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New insights in molecular analysis of gene regulation - an epigenetic overview

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ABSTRACT

The major advancements in molecular biology and genetics from the past few decades brought a lot of excitement, especially with the development of new technologies that allow for the extensive analysis and sequencing of genomes. However, despite the amount of data available about the structure and sequence of DNA and its contained genes, many questions remain unanswered when it comes to the intricate mechanisms by which these genes are regulated and the ways in which they are influenced, both by each other and by their environment. Epigenetics is a young biological science which targets to decipher the aforementioned mechanisms, in order to provide a deeper understanding of the ways in which the genetic material orchestrates cellular processes and the way in which it is inherited in biological systems. The purpose of this review is to outline novel insights into this field, emphasizing its importance in the scientific community, its applications and notable methods used to investigate it.

Keywords: *Chromatin architecture, Chromatin Immunopreciptation (ChIP), Chromosome Conformation Capture (3C), Bisulfite sequencing, Epigenetic disease.*

1. INTRODUCTION

"Epigenetics is the study of heritable changes in gene expression and phenotype that are not caused by changes in the DNA sequence" [1]. The term was used by the embryologist Conrad Waddington in 1942 in order to describe the complex of developmental processes between genotype and phenotype, by relating the term to the previous concept of "epigenesis" [2, 3]. The concept was later used by Nanney to describe several genetic regulation mechanisms and to underscore their implication in developmental processes [4]. Only in the 21st century did studies highlighted "epigenetics" rise, describing several mechanisms of chromatin changes that influence the phenotype but don't affect the DNA sequence [5-13, 15]

Discovering the epigenetic mechanisms allowed for the subsequent identification of many biological processes that are highly dependent on them, but also of several links between numerous faulty epigenetic mechanisms and a variety of human diseases and affections. All these aspects highlight the importance of studying this complex field and of developing novel technologies to tackle its complex molecular networks and pathways [14, 17].

2. CHROMATIN ARCHITECTURE AND THE MOLECULAR BASIS OF EPIGENETIC REGULATION

The chromatin fiber is a complex, dynamic molecular system, composed of DNA, protein and small amounts of RNA. The fiber can have different conformational states and levels of compaction, which are regulated by nuclear proteins in the category of histones and non-histones (Figure 1).

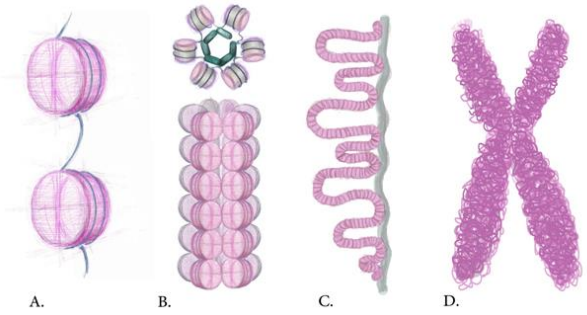

Figure 1. The different levels of chromatin compaction: A. The nucleosome; B. the 30nm fiber (the solenoid model); C. Looped domains; D. the metaphase chromosome.

The structural and functional unit of the chromatin fiber is the nucleosome. The nucleosome is an association between DNA and histones that creates a regular, low compacted form of chromatin, called the 11nm fiber. It is formed by a histone octamer around which a DNA double-helix coils itself roughly 1 and ¾ times, with a fragment of about 146 bp. The histone octamer contains two of each of the four core histones: H2A, H2B, H3 and H4. Histone H1 is associated with linker DNA found between nucleosomes and leads to higher levels of packaging. Each histone is composed of a globular domain and a tail, which extends out of the nucleosome core and is not associated with the DNA molecule. Despite that, they seem to both have a high level of conservation, from yeast all the way to humans, which highlights the important function played by both of those domains. In fact, the histone tails have been shown to play a very important role in epigenetic regulation. This role is based on the presence of several sites along the tail which are prone to posttranslational modifications such as methylation, acetylation, phosphorylation, ubiquitination and many others modifications [18].

2.1. The Mechanisms of Epigenetic Regulation. Epigenetic regulation encompasses a wide range of molecular mechanisms and processes that can affect the state and rate of gene transcription in each cell. This can be achieved, mainly, by

modifying the chromatin conformation in a way that would either facilitate or inhibit the access of transcription machinery needed for gene expression.

Epigenetic mechanisms are usually classified in one of the following categories: histone modifications; histone variants; chromatin remodeling complexes; DNA methylation; and RNA interference.

2.2. Histone Modifications. Posttranslational modifications of histone tails constitute an epigenetic mechanism that acts in two possible ways: Firstly, by suffering posttranslational modifications such as phosphorylation and acetylation, the net charge of the histone molecule is changed, which can significantly modify the affinity of the molecule to the DNA strand. A lower affinity can lead to a higher activity of the sequence, while a higher affinity leads to a less accessible chromatin structure associated with gene silencing. The second significance of histone modifications was described by Allis and Jenuwein in 2001 when they proposed the theory of the histone code. This theory claims the fact that different histone modifications and the combinations of modifications can act as epigenetic markers that can be "read" and recognized by several protein complexes implicated in transcription and regulation [1].

2.3. Histone phosphorylation is an epigenetic modification that consists in adding a phosphate group to an aminoacid residue on the histone tail. The modification is associated with transcription activation, and it can result in a more open chromatin conformation by adding more negative charges to the histone tail, which lowers the affinity towards the DNA molecule [19, 20].

Phosphorylation takes place under the activity of protein kinases which use ATP as a source of phosphate groups, and the groups can be removed by histone phosphatases [21].

2.4. Histone acetylation is a modification associated with gene activation. This modification is regulated by the antagonistic activity of two types of enzymes: histone acetyltransferases (HAT) and histone deacetylases (HDAC). By acetylation, the net positive charge of the histones is reduced, leading to a lower affinity to the negatively charged DNA backbone and a more open chromatin conformation, which allows higher accessibility for the transcriptional complexes. Acetylation can be recognized by several proteins such as those containing a bromodomain, and thus contribute to the formation of the histone code, together with phosphorylation, methylation, ubiquitination, sumoylation, ADP-ribosylation and so on [21].

An important example of the implication of hyperacetylation in a biological vital process is the histone-protamine transition during spermiogenesis. The postmeiotic stage of spermatogenesis includes several important epigenetic reprogramming events, the most important one being that of replacing 95% of all histone proteins with protamines, which are small, highly basic proteins meant to pack DNA in a way that would keep its integrity throughout the migration and fertilization processes. In this case, hyperacetylation serves as an epigenetic mark which is recognized by bromodomain-containing proteins that are implicated in the subsequent removal and replacement of histones with transition proteins, and, later on, with protamines [22, 23].

2.5. Histone methylation. Methylation can take place several times at the same site, leading to di- and tri-methylated aminoacid residues on the histone tail, all of these marks having specific functions as epigenetic tags. Methylation takes place under the coordinated activity of methylases and methyltransferases [21].

All these modifications can be considered "cis-acting" modifications of the chromatin fiber. Other modifications are considered trans-acting, like the ability of histones and histone-attached molecules to recruit several complexes or proteins or to serve as an attachment site for them [18]. An example is a bromodomain which can detect and attach to acetylated histone tails. This domain leads to the recruitment of several proteins that contain it [24]. Methylated tails can be recognized by chromodomain containing proteins, in the same way [25, 26, 27].

2.6. ATP-Dependent Remodelling Complexes. Histone modifications can play the role of recognition and attachment sites for ATP-dependent remodeling complexes. These complexes are involved in transcription regulation by remodeling the chromatin fiber in order to provide higher accessibility in the targeted areas. This is done not only by replacing or removing histones but also by nucleosome sliding.

Examples of ATP-dependent remodeling complexes include Swi/Snf complexes which use ATP hydrolysis as a source of energy in order to induce non-covalent changes in the chromatin fiber. This type of modification is crucial in regulation mechanisms [28].

2.7. DNA Methylation. A very well studied epigenetic modification is DNA methylation. DNA methylation is the process by which methyl groups are added to the 5 Carbon of a cytosine in a CpG dinucleotide, a modification associated with gene repression. Although DNA methylation on its own does not cause gene silencing, it is thought to play a role in gene repression by serving as a mark for several protein complexes that contain methylated DNA-binding domains. DNA methylation is mediated by the activity of DNA methyltransferases. This modification is the underlying mechanism behind genomic imprinting [29].

The existence of DNA demethylation in post-mitotic cells is a controversial topic. DNA methylation was viewed as an irreversible mark that could only be erased by the prevention of methylation after a round of DNA replication. The Recent evidence argues against this belief by supporting the existence of both methylation and demethylation events in mature cells, mediated by either DNMT3a and 3b or Gadd45b by a glycosylase reaction/DNA repair mechanism. This topic remains highly controversial, especially in the absence of an identified DNA demethylase [30].

2.8. Histone variants. Another mechanism of epigenetic regulation is based on histone variants. Histone variants are proteins that have a very high degree of similarity to the normal histones, but which have several different purposes and are incorporated in the chromatin fiber at specific times, replacing the regular histone proteins for a short period. Some of these variants are thought to lead to transcription activation, such as H3.1 and

H2A.Z, but others have several other roles such as H2A.X, a variant implicated in repairing DNA double strand breaks, and macroH2A, implicated in lyonization [31, 32, 33].

2.9. Non-coding RNA. Finally, epigenetic regulation can be held out by small non-coding RNA molecules such as RNAi (interference). These molecules can disrupt gene expression of complementary sequences, either by directly attaching to the DNA sequence or transcripts or by association with several protein regulatory complexes that can break down transcripts of the targeted gene [34]. Regulation mechanisms based on non-coding RNAs are present in all tissues and have a variety of important roles from genome stability and transposable element inactivation, protection against viral infections and regulation of embryonic development, to the silencing of genes and chromatin condensation [18, 12].

2.10. X - Chromosome Inactivation. A great example of a molecular process which is orchestrated by an entanglement of several epigenetic processes is the mechanism of X chromosome inactivation. In this process, of crucial importance are the non-coding RNAs Xist and Tsix [35, 36], histone variants, most importantly, macroH2A, high levels of DNA methylation and, finally, several histone modifications, notably high levels of H3K27 and H3K9 methylation, low levels of H3K4 methylation and a global hypoacetylation [37, 38].

3. IMPORTANCE

The importance of studying epigenetics is becoming increasingly clear as more and more studies are linking a variety of diseases and conditions with underlying epigenetic mechanisms. Epigenetic mechanisms have been shown to have a great implication in major, natural, biological processes such as normal embryonic development, fertility, reproduction, tissue homeostasis, immunity, learning and behavior.

3.1. Gametogenesis and Embryonic Development. Both gametogenesis and embryonic development are biological processes that are highly dependent on intricate mechanisms of epigenetic regulation. Gametogenesis, the process by which new, functional germ-line cells are produced, is based on extensive epigenetic reprogramming in both male and female cells [39]. The major events of epigenetic reprogramming can be grouped in three stages: primordial germ cell (PGC) specification and formation; the erasure of PGC-specific epigenetic code followed by the reestablishment of germ-specific epigenetic marks and; the modifications of male and female pronuclei upon fertilization [40]. Many aspects of gamete structure and function are highly dependent on the correct establishment of epigenetic mechanisms, which is why any disruption or irregularity in these events can lead to serious outcomes that affect fertility, fertilization and offspring development [41]. Epigenetic marks are thought to have very big implications in meiosis, development, cell specification, differentiation, and genome integrity [42]. For this reason, understanding epigenetic regulation in germ cells has implications for the advancement of cloning technologies, assisted reproductive technologies and human health [23, 41, 43, 44, 45].

A very important aspect of embryonic development is the totipotency of the zygote, which is strictly dependent on the nucleus epigenetic program and is a consequence of the prior and specific modifications suffered by each gamete during their formation [45]. Through epigenetic regulation, the zygote can give rise to a multicellular organism composed of different tissues and cells, but all containing the same genetic material. Epigenetic information is heritable and is implicated in establishing a selective gene expression pattern in different cells, both during early development and as an adaptive mechanism in response to environmental cues later on [46, 47].

It was initially believed that all epigenetic marks are erased and reestablished during gametogenesis [48, 49], but it has been shown in recent years that some of the epigenetic marks are conserved and transmitted to the newly-formed zygote. This was defined as *transgenerational epigenetic inheritance* and is thought to provide an additional level of phenotypic variability between individuals. It has been demonstrated that some genes show transgenerational epigenetic inheritance through the male germ line [50].

Genomic imprinting plays a major role in several important processes which include embryonic development and in metabolic health during early postnatal life. The monoallelic expression of specific genes allows for the maintenance of a balance that is susceptible to damage in a variety of ways, leading to several health consequences. This can happen through chromosome deletions, uniparental disomy (UPD), or alterations in the imprinting center. Diseases caused by imprinting defects include: Russell-Silver syndrome, Beckwith-Weidermann syndrome, Albright hereditary osteodystrophy, pseudohypoparathyroidism, transient neonatal diabetes mellitus, Prader–Willi syndrome, Angelman syndrome [41].

Global demethylation occurs in primordial germ cells and later in fertilized zygotes and it is followed by the establishment of new patterns of gene methylation necessary for the existence of the organism. It has been demonstrated that the establishment of these new patterns can be influenced and changed by environmental factors. A study which described this phenomenon consisted of altering the expression pattern of the agouti gene in mice by changing the level of methyl donors in the maternal diet [51].

The epigenetic landscape appears to be highly dynamic throughout an individual's life, is strongly shaped by its environment and diet. Most of an individual's genome is not imprinted and is rich in a diversity of epigenetic marks that are definitely not static. Monozygotic twin studies show that epigenetic profiles change a lot in time, being almost identical in early childhood, but strikingly different by the age of 50 [52, 14].

The alteration of the culture environment for *in vitro* grown cells has significant effects on the global epigenetic landscape [53]. This highlights the possible implications for Assisted Reproductive Technologies that require an *in vitro* step [14]. These changes influence not just disease susceptibility but also regulate the aging process.

3.2. Cellular Age. An important process that is correlated with several epigenetic modifications is cellular aging and senescence. Several studies have shown that DNA methylation profiles can be used to determine the exact age of specific tissues inside an organism. Such estimations have been named "epigenetic clocks" and two such examples are Horvath's clock [54], which can be applied to any human tissue and Hannum's clock [55], which can be used for blood samples.

3.3. Health Implications – Epigenetic Factors in Human Disease. Considering the multitude of important biological processes in which epigenetic mechanisms are implicated, it is pretty obvious that any disruption or deviation of the normal way in which these mechanisms naturally unfold can lead to a variety of serious consequences.

3.4. Cancer. One of the most studied diseases that have a strong epigenetic factor is cancer. Cancer is both a genetic and epigenetic disease, associated with the disruption of normal DNA methylation and histone modification patterns, faulty expression of miRNAs, and with the aberrant dysregulation of various epigenetic machinery proteins [17].

Both hypomethylation and hypermethylation can be implicated in the onset of cancer [56]. Hypomethylation of DNA leads to a highly active state in which many genes can become overexpressed, including the genes implicated in proliferation, cell cycle regulation and DNA repair. On the other hand, hypermethylation turns genes in an inactive state, but sometimes tumor suppressor genes can also be inactivated through hypermethylation, which can result in a state of high sensitivity that can easily lead to a neoplasic evolution [57, 58].

Histone modifications are also often responsible for the malignant transformation of cells. For example, carcinogenesis has been frequently associated with the histone methyltransferase loss of function. Changes in the functions of methyltransferases are thought to contribute to heritable changes that could lead to sporadic cancer [59].

3.5. Autoimmune Diseases. As mentioned earlier, epigenetic mechanisms are responsible for the proper unfolding and maintenance of many vital biological processes, including both innate and adaptive immune responses. The regulation of such processes takes place both at the transcriptional and posttranslational level. It has been shown that epigenetic regulation underlies many immunological mechanisms including T cell differentiation, cytokine production, antigen receptor arrangement, inflammation and normal immune response regulation [60]. Additionally, faulty epigenetic mechanisms have been widely associated with inflammatory and autoimmune diseases. Specific epigenetic alterations have been associated with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), ankylosing spondylitis (AS), Sjo¨gren's syndrome (SjS), inflammatory bowel disease (IBD), psoriasis, type 1 diabetes (T1D), and primary biliary cirrhosis (PBC) [61].

For instance, in SLE and other autoimmune disorders, the presence of multiple chromatin components such as histones and DNA has been observed in the bloodstream, which leads to the idea that these, naturally intracellular components, can act as antigen triggers for the onset of autoimmune disease. The presence of these components in the peripheral circulation has been explained by defective apoptosis or faulty clearance of cell debris and apoptotic material [60]. Furthermore, it has been observed that lymphocytes of SLE patients present globally hypomethylated DNA compared to normal patients. This aspect led to a theory which claims that the hypomethylated DNA might mimic microbial DNA in the serum of the patients, which could then lead to the production of anti-DNA antibodies [60, 62]. Expression of methylation-related genes, downregulation of DNMT1 and MBD4, and overexpression of MBD2 and MeCP2 in CD4+ T cells have also been documented in SLE [63].

3.6. Neural Development and Cognitive Health. Epigenetic regulation plays an important role in the development and plasticity of neural structures in humans and other animals, both in early development and in later life. Research shows that many neuronal pathways involved in memory formation and CNS plasticity target epigenetic modifications, such as histone modifications and DNA methylation [30]. Moreover, many aberrant epigenetic mechanisms have been identified in diseases and affections of the nervous system, including neurodevelopmental disorders, such as Rett Syndrome; neurodegenerative diseases, most importantly Alzheimer's Disease and Huntington's Disease; psychiatric disorders, a notable example being depression, which has an epigenetic component sustained by a significant body of research, and psychotic disorders, the best example being the most common form of psychosis, schizophrenia [64].

3.7. Learning and Memory. The hypothesis that certain histone posttranslational modifications play a role in long-term memory formation has been supported by a vast amount of evidence. Some studies identified the implication of H3 acetylation and phosphorylation in long term memory formation, like the acetylation of histones associated to promotor I of BDNF (the brain-derived neurotrophic factor gene) which was detected following NMDA treatment of hippocampal neurons. Furthermore, it was shown that the activity of histone deacetylases (HDACs) such as sodium butyrate and trichostatin A improved long term potentiation and fear memory. These were some of the first studies to suggest the existence of a link between epigenetic alterations and long-term memory formation, and the possibility to alter memory capacity by the manipulation of epigenetic processes [65, 66, 67, 68, 69, 70, 71, 30].

3.8. Epigenetics and Metabolic Disorders. Epigenetic mechanisms have been strongly correlated with several metabolic disorders. These disorders are characterized by the inability of the body to absorb and use different nutrients, an inability associated with defective metabolic pathways of lipids, proteins and/or carbohydrates. These disorders are complex diseases, associated both with a genetic component and an environmental one. Lifestyle and diet can have a great influence on the onset of these disorders, but it turns out that the effects extend to several generations where they manifest as a predisposition observed in individuals with affected parents. Very important examples include Metabolic Syndrome and Type 2 Diabetes. Metabolic Syndrome describes a complex of metabolic disorders including inflammation, obesity, dysfunctional glucose metabolism, insulin resistance, hypertension and dyslipidemia [72].

4. METHODS USED IN THE STUDY OF EPIGENETICS

The study of a complex branch of biology such as epigenetics requires developing specific methods of analysis, in order to decipher the intricate ways in which the mechanisms of epigenetic regulation and inheritance unfold, both in a normal and pathological organism.

The first methods of chromatin studies include chemical analysis and optic microscopy. Such methods fall short in describing the architecture of chromatin, because chemical analysis can only provide information about the components of the chromatin fiber, not the way they are spatially arranged, the interactions they take part in, or the changes they undergo and optic microscopy can only provide a very superficial image of chromatin's high levels of packaging, being able to reveal only structures such as the metaphase chromosomes and nothing of a higher resolution.

The foundations of important chromatin discoveries began in the second half of the 20th century when, through a combination of methods including the use of restriction enzymes, physical methods, such as X-ray diffraction and, finally, thanks to the development of electron microscopy, the structure of the nucleosome was described by several authors through independent work [6, 7, 8]. A great and fast advancement followed that eventually gave birth to the new field of epigenetic studies. In the past few years, development of high-throughput genomic analysis technologies and bioinformatics tools significantly contributed to the growth of the field. The use of microscopy in Chromatin analysis requires other methods of identification that can allow the visualization and differentiation of the analyzed structures by marking them.

4.1. Immunohistochemistry and Immunocytochemistry. In the category of immunological methods, most notable are the two methods known as Immunohistochemistry and Immunocytochemistry. The two methods are widely used to detect and localize antigens in the desired tissue or cell samples, and can easily be adapted for the extensive analysis of chromatin components by coupling with high-resolution microscopy analysis [73].

The specificity of the method varies depending on the used antibody and on the number of reactions. An increased specificity is achieved by the use of what is known as an indirect reaction rather than a direct one. The direct reaction consists of using a single type of antibody, which is specific to the antigen-target and is labeled to allow the direct visualization of the complex. This type of reaction is limited when it comes to antigens that are found in small numbers, or that are hardly reached by the antibody. For this reason, the indirect reaction is preferred for increased specificity and accuracy. The indirect reaction includes a series of subsequent steps, each including a different immunological antibody-antigen reaction. The first reaction is between the primary antibody and the antigen that is detected, the subsequent reactions include using marked antibodies specific to the last added antibody. Marking or labeling the antibodies allows for microscopic analysis and detection of the immunological reactions, signaling the presence of the antigen in that region of the sample. Most commonly, fluorescent or enzymatic labeling is used. The method combined with fluorescent labeling is commonly known as immunofluorescence and requires the use of a fluorescence microscope. Immunohistochemistry is the method used to detect antigens in slices of entire tissues meanwhile Immunocytochemistry samples are cells that were either isolated from their surroundings by extraction or have been grown in a lab culture [74].

4.2. Chromatin Immunoprecipitation (ChIP). A very common method used to study chromatin is the Chromatin Immunoprecipitation Assay (ChIP). The method is also based on the use of antibodies, but in this case, the antibody is used to isolate DNA fragments bound to a target antigen and detecting possible DNA-protein interactions. Commonly targeted proteins include histones, histone variants and transcription factors. Chromatin immunoprecipitation was developed and first used by Varshavsky et al in 1988 when they demonstrated the attachment of histone H4 to a *Drosophila melanogaster* gene called HsP70 (Heat shock Protein 70) [75].

The ChIP protocol has a few common steps in two major variations, known as x-ChIP and n-ChIP (the conventional method and, respectively, the native method). The two major variations differ only in the first step of the protocol which, in the conventional method, requires the use of a cross-linking agent, such as formaldehyde, in order to keep the fixed interactions from breaking apart; the native variation lacks this step and relies solely on the strength of the natural bonds as they genuinely occur in the chromatin fiber. As expected, the native method is not suited for analyzing the interactions of the proteins that occur in very small amounts or those that interact weakly with DNA. However, despite the fact that the conventional method overcomes this error, it comes with disadvantages of its own. The biggest disadvantage is the false positives that result from crosslinking errors between neighboring regions. In order to minimize the errors, the protocol has to be optimized for each experiment.

The next steps are common and include: Fragmentation of the chromatin fiber through sonication or by using Micrococcal nuclease; Antibody enrichment and precipitation of the desired fragments that will be selected by the specific antibody (which is bound to beads/ magnets/a specific surface); and finally reverse cross-linking and DNA elution (fig.6). The eluted DNA can then be analyzed in a variety of ways, all of which resulted in different variations of the ChIP protocol (Figure 2)[76].

4.3. MeDIP. Although ChIP is mostly used to detect chromatin changes that include proteins, the method has been optimized to encompass a wider range of modifications. One such modification is DNA methylation which can be detected by using a ChIP variation called MeDIP. The method is based on a protocol which uses methyl-cytosine-specific antibodies in order to selectively precipitate methylated chromatin fragments and to subsequently analyze them using a variety of methods such as microarray or sequencing (MeDIP-chip, MeDIP-seq). The method can be utilized to detect any methylation profile from those implicated in ontogeny or cancer, to those found in normal or pathological neural tissues [76, 77, 78].

4.4. ChIP-PCR. The first method of ChIP DNA analysis was ChIP-PCR, which is based on the amplification of a hypothetical binding site sequence upon co-precipitating with the binding protein, by using only primers specific to that proposed sequence. The sequence is confirmed as a binding site if the reaction yields proper amplification products. This method is highly limited as it only provides information about a single known DNA sequence that is thought to be a binding site for the analyzed protein. In order to overcome this limitation, a different DNA analysis protocol was coupled with ChIP, giving rise to a new method known as ChIP-on-chip or ChIP-chip [76, 79].

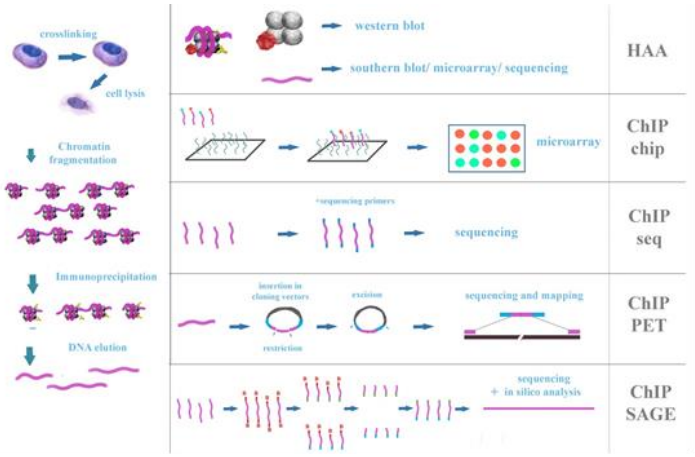

Figure 2. Variations of the Chromatin Immunoprecipitation protocol.

4.5. ChIP-chip. ChIP-chip is simply a combination of ChIP with the microarray method. This combination extends the DNA fragment examination to a genome-wide level. Both precipitated and non-precipitated DNA are fluorescently labeled in order to be easily differentiated. The DNA samples are then added to the microarray chip and allowed to hybridize with the complementary probes on its base. The oligos on the chip are an array of genomic sequences that could be possible binding sites for the protein. They are usually represented by promotor regions or CpG islands. Based on the differential labeling, it can be determined whether any of the sequences present on the chip are binding sites or not. The results are interpreted *in silico* through a detailed analysis of the intensity of each label, a process designed to yield a most accurate interpretation [79, 80]. This method was the first one to allow for an extensive mapping and examination of genome-wide protein-DNA interaction sites. Since its development in 2000 [81], many proteins have been analyzed using this method. Examples include p53, c-myc, Oct4, Nanog, Sox2 [76].

All in all, several difficulties and limitations were faced with this method. Firstly, the genome-wide character of the analysis is directly linked to the amount and type of oligos on the chip. Repetitive regions and other attachment sites that were not included in the array will not be detected as attachment sequences.

4.6. ChIP-DSL. Another limiting factor is represented by the need to have a large number of starting material in order to get optimal results. Proteins such as histones, which occur quite frequently, will yield large amounts of precipitated DNA that can be easily viewed after hybridization on the chip, but other proteins that have very few attachment sites can barely be detected [79]. For this reason, the precipitated DNA is often amplified through PCR, but this approach brings about another difficulty which is the preferential amplification of certain samples. The difficulties were overcome by developing a method called Chip-DSL, which is based on the combination of ChIP, DNA selection and ligation and the microarray method, and allow for a more specific analysis of the immunoprecipitated samples, without the need to analyze a large number of cells [82].

4.7. ChIP – sequencing. Several ChIP methods coupled with sequencing have been developed, increasing the potential of ChIP protocols in genome-wide analysis. The major addition of these protocols is the fact that they don't require the prior knowledge of the DNA sequences. Several techniques are part of this category: ChIP-seq, ChIP-SAGE, ChIP-PET [79].

4.8. ChIP-PET. In order to map the transcription-factor binding sites of p53 in the human genome, the method ChIP-PET was introduced [83]. This method was developed by the coupling of the ChIP protocol with the Paired End Tags method, resulting in an approach based on the isolation of small DNA marginal fragments that allow, through sequencing and alignment, the identification and subsequent mapping of protein binding sites throughout the entire genome. For each binding site sequence, two short marginal fragments of the immunoprecipitated DNA are obtained through insertion in cloning vectors, cleaving with restriction enzymes and ligation. The two fragments are short enough to increase specificity and decrease cost and labor, but long enough to be specific only to the sequence of origin. Genome libraries are then obtained which are used to clone and sequence the fragments for later analysis. Mapping and alignment are run *in silico* with the use of specific software [76, 79, 83].

4.9. ChIP-SAGE. Serial Analysis of Gene Expression is an approach generally used to examine the differential expression of genes contained in a genome at a certain point in time, by the isolation and analysis of mRNA. The approach is similar to PET in the fact that it uses only fragments of the RNA instead of the entire molecule. In the end, a concatenated sequence of all the obtained fragments, spaced by adaptors, is obtained by restriction and ligation, then, the whole thing is sequenced. The frequency of each fragment is then determined *in silico*, along with the corresponding RNA for each fragment, and then the relative expression of each gene is deduced [84].

The approach was adapted to be used in combination with Chromatin Immunoprecipitation, in which case the analyzed fragments are represented by the immunoprecipitated DNA. After sequencing the concatemer, the fragments are mapped and their origin is determined. This allows for the detection of protein-binding sites all over the genome [85, 79].

4.10. ChIP-seq. ChIP-seq is an important method of chromatin analysis. The protocol is pretty straight-forward: DNA fragments are obtained through Chromatin Immunoprecipitation and adapters are added to their ends. The DNA is then amplified and sequenced with high-throughput methods. Millions of accurate reads can be obtained in just a few days. For this reason, ChIP-seq has a few advantages over other ChIP-based methods of chromatin analysis: Prior knowledge of the analyzed sequences is not needed, it yields fast, highly accurate results compared to other methods and it's not as labor-intensive [76, 79].

However, all the described ChIP methods, including ChIP-seq have some common difficulties. DNA contamination and DNA fragmentation heterogeneity can produce false positives and imprecision in mapping and, in consequence, stringent filtering of data can produce false negative results. To overcome these limitations, the method ChIP-exo was developed [86].

4.11. Histone Association Assay (HAA). The histone Association Assay is another variation of ChIP which allows the detection of chromatin binding proteins *in vivo.* The protocol typically uses antibodies that are specific to the H3 histone, and thus allow for the Immunoprecipitation of the entire nucleosomal particle. Prior to the immunoprecipitation, the chromatin is both cross-linked and fragmented, which causes the chromatin-binding proteins to stay attached to the nucleosomes and co-precipitate along with them, regardless of the innate solubility of those proteins. In this way, DNA can later be separated from the proteins through reverse cross-linking and elution, after which both parts can be analyzed. DNA can be analyzed through a variety of ways including southern blotting, microarray, or sequencing meanwhile the proteins are typically examined by SDS-PAGE and western blot.

This technique provides a complete picture that includes both histone and non-histone proteins interacting with a given DNA sequence in the chromatin fiber, which can be very useful in studying several molecular and cellular processes in which these molecules are involved [87].

4.12. ChIP-chop. Another variation which is not as commonly used is the ChIP-chop method. This method was developed in order to detect proteins that specifically bind to repetitive regions, and by later determining the methylation status of the binding region, differentiating the repetitive sequences that are active from the ones that are inactive. The methylation is detected by using methylation-specific Restriction Enzymes on the precipitated DNA fragments [88].

4.13. RIP-PCR. In addition to the identification and mapping of protein binding sites throughout the genome, the ChIP Assay can be used to identify RNA-Protein interactions, interactions that include non-coding RNA which plays an important role in nuclear RNA-directed epigenetic control [79]. By combining RNA-binding protein immunoprecipitation with PCR, a new method was developed for the extensive analysis of RNA-protein interactions, a method called RIP-PCR that can be used to analyze any kind of RNA interacting with proteins, such as mRNA [89], or non-coding RNA [90, 91, 92].

4.14. Chromatin Accessibility. One of the basic principles that chromatin architecture studies rely on is the Chromatin Accessibility Regions. Based on this principle, any sensitive or accessible regions along the chromatin fiber are regions of "naked" DNA, lacking any associated proteins, making them possible regulatory sequences. Many approaches were developed based on this idea, resulting in methods targeting open chromatin in different ways, most commonly through the use of restriction enzymes or DNase.

Such methods include the DNase-seq(chip) and FAIRE-seq. These methods have as a goal the identification and sequencing of regulatory DNA elements by detecting sites that present a higher sensitivity to the DNase enzyme [93, 94, 95, 96, 97]. Similar methods include older approaches like using the micrococcal nuclease: MNase-seq and MACC (MNase Accessibility)[98].

4.15. ATAC-seq. Other methods target open chromatin in different ways. This is the case for the method called ATAC-seq which is based on the use of a modified, hyperactive transposase which introduces adapters in regions of high-accessibility, allowing for the subsequent identification and mapping of those regions by sequencing [99].

4.16. DNA-footprinting. DNA footprinting is a method meant to determine the attachment regions of certain proteins. The protocol starts with the amplification and labeling of DNA fragments by PCR and then separating the amplicons into two samples. One sample will be mixed with the tested DNA-binding protein; the other sample will be kept for later comparison. After this step is complete, a cleaving agent is added to both samples, so the DNA is randomly cut. This step is controlled, so every DNA fragment is only cleaved once. By running the samples in an electrophoresis polyacrylamide gel, the fragments become aligned in a ladder-like formation, based on size. Assuming the tested protein got attached to its target sequence, that sequence will be protected against the action of the cleaving agent, which will result in a missing spot in the electrophoresis gel ladder, corresponding to the nucleotides that are part of the binding site that was protected. The missing spot in the ladder is called a footprint [100].

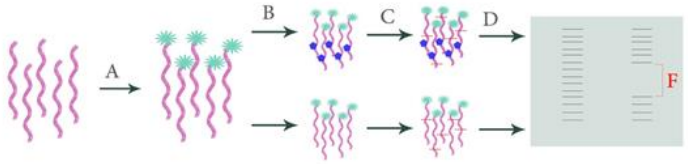

Figure 3. DNA footprinting steps: A. PCR amplification and labeling of binding site-containing DNA fragments. B. Separating into two samples and adding the protein of interest to one of the samples. C. Random fragmentation of both samples. D. Running in gel and identifying the missing spot from the protein-enriched sample. (F = footprint).

4.17. Chromosome conformation capture methods. The Chromosome Conformation Capture methods are a great way to analyze chromatin architecture but also to detect and quantify the interactions between regulatory elements. These methods allow for the detection of DNA interactions, such as those that occur between in-cis regulatory sequences. Such sequences are regions that can be in close proximity spatially but quite distant on the DNA molecule itself.

Like the ChIP methods, all 3C methods are based on the same steps, but the DNA is generally analyzed in different ways. The common steps start with the cross-linking of chromatin, followed by the fragmentation, either by sonication or under the action of restriction enzymes, the resulted fragments are then ligated at one end, based on the principle of proximity ligation, which results in a linear molecule containing both loci (fig.8). From this point, the obtained DNA fragment can be analyzed in different ways, depending on the 3C method used [101, 102,103,104].

3C – In this approach, primers are used to amplify both loci in a fragment to confirm possible interactions between them. This

allows for the identification of only one interaction in the entire genome.

4C – This method extends the analysis by using inverse PCR to detect all the existent interaction sites for a single known sequence. The use of circularization and inverse PCR requires one single primer specific to the tested sequence [105].

5C – In this protocol universal primers are attached to all the obtained fragments, then all of them are amplified and analyzed for identification and mapping [106].

4.18. Hi-C – This method identifies all possible interactions by directly adding adaptors to the fragments and sequencing them all by ultra-high-throughput sequencing [107].

Some new methods have been recently developed with the purpose of overcoming certain limits encountered both with 3C and ChIP. The resulting methods proved to be quite promising, allowing both the identification of interacting loci throughout the genome, as well as the detection of proteins that regulate these interactions.

4.19. ChIP-LOOP – A method resulting from the combination of the chip protocol and the 3C (one vs. one) protocol. Ligated, cross-linked chromatin fragments are immunoprecipitated and then PCR amplified with two specific primers [102].

4.20. ChIA-PET – In this method, after fragmentation, the fragments are ligated and labeled with special adapters between them which are important for later sequencing and *in silico* analysis of short DNA ditags that are used to map and identify the loci, in a way similar to the ChIP-PET protocol [108].

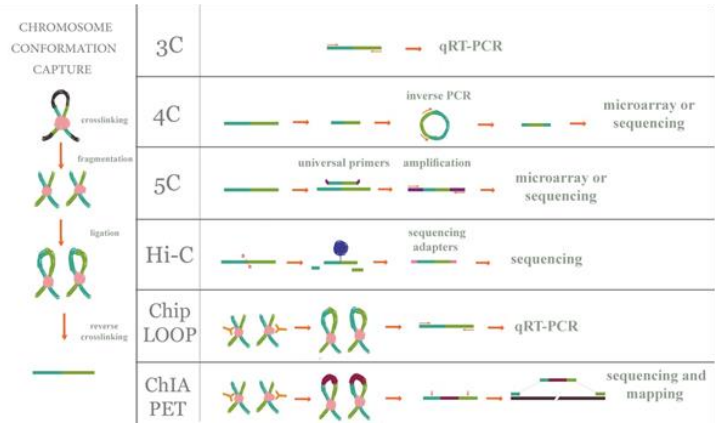

Figure 4. Chromosome conformation capture variations: Common and particular steps.

4.21. Bisulfite sequencing. One very useful way to extensively examine DNA methylation is a method developed by Fromer et al., which is based on treating the DNA with sodium bisulfite. By doing so, the bisulfite converts all cytosine bases present in the DNA into uracil. After amplification, the uracil will be replaced by thymine. This conversion effect seems to be prevented by the presence of a methyl group on the 5 carbon of cytosine. Therefore, after bisulfite treatment, all sequences in the DNA containing cytosine will be probable methylation sites. Those can be later identified by using a variety of methods [109].

4.22. Pyrosequencing. A very commonly used method to analyze bisulfite-treated DNA is pyrosequencing, but other sequencing methods such as Illumina are used as well. Sequencing is a very accurate way to determine the methylation level of DNA by detecting the presence of cytosine in the sample [110, 111, 112, 113].

4.23. Methylation Specific PCR (MSP). Another less laborious method was developed by coupling PCR with bisulfite sequencing and is commonly known as Methylation-Specific PCR or MSP. This method is based on a protocol that uses methylation specific primers, compatible only with unconverted CpG sequences. In this way, only methylated CpG islands will yield any products upon amplification, which can then be sequenced to determine the exact genomic region [110, 114, 115].

4.24. Combined Bisulfite Restriction Analysis (COBRA). As described earlier, restriction enzymes are a useful tool in epigenetic studies. This proves to be true with bisulfite treatment as well, as this method has been coupled with restriction enzymes to develop a new method used in methylation analysis called COBRA or Combined Bisulphite Restriction Analysis. As the name suggests, COBRA combines sodium bisulfite treatment, followed by PCR, with the use of CpG specific restriction enzymes. After bisulfite conversion, the enzymes will only cleave the methylated CpG sequences, leaving the unmethylated sequences intact. The samples are then analyzed through gel electrophoresis [116].

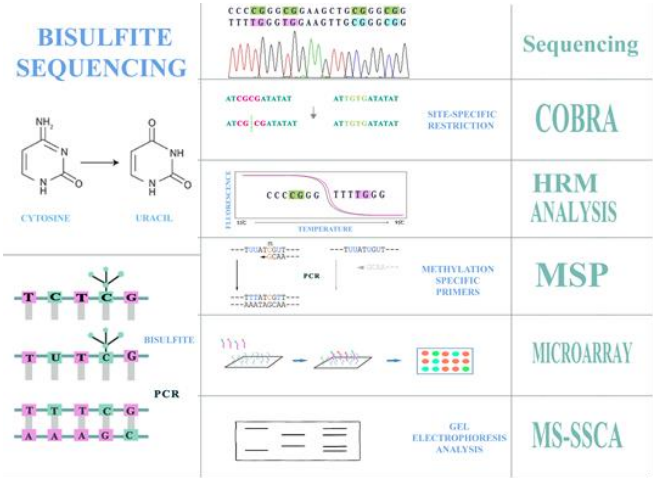

Figure 5. The various methods of analyzing bisulfite treated DNA.

4.25. HRM analysis (High resolution melt analysis**).** This technique is used to distinguish between the sequences of two double-stranded DNA molecules, based on the speed at which the two strands separate while being gradually heated up past their melting point. With the use of an intercalating dye, the process can be detected in real-time by a special camera, (as the double strands break apart, the dye is no longer bound to them and its fluorescence drops significantly). The data can be analyzed by a computer which will translate the rate of fluorescence intensity decrease into a curve. The curve is plotted on a graph by the computer. Each DNA double-stranded sequence will have a specific melting curve. Thanks to the high-resolution capacity of the camera, differences of up to one nucleotide can be detected.

The method is normally used to detect polymorphisms, mutations and differences in double-stranded DNA samples. In this case, the method can be coupled with bisulfite treatment in order to differentiate between two molecules with the same sequence but with a different methylation profile (such as sequences originating in cells from different tissues of the same

organism). It can also be used to identify the methylation sites, by comparing a bisulfite treated double-stranded DNA to an untreated one [117, 118, 119].

4.26. Methylation Specific Single Strand Conformation Analysis (MS-SSCA). Another way to analyze sequence differences following bisulfite conversion is by using electrophoresis. A method called Methylation-Specific Single-Strand Conformation Analysis or MS-SSCA is a variation of the SSCA which detects sequence differences between two single-stranded DNA molecules by running the two in an electrophoresis gel. Due to conformation differences induced under certain specific lab conditions, but which are dependent on the nucleotide sequence, the two strands will migrate differently in the gel. Based on the protocol and the type of gel used, the resolution can vary, but can be improved up to single-nucleotide difference identification [120, 121].

4.27. Microarray. Microarray is another useful method to distinguish between methylated and unmethylated sequences. This is achieved by treating the samples with bisulfite prior to hybridization with the oligos. The probes are an array of sequences complementary to both converted and normal CpG sequences. Samples are differentially labeled before hybridization and, finally, data analysis takes place *in Silico* [122].

4.28. Bisulfite sequencing limitations. Although this method is quite useful for genome-wide investigations of DNA methylation, it proved to have a few limitations and difficulties as well. The most significant limitation of the method is the inability to distinguish between the 5-methylcytosines and 5-hydroxymethylcytosines (5hmC), the latter being a recently identified epigenetic mark in mammals [123]. The two modifications seem to both be read as C after bisulfite treatment. Some solutions have been developed to overcome this limitation, one of which implicates running an additional step prior to the bisulfite treatment which converts 5hmC into uracil, making sure that the only detected cytosines will be the methylated ones. The additional step is a specific oxidation of 5hmC into formylcytosine, which is converted by the bisulfite treatment. The method is known as the oxidative bisulfite sequencing [124].

4.29. hmeDIP. Another solution is a method which is another variation of ChIP: Similar to meDIP, hmeDIP uses hydroxymethylcytosine-specific antibodies to precipitate and detect hydroxymethylated DNA sequences. Purified and sequenced DNA is then positive for hydroxymethylation. In this way, the 5mC and 5hmC can be distinguished experimentally [123].

4.30. DamID. This method has also been developed for the purpose of detecting protein attachment sites throughout the genome, but the protocol is quite unique [125]. The method is not based on immunological principles or restriction enzymes, nor does it require prior treatment with cross-linking agents, instead, it relies on the use of a bacterial methyltransferase, isolated from *Escherichia coli*. In bacteria, unlike in eukaryotes (with a few exceptions), methylation occurs at the adenine base, not the cytosine. Based on this principle, the methyltransferase is conjugated to the examined DNA binding protein, making sure the attachment region is a structural protein domain and does not affect the functionality of either protein, and the obtained complex is added to the DNA. Later, through different approaches, the methylated adenines can be detected (MedDIP, restriction enzymes), and the region containing the methylated adenines will be considered a possible attachment site, because, upon attachment to the DNA, the DNA binding protein will bring the methyltransferase in the proximity of the attachment region, which the enzyme will proceed to methylate.

This method is quite specific and yields good results, can be used *in vivo* and will be able to detect attachment sites even in weak DNA-protein interactions; it can be used both in living organisms or cell cultures [125].

5. CONCLUSIONS

Epigenetics is still a young field which, despite having many informational gaps, proves to be quite useful, promising and, undoubtedly, will majorly contribute to the advancement of medicine and human health by accelerating and improving the development of new personalized therapies and overcoming several barriers encountered in the past.

Research technologies in this field are diverse and in a dynamic and continuous growth, and the enthusiasm among scientist is increasing interest in epigenetic research, which will most likely lead to a fast growth in the following years, a growth supported by the advancement of computational technologies as well, which allow for the accurate and statistical analysis of great amounts of accumulated data in a short amount of time. The high-throughput sequencing technologies and the sophisticated algorithms designed to analyze a large amount of data will provide a chance to discover the epigenetic marks associated with specific disease and will help to diagnose patients.

The advancement of epigenetics will be a stepping stone towards perfecting cloning technologies, better-targeted treatments for complex disease, Assisted Reproductive Technologies and metabolic or endocrine disease therapies, which is why it is crucial to understand the implications and importance of a deep understanding of the molecular basis of epigenetic mechanisms.

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