve cell destiny and genomic integrity [46–49].

2.2.8 Other Histone Modifications

Phosphorylation of histones happens most often on N-terminal serine, threonine, and tyrosine residues. Phosphorylation of histones gives them a negative charge, which leads to a less closed chromatin structure. Thus, it is linked to gene expression and plays a role in chromatin remodeling and DNA damage repair. Histones may acquire a negative charge by the reversible mono- and poly-ADP ribosylation of glutamate and arginine residues, which aids in maintaining a state of relaxed chromatin. When DNA is damaged, these alterations become more common and play a role in the body's repair process [50–53]. A large ubiquitin moiety (a polypeptide of 76 amino acids) is attached to lysine residues in histone ubiquitylation. Polyubiquitylation of histones marks them for proteolytic breakdown, whereas monoubiquitylation may result in either gene activation or repression. The result of sumoylation is the covalent binding of small ubiquitin-like modifier molecules to histone lysines, as with ubiquitylation. Sumoylation, which may occur on any of the four core histones and blocks the acetylation or ubiquitylation of lysine residues, is responsible for gene repression [54, 55].

2.2.9 The "Histone Code": Its Writers, Readers, and Erasers

Remarkable nucleosomal variety results from covalent changes happening at various and specific places on the histones. Genetic changes in gene expression are regulated and determined by the chromatin structure, which may alter various histone modification combinations. The "Histone Code Hypothesis" describes this theory. This theory proposes that changes to histones serve as a platform for signals that may activate or silence genes by affecting the chromatin states. Histone modifications are performed by enzymes known collectively as the "writers" of the histone code. The enzymes in question include histone deacetylases (HATs), methyltransferases (HMTs), and histone kinases [56, 57]. Erasers of histone codes refer to enzymes like HDACs and KDMs that remove histone modifications. More than just changing

the chromatin state, these modifications also create binding or recognition sites for a variety of effector proteins called "readers" of the histone codes, which in turn recruit additional co-regulator complexes to affect even more dramatic changes in chromatin structure and, ultimately, gene expression [58–61].

2.2.10 Noncoding RNAs

Only a tiny percentage of the human transcriptome (2–5%) was encoded for proteins, and the functions of the other transcripts were not understood before the Human Genome Project. New sequencing methods have shown that 75% of the genome is transcribed. There has been a reevaluation of the idea that most of the genome is "junk DNA" since it is not transcribed, but the argument remains heated. Classification of RNA types has undergone significant revision in recent years. It now falls into two basic categories: RNAs that code for proteins (messenger RNAs) and those that don't (noncoding RNAs) [62, 63]. Promoter-associated RNAs (PARs), Small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), circular RNAs (circ RNAs), long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and enhancer RNAs (eRNAs) are just some of the numerous noncoding RNAs that have been discovered in recent years. Recent years have seen much research into the activities of ncRNAs, and it has become clear that they serve several structural and regulatory functions [26, 64, 65].

What's more, noncoding RNAs have recently been recognized as a distinct epigenetic mechanism for controlling gene expression. Changes in chromatin states may be effected through cis or trans processes, and the scaffolding for chromatin-remodeling and -modifying enzyme complexes is an important function of ncRNAs. They may also facilitate the recruitment of co-repressors and other factors involved in gene silencing or transcriptional activation. These ncRNAs may offer the required sequence specificity to guide chromatin-modifying complexes to their targets. ncRNAs, whose roles are gradually revealed, are an essential component of epigenetic control [66, 67].

2.2.11 RNA Modifications

Covalent modification of RNA may be induced by a broad range of chemical additions to its sugar and nucleotide groups. However, although alterations at the nucleotide base impart new regulatory activities, RNA is largely stable because of modifications to its sugar backbone. The vast majority of coding and noncoding RNA undergo post-translational modifications; methylation of adenosine at position N(6) (m(6)A) is the most common of these modifications. The 30UTRs and stop codons are the most common places where this happens. Different stages of mRNA metabolism, including RNA polyadenylation, microRNA-mediated degradation, and pre-microRNA processing, are regulated by RNA methylation and engaged in translation and RNA degradation [68–70].

Additionally, it regulates alternative splicing for a certain group of messenger RNAs (mRNAs) and long noncoding RNAs (lncRNAs). The METTL3 RNA methyltransferase complex initiates this modification, while the FTO (fat mass and obesity-associated) RNA demethylase complex reverses it. This alteration has a critical function in development, metabolism, and fertility, and its amount is dynamically regulated by the activity of these enzymes [71, 72].

2.3 Techniques for Epigenetic Analysis

Initially, epigenetics relied on specialized nucleases (such as restriction enzymes, DNase I, and MNase); DNA- and histone-modifying enzymes were just recently discovered, yet they've already yielded a plethora of fresh data. The last several years have introduced novel methods that directly evaluate interacting proteins, chromatin modification, nucleosomal occupancy, and DNA methylation patterns [73, 74]. Understanding epigenetic processes have been sped up by the active development of new tools to examine genome-wide DNA methylation patterns and chromatin structure in the contemporary age. Following is a discussion of the many genome-wide and loci-specific methodologies built to identify epigenetic markers and evaluate their functioning [75, 76].

2.3.1 DNA Methylation

From restriction, endonuclease-based southern blotting to epigenomic microarrays and methylation-specific polymerase chain reaction to targeted or whole genome bisulfite sequencing using next-generation sequencing technology, the methods for detecting DNA methylation have progressed significantly in recent years [77, 78]. The methodology used is crucial to gain an objective response to the study topic. The sensitivity and specificity requirements, the study's goal, the volume and quality of the DNA sample, the availability of reagents and equipment, and the technique's cost-effectiveness and ease of use all play a role in determining which method will be used. Whether the information sought is genome-wide or locus-specific is another consideration when deciding on a methodology. Whether or not the candidate genes are known may also play a role in deciding which approach to take [79, 80].

2.3.2 Histone Modifications and Chromatin Remodeling

The study of histone modifications and chromatin remodeling throughout the whole genome has come a long way in the last three decades. Improvements in high-throughput sequencing in tandem with chromatin immunoprecipitation assays (ChIP) and DNA microarrays (DNA chips)—for example, ChIP-on-chip, Chromatin

Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET), and ChIP-Sequencing (ChIP-Seq)—have aided in deciphering the human epigenome [81, 82].

Studying the kinetics of histone methylation is made possible by chromatin immunoprecipitation (ChIP), a potent method for analyzing protein-DNA interactions. The idea of chromatin immunoprecipitation (ChIP) is to enrich the area of DNA of interest (antigen) by immunoprecipitation, then amplify the enhanced region by polymerase chain reaction (PCR) to acquire an adequate amount of the enriched fraction. Also, southern blotting, polymerase chain reaction (PCR), and genome-wide approaches are used for the study [83, 84].

Two kinds of ChIP-on-chip methods exist, each tailored to the specific contents of microarrays: (1) promoter tiling arrays and (2) genome tiling arrays. Since the probes in promoter tiling arrays are tailored to target certain promoters and other genomic elements, some important details may be overlooked. Genome tiling arrays use probes that span the whole genome, allowing for comprehensive genomic studies. Histone alterations have been previously studied using the ChIP-on-chip technique in yeast and *Drosophila melanogaster*. Analysis of histone modifications in the human genome using ChIP-on-chip has recently proven fruitful [85, 86].

Thirdly, ChIP-seq utilizes next-generation sequencing techniques to provide an additional way for evaluating histone methylation and chromatin remodeling. To do ChIP-seq, DNA ends must be repaired and ligated to a set of adapters. The oligonucleotides are attached to the surface of the flow cell after the DNA has been amplified. These oligonucleotides are specific to adapter sequences ligated to DNA. During solid-phase PCR, the DNA sequences are read by the genome analyzer and then mapped to a reference genome to determine positions. This method helped researchers overcome the ChIP-chip technique's mediocre resolution and distracting background noise [87–89].

Mass spectrometry allows for the quantitative analysis of protein expression and the differential expression of protein modifications. Recently, chromatin affinity purification in conjunction with MS (ChAP-MS) and chromatin proteomics (ChroP)/ChIP-MS was developed to overcome the problem of MS's inability to map alteration patterns to particular promoter regions. Accordingly, it is now possible to examine histone marks and binding proteins in functionally different chromatin regions concurrently [90]. Different mass spectrometry approaches, named bottom-up, middle-down, and top-down, are distinguished by the level of the histone sequence at which they are applied. In the standard "bottom-up" approach, protease enzymes digest the target protein into shorter peptides (5e20 aa) before MS analysis. The "top-down" technique is used to characterize full-length proteins, whereas the "middle-down" method is used to analyze and characterize big peptides with less than 50 N-terminal amino acid residues of histone tails [91, 92].

2.3.2.1 Technologies for Capturing the Conformation of Chromosomes

Many methods of capturing chromosomes in their native 3D shape have been developed so that the genome's spatial organization may be analyzed.

Methods such as (3C) and (4C) chromosomal conformation capture, (6C) combined 3C-ChIP-cloning, (GCC) genome conformation capture,

(ChIA-PET) chromosome in situ hybridization, (5C) chromosome conformation capture carbon copy, etc. are all examples. Formaldehyde crosslinks the cells, a hypotonic buffer and protease inhibitors lyse the cells, and sodium dodecyl sulfate (SDS) solubilizes and digests the chromatin. Ligase is then used to relink the chromatin under diluted circumstances. This is the 3C technique. In addition, 3C libraries are produced by reverse cross-linking and purification. Higher resolution and throughput studies of the 3D structure of chromatin at a specific locus are made possible by using the 3C libraries to build 5C libraries [93, 94].

The disadvantage of the ChIP-chip technology is the development of microarrays (6, ChIA-PET). A 3C-based method called Chromatin Interaction Analysis by paired end tag ((ChIA-PET)) was developed to solve this issue. In this technique, DNA "tags" that have been immunoprecipitated are cloned into a plasmid library and then sequenced [95].

2.3.3 Methods to Analyze Methylation of RNA and ncRNA Species

Purifying RNA according to standard techniques allows for analysis of RNA modifications such as N6-methyladenosine (m6A), N1-methyladenosine (m1A), 20-OMethylation (20OMe/Nm), and 5-Methylcytosine (m5C). Multiple variables, including the number and kinds of modifications present and the RNA sequence itself, influence the analysis and characterization of RNA [96, 97]. The following are some of the methods that have been used to learn about RNA methylation.

Incorporating radioactive isotopes into RNA is one method used in radioisotope incorporation tests, which may be used to measure RNA methylation levels. Methyltransferase activity is assessed via scintillation because of the radioactivity of the methyl group added from the donor to the nucleoside. The methyl donor, S-adenosyl methionine, is tritium-labeled.

Another method for recognizing most RNA alterations is two-dimensional thin-layer chromatography. The nucleotides are spread out over the cellulose substrate in a two-dimensional RNA separation based on their charge and hydrophobicity. The disadvantage of 2D-TLC is that it offers an overall methylation status of the transcriptome. Ultraviolet light may then be used to create an image of the pattern. Site-specific cleavage, radioactive tagging, ligation-assisted extraction, and thin-layer chromatography (SCARLET) may be used to investigate stoichiometry when the sequence is known [98, 99].

Nucleotides are characterized by their mass-to-charge ratio in a conventional MS-based comparison approach. MS is comparable to chromatography-based techniques. However, it doesn't need a radioisotope or label. A key downside of MS-based techniques is the need for substantial amounts of RNA and priori-sequence information.

Sequencing using bisulfite, a thymine mutation occurs during reverse transcription. This mutation arises because sodium bisulfite converts cytosine to uracil, which is subsequently reflected in the final sequencing data set. Methylated cytosine is protected against delamination. The bisulfite sequencing method works this way,

yielding information on RNA methylation at the level of a single base pair. Bisulfite sequencing has several limitations, including the need for large amounts of RNA and the resistance of surrounding changes and double-stranded areas to bisulfite treatment [100, 101].

Method number five involves antibodies; they may be purchased and used to detect methylated RNA residues like m6A, m1A, or m5C. Estimates of m5C, m1A, and m6A are also being made using antibodies directed toward the changes. "Methylated RNA immunoprecipitation sequencing" ("MeRIP-seq") is the most well-known and widely used method for mapping these modifications. The technique of N6-methyladenosine and m6A crosslinking immunoprecipitation sequencing (m6A-CLIP Seq/mi-CLIP) enables high-resolution mapping of m6A modifications on as little as 1 mg of poly(A)-selected mRNA [102, 103].

2.4 Understanding the Roles of Epigenetic Modulating Enzymes

Western blotting, enzyme-linked immunosorbent assays, chromatin immunoprecipitation (ChIP) tests, and coimmunoprecipitation are often used to investigate the expression and analysis of epigenetic modulators such as DNMTs, HDACs, and MeCPs. Co-immunoprecipitation is often employed to investigate the relationships between epigenetic regulators. In addition, imaging techniques in living organisms are being utilized to investigate the pharmacokinetics, direct binding, and activity of HDAC inhibitors [104, 105].

2.5 Single-Cell Epigenomics

Current epigenetics knowledge is based on population-level data that predominantly related epigenetics to transcriptional activation or silencing. These simplifications are a major reason why so many pressing questions in biology have yet to be resolved. Epigenetic controls must be studied at the single-cell level, where changes across cells may be investigated, to provide a fuller picture of cellular activities and dysfunctions. Recent advances in single-cell technology have shown conclusively that even seemingly similar cell populations display different degrees of gene expression, likely due to epigenetic heterogeneity. Single-cell epigenomics is a cutting-edge field of study that has tremendously helped our comprehension of gene control and associated molecular disorders. This exciting new method has the potential to advance our understanding of epigenetic regulation [106, 107]. However, concurrent analysis of genomic, transcriptomic, and epigenomic data may reveal the full potential of single-cell epigenetic research. When epigenome data is included in multi-omics measures, it may further strengthen molecular linkages between functional output and genome. In the realm of single-cell epigenetics analysis methods, the primary step involves isolating a single cell from a culture or dissociated tissue, followed by lysing the cell for subsequent epigenetic analysis. This may be done in

various ways, including droplet encapsulation, fluorescence-activated cell sorting (FACS), and manual manipulations. New microfluidics technologies allow for the isolation of cells in chambers, followed by lysis and RNA-seq library preparation [108].

2.6 DNA Methylation and Other Modification

Single-cell sequencing at a single-nucleotide resolution has made it possible to investigate a variety of DNA modifications, including hydroxymethylation (5hmC), formyl cytosine (5fC), and methylation (5mC). Enrichment of CpG dense places (like CpG islands) and restriction digestion form the basis of a method called reduced representation bisulfite sequencing (scRRBS), which was first used to quantify 5mC throughout the whole genome in a single cell. Despite its ability to assess a larger proportion of CpG sites in the promotor region, it does not cover many crucial regulatory areas. The technological developments in single-cell whole-genome methylation sequencing are based on a post-bisulfite adapter-tagging (PBAT) strategy, where bisulfite modification is performed before library formation. Microfluidics has recently given rise to a new method for producing single-cell libraries. This approach has greatly improved library preparation throughput, especially when combined with cell-specific barcoding and the pooling of adapter-tagged fragments. This method can determine methylation at 50% of CpG sites in a single cell. Because of this, we have identified large differences in distal enhancer methylation across individual cells, a phenomenon often missed by scRRBS. Current technologies such as AbaSI (restriction enzyme) and TET-assisted bisulfite sequencing (TAB-seq) coupled with sequencing (Aba-seq) may be tweaked for hydroxymethylated cytosine single-cell research (5hmC) [96, 109].

2.7 Histone Modifications and Transcription Factor Binding

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is used to map histone modifications; nevertheless, ChIP-seq at the single-cell level is very difficult to conduct. Background noise from nonspecific antibody pull-down is an issue with single-cell ChIP-seq that worsens at lower target antigen concentrations. The use of barcoding and micrococcal nuclease (MNase) digestion has recently replaced this method, allowing for the efficient processing of thousands of cells. This method employs microfluidic droplet technology to simultaneously process vast swaths of cells [92]. Low-level expression of a fusion protein of *Escherichia coli* deoxyadenosine methylase (Dam) and the protein of interest in a cell line paves the way for investigating protein-DNA interactions in single cells using the DamID method. This Dam-based method employs adenine methylation adjacent to protein-binding sites, followed by cleavage of the DNA using the enzyme DpnI, sensitive to methylation, and ligation of sequencing adapters. The current state of this method prevents it from mapping transcription factor binding sites in single cells due to its

low resolution (100 kb). Combining Dam fusion with targeted histone readers or modifiers might also allow for genome-wide investigation of histone changes utilizing single-cell DamID [89, 101].

2.8 Chromatin Structure and Chromosome Organization

A transposase test (ATAC-seq) measures chromatin structure in single cells. With this method, DNA is fragmented, and adaptor sequences are attached to it simultaneously using an enzyme called Tn5. ATAC-seq achieves single-cell resolution using the "combinatorial indexing" method of giving a unique barcode to each of 96 pools containing several thousand nuclei [95, 105]. Another described method for single-cell ATAC-seq involves using a piece of commercially available microfluidics equipment to carry out the transposition reaction on individual cells. By mapping an average of 70,000 reads per cell, the resolution gains from this combinatorial indexing method are substantial. DNase-seq is a method used to map DNaseI hypersensitive regions, which have been used to study open chromatin genomic regions in single cells. If the resolution of single-cell DNase-seq is improved to 300,000 mapped reads per cell, the mapping proficiency is only 2%, and the throughput is even lower [100, 109].

Recent technological advances have made it feasible to use an HiC-based approach to evaluate chromatin structure and chromosomal conformation in a single cell. An extension of chromosomal conformation capture (3C), Hi-C can detect long-range interactions throughout the whole genome. Despite the current limitations of single-cell HiC, it is possible to portray chromosome structure, including interchromosomal connections and compartmentalization [75, 78].

2.9 Epigenome Manipulation and Editing

Eukaryotic cells' epigenomes are intricate and tightly linked to essential cellular functions. If they aren't checked, it may lead to pathology and abnormal gene expression. Increased understanding of epigenetics' role in gene regulation and the ability to manipulate cell phenotype for research or therapeutic reasons are only two potential benefits of epigenome editing. Several epigenome editing platforms are available now, thanks to recent developments in genome engineering that use DNA-targeting techniques to properly adjust epigenetic modifications in a locus-specific manner [63, 66] (Table 2.1).

2.10 Epigenetic Manipulation Techniques

Classical genetic techniques can be used to simulate the perturbation of individual components of the epigenome. These methods include gene knockouts and domain deletions, point mutations, inducible expression constructs, ectopic expression of

Table 2.1 Analysis of DNA methylation using a variety of methods

Class	Method	Principle	Features	Limitations
Differentially methylated regions	Array or bead hybridization	To hybridize with a microarray, methylated DNA subsets are used	Capable of methylation-spot identification at individual loci	Potentially produces false positive results owing to cross-reactivity
	Bisulfite sequencing	The "gold standard" technique for DNA methylation studies, validated through polymerase chain reaction (PCR) amplification and Sanger sequencing, involves bisulfite treatment to facilitate the deamination of cytosine into uracil, which is then read as thymine. This treatment does not lead to uracil formation from cytosine-5 mC residues.	Resolution down to the nucleotide level	It is possible that partial uracilization of unmethylated cytosine results in bias
	Endonuclease digestion followed by sequencing	Methylation-sensitive endonucleases are used to cut off unmethylated sections of DNA in the genome, and then adapter ligation occurs	To isolate methylated CpG islands, use next-generation sequencing	It's crucial to use high-quality DNA
DNA methylation analysis	Bead array	An assay using an extension technique	Profiling of up to 384 unique CpG sites is conceivable with this method, and it is applicable to non-human animals	Costly
	COLD-PCR	The PCR's denaturation temperature is lowered such that only unmethylated fragments (those with a lower concentration of GC) are amplified and identified	Methylation levels in DNA are so low that they may be identified at the 0.1% level	Using a denaturing gradient, such as polyacrylamide, is essential
	Methylation-specific PCR	To accomplish DNA amplification, you'll need two sets of primers, one set for methylation DNA, and another set for unmethylated DNA. Two separate qPCR reactions are run on each sample, and the difference in Ct values is used to determine the level of methylation	An effective procedure that can rapidly profile a large number of samples	The methylation status of one or two CpG sites is evaluated all at once

(continued)

Table 2.1 (continued)

Class	Method	Principle	Features	Limitations
	PCR and sequencing	Amplification of region-specific DNA that has been bisulfite-treated and sequenced	Direct sequencing of PCR products is best for detecting significant methylation changes	Uses nested PCR to reduce nonspecific amplification
	PCR with high-resolution melting	DNA methylation status may be inferred from the fact that high-Tm DNA melts at a lower temperature than unmethylated DNA (low Tm). After attaching to DNA, intercalating dyes like SYBR and Eva green emit a fluorescent signal. Reduced fluorescence is a consequence of DNA dissociation as temperature increases	Depending on the quality of the PCR result, we are able to identify subtle changes in methylation (5–10%)	The need for a clean PCR product
	Pyrosequencing	Short-read pyrosequencing reaction (w100 bp) is carried out when primers are constructed, PCR products are acquired, and the reaction is initiated. Signal strengths for integrated dGTP and dATP are used to infer the methylation status of individual CpG sites within the sequenced area	Appropriate for low-throughput applications; efficient method for diverse samples; sensitive enough to pick up methylation variations as tiny as 5%	Poor proofreading and the presence of repetitive DNA make genome assembly difficult
Profiling whole genome methylation	AFLP (amplification fragment length polymorphism) and RFLP (restriction fragment length polymorphism)	Differentially methylated fragments are identified using polymerase chain reaction	Affordable, universally applicable across all species	Evaluates a negligible fraction of the whole DNA methylation landscape
	ELISA-based methods	Uses a tagged secondary antibody and colorimetric/ fluorometric detection with a primary antibody produced against 5 Mc	Fast and cheap	High in error, yet adequate for providing a ballpark estimate of DNA methylation

	Global DNA methylation assay-LINE-1 (long interspersed nuclear elements-1)	Using biotinylated LINE-1 probes, DNA fragments are hybridized and then immobilized on a streptavidin-coated plate. Methylated cytosines were counted with the use of an anti-5 mC antibody, a secondary antibody coupled with HRP, and chemiluminescent detection reagents	Detects very subtle DNA methylation changes	Since pyrosequencer detection requires extensive amplification of target sequences, PCR amplification bias is a real possibility
	HPLC-UV (high-performance liquid chromatography—ultraviolet)	Reducing DNA to its nucleoside bases by hydrolysis; separating 5 mC and dC bases using chromatography; measuring fractions; and calculating the 5 mC/dC ratio	Deoxycytidine (dC) and methylcytosine (5 mC) test is the gold standard for measuring DNA content in hydrolyzed DNA samples	Requires a lot of DNA (3–10 μg) and specialist lab equipment
	LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry)	MS is used for both evaluation and quantification, like HPLC, but using spiked internal standards instead	Because less hydrolyzed DNA is needed, low-quality DNA has little impact and high-throughput methylation analysis may be performed at a lower cost	It's expensive to get started, and it costs much more to buy internal standards
	LUMA (luminometric methylation assay)	DNA was cut using restriction enzymes that are sensitive to methylation, and the polymerase extension test was performed using pyrosequencing	Easily scalable, quantitative approach with excellent reproducibility	Necessitates prime DNA
	Methylated DNA immunoprecipitation (MeDIP)	Involves the use of a 5mC-specific antibody and subsequent genome-wide PCR microarray analysis of the DNA that was immunoprecipitated from the antibody	Highly repeatable and suitable for genome-wide methylation pattern study during the course of development	Requires high-grade and cross-reactive 5mC antibodies
	Reduced-representation bisulfite sequencing (RRBS)	The CpG-rich areas close to the restriction enzyme's recognition sequence are enriched, suggesting that these regions are particularly important	Cost-effective	Intergenic and distal regulatory components are not always fully covered

(continued)

Table 2.1 (continued)

Class	Method	Principle	Features	Limitations
	Whole-genome bisulfite sequencing (WGBS)	This should, in theory, include all there is to know about the C programming language. Genomic DNA is purified, then snipped into pieces and their ends mended. Sequencing is performed after a tailing (adenine base addition at 30 end), methylated adapter ligation, sodium bisulfite treatment, polymerase chain reaction, and sequencing of DNA fragments	Features genome-wide coverage and single-nucleotide resolution	Expensive and demanding careful planning

vectors, targeted knockdowns of a transcript, and various screens for gain or loss of function that alter genome structure or gene expression. These methods have been critical in laying the groundwork for our present understanding of epigenetics. However, these methods also cause widespread changes to the epigenome, which might potentially skew the findings of experiments [56, 70].

2.10.1 Small-Molecule Inhibitors

Anticancer medications and research use a class of small-molecule inhibitors that target specific epigenetic markers. The hallmarks of these medications are the histone deacetylase (HDAC) inhibitors romidepsin and suberoylanilide hydroxamic acid (SAHA), and the irreversible DNMT1 (depsipeptide or FK228) and DNMT3 inhibitors decitabine (5-aza-2 -deoxycytidine) and azacitidine (5-azacitidine). Histone-modifying enzymes are only one example of an epigenetic component that a variety of small-molecule inhibitors may target. Even though these chemicals aren't selective for any one kind of tissue or cell, they have demonstrated extraordinary effectiveness in various models when used in a narrow dosage range [63].

2.11 Targeted Epigenome Manipulations

Transcription activator-like effectors (TALEs), zinc finger proteins, and CRISPR-Cas systems are the three most essential molecular tools for targeted epigenome editing.

2.11.1 Zinc Finger Proteins

When manipulating nucleic acids with a precise focus on a certain sequence, zinc finger proteins are one of the best-studied systems. One method of editing the epigenome involves the combination of DNA-binding zinc finger proteins with effector domains that are either catalytically active or scaffolding. As a result, the chimeric proteins behave as artificial transcription factors (ATFs), altering gene expression patterns [73].

2.11.2 TALEs

TALEs' ability to recognize DNA results from its 33–35-residue-long core tandem amino acid repeat domain. TALEs' tandem repeat sequence encodes a DNA specificity that may shift depending on the context. In contrast to zinc fingers, which need triplet sequence recognition sites, TALEs could address a single nucleotide at the moment throughout its repeat variable di-residues (RVDs). This feature makes

TALEs more amenable to engineering, which has allowed the logical construction of artificial TALEs for use in epigenome editing [63].

2.11.3 CRISPR/Cas9 System

Because of its versatility and simplicity, the CRISPR/Cas9 system has been the primary focus of the new epigenome editing technologies. Several methods have been used to determine the best CRISPR/Cas9 targeting locations. Some research has successfully used innovative epigenome editing methods by simultaneously manipulating many nearby genes in the same area of the genome. In addition to the potential for additive effects, such an approach may raise the likelihood of an elevated number of off-target sites and complicate steric effects like suppression by catalytically inactive variations. Alternatively, the CRISPR-based method may identify the most effective gRNAs that modify transcription or protein expression by screening libraries containing hundreds of unique gRNAs. Cost and complexity are the only real issues with this method. Since it relies on assessing RNA or protein expression levels, which may or may not reflect epigenome alterations. CRISPR/Cas9 and other targeted epigenome editing methods provide unique platforms for the easy and flexible targeting of several epigenetically active regions of the genome [68, 74].

2.12 Conclusion

Only a tiny percentage of potential epigenetic targets have been explored, and the field of epigenome editing remains in its infancy. The development of cutting-edge technologies is expected to lead to a meteoric rise in the capacity of epigenome editing, enabling the production of novel epigenetic states. These advancements will facilitate the study of epigenetic processes responsible for altering epigenetic marks.

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