

A Primer on Epigenetic Changes: The More We Know, the More We Find in Fetuses and Infants

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ABSTRACT

Epigenetics is the study of heritable traits that happen without changes to the DNA sequence. The Greek prefix *epi-* implies features that modify the traditional genetic mechanisms of inheritance. Increasing information underscores the importance of epigenetic changes during the fetal period and infancy. The most frequently seen epigenetic changes are mediated via DNA methylation, changes in gene expression due to non-coding RNAs, and post-translational modifications of histone proteins. DNA methylation can be confirmed using methods such as bisulfite treatment, enzyme sensitivity assays, and antibody specificity-based techniques. Histone modifications are typically detected through antibody recognition. Chromatin immunoprecipitation (ChIP) is an antibody-based technology to selectively enrich specific DNA-binding proteins along with their DNA targets. Since epigenetic alterations are often reversible, modifying epigenetic marks contributing to disease development may provide an approach to designing new therapies. Gene hypermethylation and histone hypoacetylation are attractive targets for the treatment of epigenetic diseases because these epigenetic alterations are reversible. The first 1000 days of life, from conception through infancy, comprise the most-likely time-period for environmental exposures and nutrition to exert beneficial/potentially harmful epigenetic effects. During this period, a typical metabolic reprogramming induced by extrinsic factors such as allergens, viruses, pollutants, diet, or microbiome might drive cellular metabolic dysfunctions and defective immune responses in allergic diseases. Epigenetics also plays a role in the developmental origins of adult metabolic diseases.

Keywords: DNA methylation, Epigenetics, Genomic Imprinting, Histones, Infant, miRNA, Neonate, Newborn, RNA silencing.

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KEYPOINTS

- Epigenetics is the study of heritable traits that happen without changes to the DNA sequence. Here, the Greek prefix *epi-* implies features that modify the traditional genetic mechanisms of inheritance. Increasing information underscores the importance of epigenetic changes during the fetal period and infancy.
- The most-frequently seen epigenetic changes are mediated via DNA methylation, changes in gene expression due to non-coding RNAs, and post-translational modifications of histone proteins.
- The histone tails on the nucleosome surface can undergo several enzyme-catalyzed post-translational modifications. These altered histones contain specialized structural folds in the *N*-terminal tail domain, which changes the interaction with DNA-binding proteins such as transcription factors and other binding proteins.
- Other than methylation, histones can undergo various other covalent modifications such as acetylation, ubiquitination, the addition of small ubiquitin-like modifiers (SUMO; SUMOylation), glycosylation, hydroxylation, phosphorylation, sulfation, acetylation, citrullination, crotonylation, malonylation, and ADP-ribosylation.
- The first 1000 days beginning from conception comprise the most-likely time-period for environmental exposures and nutrition to drive cellular metabolic dysfunction and defective immune responses in allergic disease. Epigenetics also play a role in the developmental origins of adult metabolic diseases.

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INTRODUCTION

In the 1940s, Conrad Waddington coined the word epigenetics, referring to indefinite genetic principles.^{1–4} He described it as the causal interactions between genes and their products, thereby modulating the expression of a genotype into a particular phenotype. Nowadays, it also includes non-genetic heritable (mitotically and/or meiotically) genomic modifications involved in gene expression regulation, but not changes in DNA sequence.^{5,6}

Epigenetic changes carry heritable information obtained during cell division, apart from the actual DNA sequence consisting of chemical tags that alter DNA structure and expression of genes.^{7,8} These are perceived as a link with the immediate environment that enables adaptation to changing environments through extra- and intracellular cues.⁹ Increasing information underscores the importance of epigenetic changes during the fetal period and infancy.

Molecular Mechanisms of Epigenetics

Most epigenetic changes occur more rapidly than genetic mutations, especially in response to environmental changes. *De novo* epigenetic mutations are seen more often than somatic DNA mutations because of the inherent, higher error rates.^{10–12} Therefore, epigenetic biomarkers are mitotically and/or meiotically heritable but reversible, functional, and biologically relevant biochemical modifications of the chromatin carrying the information but not changing the nucleotide sequence of the genome.^{13–17}

Table 1 lists various epigenetic mechanisms that are known to regulate gene expression. The most frequently seen epigenetic changes are mediated include DNA methylation, changes in gene expression due to non-coding RNAs, and post-translational modifications of histone proteins.¹⁸ DNA methylation and histone modifications are the two most frequently evaluated “classical” epigenetic mechanisms.^{19,20} These can alter the accessibility of genes to the transcriptional machinery and consequent regulation of gene expression, cellular hemostasis, and responses to DNA damage.^{21,22} Figure 1 shows the nucleosome solenoid model. In Figure 2, euchromatin and heterochromatin are depicted.

DNA Methylation

These epigenetic changes refer to the addition of methyl groups to DNA; these frequently alter cellular reprogramming, tissue differentiation, and development.^{23,24} Cytosine, and sometimes adenine, bases in DNA can undergo methylation. The 5' methylation of cytosine in CpG dinucleotides shows as the 5-methylcytosine (5mC) bases. The CpG or CG sites are regions in DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' → 3' direction. Around 60%–90% of CpGs are methylated, whereas unmethylated CpG-rich sequences are seen more frequently in the “CpG islands” located in gene promoters. The methyltransferases DNMT3a and DNMT3b carry out cytosine methylation of CpG sites, and the maintenance methyltransferase DNMT1 duplicates pre-existing methylation patterns during DNA replication.

The CpG islands (CGIs) are short-interspersed DNA sequences that are GC-rich, CpG-rich, predominantly non-methylated gene regulatory hubs. These serve as transcription initiation sites and are associated with gene promoters.²⁵ Transcription in more than 50% of human genes is initiated from CpG islands. Silencing of CGI promoters is achieved by CpG methylation or polycomb recruitment.²⁶ The CGI promoters are loaded with polymerases

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Table 1: Mechanisms of epigenetic changes¹²

<i>Epigenetic mechanisms</i>	
DNA methylation	
RNA silencing/Non-coding RNAs	<ul style="list-style-type: none"> • Small interfering RNAs (siRNA) • MicroRNAs (miRNA)
Post-translational modifications (PTMs) of histone proteins	<ul style="list-style-type: none"> • Acetylation • Ubiquitination is the addition of a small ubiquitin-like modifier (SUMO; SUMOylation) • Glycosylation • Hydroxylation • Phosphorylation • Sulfation • Acetylation • Citrullination • Crotonylation • Malonylation • ADP-ribosylation
Prions	
Chromatin remodeling	
Nucleosome positioning	
Sex-specific epigenetic changes	<ul style="list-style-type: none"> • Genomic imprinting • Chromosome inactivation

that create short abortive transcripts even when the associated gene is inactive.²⁷ This protects CGIs from the action of DNA methyltransferases, allowing these “silent” promoters to exclude DNA methylation.

Polycomb-group (PcG) genes encode multimeric chromatin proteins that bind specific histone modifications to prevent gene activation and maintain repressed chromatin domains. These multifaceted proteins were first discovered as epigenetic, global transcriptional repressors of homeotic (Hox) gene expression in *Drosophila* during development and differentiation.²⁸

The DNA methylation of a promoter region can repress transcription by blocking transcriptional activators and through methyl CpG binding proteins (MBPs).²⁹ The MBPs can recognize methylated DNA and recruit co-repressors, such as HDACs, to repress gene expression.²⁹ The enzymes that establish, recognize, and remove DNA methylation can be categorized as writers, erasers, and readers. Writers are the enzymes that catalyze the addition of methyl groups onto cytosine residues and are comprised of Dnmts. Three members of the Dnmt family directly catalyze the addition of methyl groups onto DNA: Dnmt1, Dnmt3a, and Dnmt3b.²³ Erasers modify and remove the methyl group while readers recognize and bind to methyl groups to ultimately influence gene expression. DNA demethylation is characterized as either passive or active. Passive DNA demethylation occurs in dividing cells. Inactivation of Dnmt1 leads to unmethylation of newly incorporated cytosine, reducing the overall methylation level after each cell division, thereby causing erosion. Active DNA demethylation can occur in both dividing and

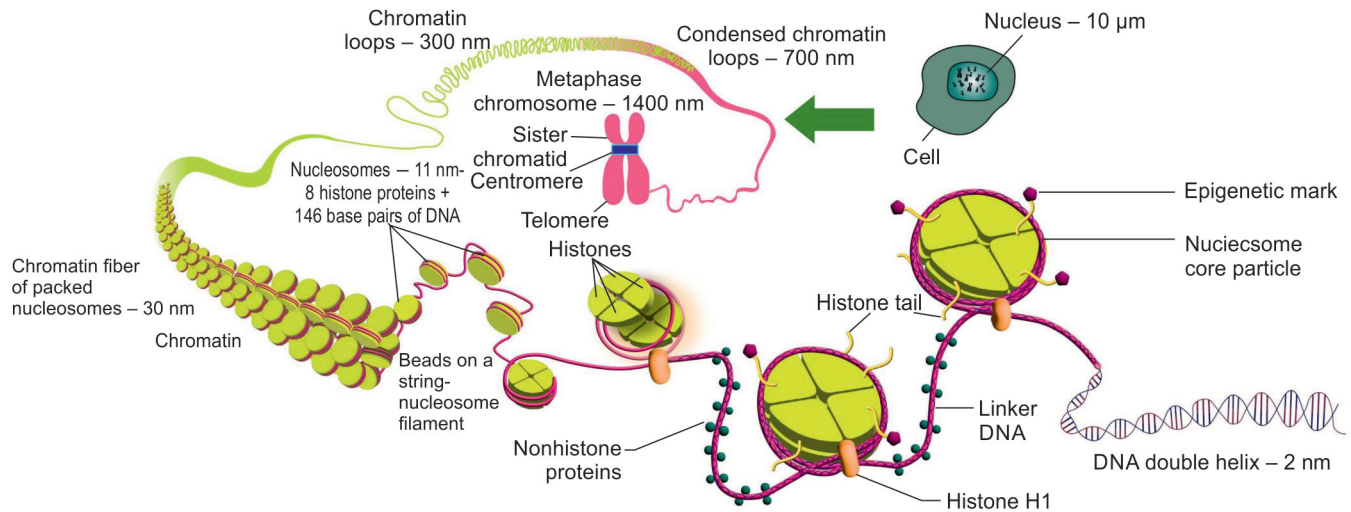


Fig. 1: Nucleosome solenoid (30 nm DNA fiber of chromatin, resulting from helical winding of ≥ 5 nucleosome strands). This model of chromatin organization was proposed by Kornberg and Thomas in 1974

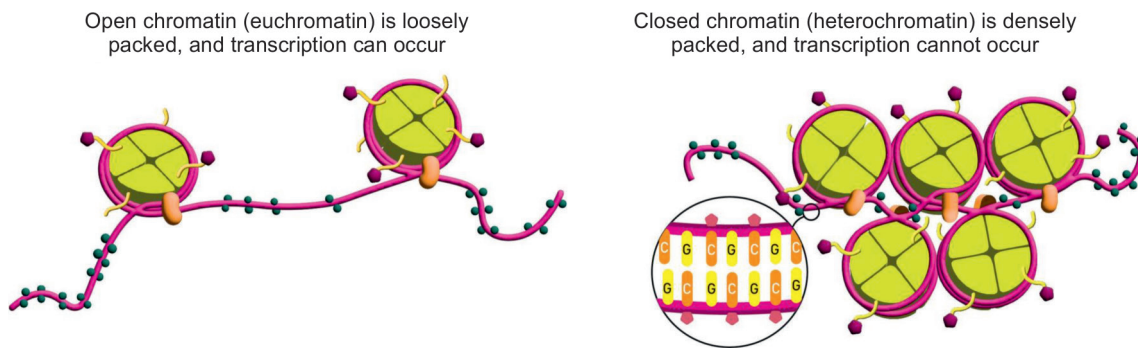


Fig. 2: Differences between euchromatin and heterochromatin

nondi-viding cells but requires enzymatic reactions to process the 5mC in order to revert it back to a naked cytosine. Reading DNA methylation can be done by the MBD, the UHRF, and zinc-finger proteins.²³

Epigenetic Crosstalk

There is a crosstalk between DNA methylation and other epigenetic mechanisms, such as histone modifications and microRNA (miRNA) to regulate transcription.²³ Multiple epigenetic mechanisms interact to activate or silence gene expression and regulate transcription. Methylation is regulated by proteins such as Dnmt, and their catalytic activity is enhanced by their association with histone tails and Dnmt3L. In regions of DNA with activated transcription, Tet removes DNA methylation, and histone tails in this region often contain H3K4me3 that inhibits Dnmt binding to unmethylated CpG sites and maintains a permissive environment for transcription. Methyl binding proteins are the strongest link between DNA methylation and histone modification. Both the MBDs and the UHRF proteins interact with methylated DNA and histones to enhance gene repression.^{30–32}

Non-coding RNAs

The non-coding RNAs (ncRNAs) are RNA transcripts that do not encode proteins like mRNAs but regulate post-transcriptional gene expression in physiological processes such as cellular differentiation

and organ development.³³ These are epigenetic regulators of gene expression through actions on chromatin; and are categorized into (a) housekeeping or infrastructural ncRNAs that regulate cellular functions and are constitutively expressed and (b) regulatory ncRNAs which alter gene expression through complex molecular and cellular processes.³⁴ Infrastructural ncRNAs include ribosomal, transfer, small nuclear, and small nucleolar RNAs. Regulatory ncRNAs can be classified into microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and long non-coding RNAs (lncRNAs).^{33–36} Small ncRNAs such as miRNA, siRNA, and piRNA have less than 200 nucleotides, whereas long ncRNA contains more than 200 nucleotides.³⁷ They can effectuate post-transcriptional silencing of gene expression by RNA processing, chromatin structure, RNA stability, chromosome segregation, transcription, and translation.^{38,39} Only 3–4% of the transcripts in the genome encode for proteins, while the remaining are ncRNAs. During evolution, the spectrum of protein-coding genes has been conserved but the number of non-coding sequences has increased in proportion to the organism complexity.⁴⁰ Table 2 presents a description of the regulatory ncRNAs.

Micro (Mi)-RNAs

Micro-RNAs (Mi-RNAs) are evolutionarily conserved, small single-stranded molecules (20–24 nucleotides) that regulate the expression of around 50% of the genes in a cell at the

Table 2: Regulatory ncRNAs³⁵

Type		Length	Characteristic	Function
miRNA	Micro RNA	20–24	miRNA produced in the nucleus as capped and polyadenylated ssRNA with an imperfectly paired stem-loop structure. Processing by Drosha and Dicer lead to the production of mature dsRNA with exact ends. The effector phase occurs primarily in the cytoplasm mediated by Ago proteins.	Perfect complementarity – Ago2-mediated cleavage of mRNA. Non-perfect complementarity – Suppression of translation or mRNA degradation (deadenylation, decapping, and exonucleolytic degradation). Transcriptional silencing and translational activation. Minor functions in transcriptional silencing and translational activation.
piRNA	PIWI-interacting RNA	24–31	Precursor ssRNA, which is modified to contain 3'-terminal 2'-O-methyl Strong preference for uridine at the 5' end.	Silencing of transposable elements in the germline.
siRNA	Small interfering RNA	20–24	Canonical siRNAs form long, linear, perfectly base-paired dsRNA. Processed by dicer into mature siRNA with heterogeneous end-composition. Effector functions occur primarily in the cytoplasm supported by Ago proteins.	Perfect match with endonucleolytic cleavage. Non-perfect match or endonuclease-inactive RISC: Translational repression or exonucleolytic degradation Induction of heterochromatin formation Silencing of the same locus from which they are derived.
lncRNA	Long non-coding RNA	>200	Precursor of ssRNAs Many lncRNAs are subject to splicing, polyadenylation, and other post-transcriptional modifications.	Chromatin remodeling. Transcriptional regulation. Post-transcriptional regulation (splicing, TF localization). Precursors for siRNAs. Component of nuclear organelles (paraspeckles, nuclear speckles).
eRNA	Enhancer RNA	100–9000	ssRNA produced bidirectionally from enhancer regions enriched for H3K4me1, Pol II, and co-activators such as p300 Short half-life Evolutionarily conserved sequences Dynamically regulated upon signaling Expression correlates positively with nearby mRNA expression.	Mostly unknown but plays a role in transcriptional gene activation.
PAS (PASR, TSSa-RNA, tiRNA, PROMPT)	Promoter-associated RNA	16–200	Weakly expressed ssRNAs short-half life Bidirectional expression reflecting Pol II distribution.	Transcriptional regulation (interaction with Polycomb group of proteins).

PASR, promoter-associated small RNA; PROMTs, promoter upstream transcript; tiRNA, transcription initiation RNA; TSSa-RNA, transcription start site-associated RNA

post-transcriptional level.⁴¹ These regulate immune functions and inflammatory responses. miRNAs are derived from transcripts with distinctive hairpin structures. Processing of the hairpin into the mature miRNA by Drosha and Dicer allows interaction with argonaute (Ago) proteins to form an RNA-induced silencing complex (RISC).^{42–45} Strand selection for RISC depends on thermodynamic stability, with the 5' terminus favored at the less stable end of the duplex.⁴¹ The miRNAs then pair with mRNAs, most favorably to the 3' untranslated region (UTR), to guide their translational repression or deadenylation and degradation. Dysregulation of miRNA expression and consequent epigenetic disruption have been correlated with altered development.⁴⁶

Small Interfering RNA

Small interfering RNA (siRNA) is a linear, perfectly base-paired dsRNA, which is processed by Dicer into 20–24 nucleotide siRNAs

that direct silencing when loaded onto RISC. The siRNAs mediate post-transcriptional silencing through RNA interference (RNAi) processes; but in contrast to miRNAs, guide strand recognition is indistinguishable.^{35,38} In addition to post-transcriptional gene silencing (PTGS), siRNAs have also been found to direct sequence-specific transcriptional gene silencing by increasing epigenetic marks characteristic of heterochromatin.^{38,47}

PiRNAs

Piwi (*P*-element *Induced W*lmpy testis in *Drosophila*)-interacting RNA (piRNA) is the largest class of small non-coding RNA molecules expressed in animal cells. These are usually 21–35 nucleotides long, small regulatory ncRNAs. During germline development, these bind PIWI proteins to form piRNA-induced silencing complexes.^{48,49} The PiRNAs can interact with mRNA with enhanced silencing of gene transcription through RISCs-mediated mRNA degradation.^{35,50}

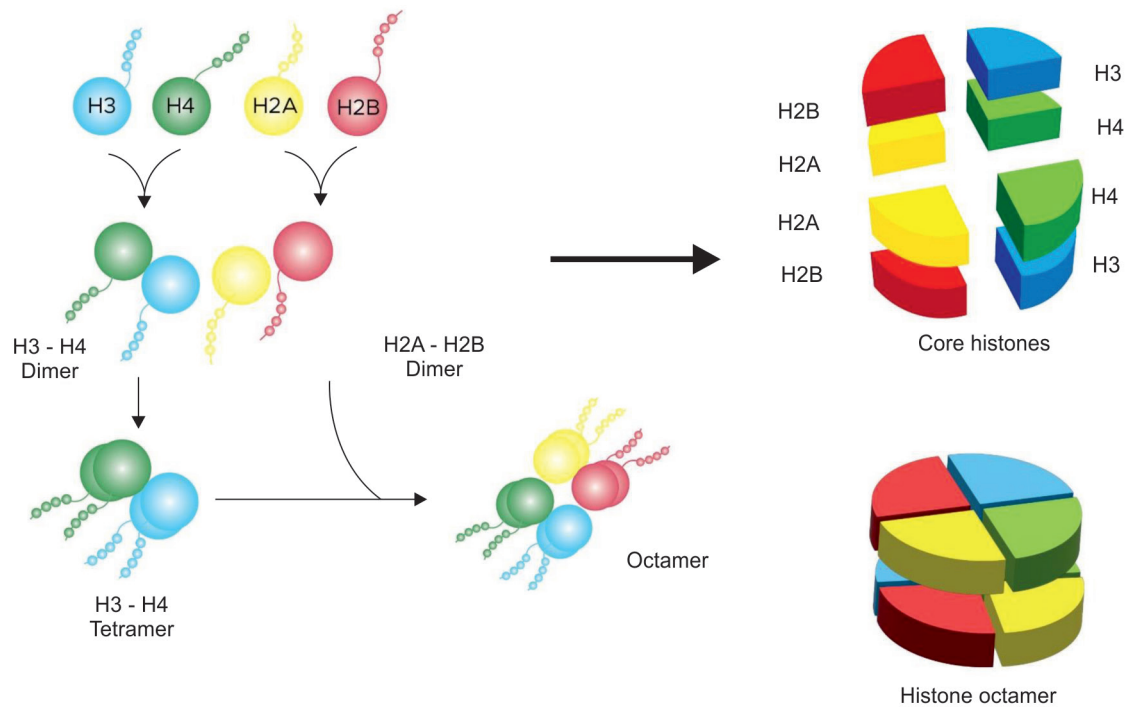


Fig. 3: Formation of histones

There is also increased suppression of transposon activity during germline development.⁵¹ Some single-stranded precursors can mature into antisense (AS) piRNAs, which then recognize and target the cleavage of transposons by associated PIWI-proteins. This sequence generates additional sense-piRNAs from the target transposon sequence, mostly in germline and sometimes in somatic cells.^{52,53} The piRNAs may also have a role in the regulation of the cell cycle of mesenchymal stem cells.⁵⁴

Long Non-coding (Lnc)-RNAs

Long non-coding RNAs (Lnc-RNAs) comprise the largest subgroup of non-protein-coding transcripts. These are typically more than 200 nucleotides in length. Based on the proximity to protein-coding genes, Lnc-RNAs are usually classified into five categories: sense, AS, bidirectional, intronic, and intergenic.³⁶ A subgroup of LncRNAs, named large intergenic non-coding RNAs (lincRNAs), has been described based on a distinctive chromatin signature that marks actively transcribed genes.^{55,56} These are transcribed by RNA polymerase II. There is considerable mechanistic diversity due to variations in interaction with RNA binding proteins (RBPs) at specific DNA regions.⁵⁷ Due to the presence of a poly(A) tail, these can be detected by qRT-PCR through a poly-A tailing method.

Table 2 summarizes the role of regulatory non-coding RNAs.

Post-translational Modifications (PTMs) of Histone Proteins

The histone tails on the nucleosome surface can undergo several enzyme-catalyzed PTMs.^{58,59} These modifications can alter the net charge, inter-nucleosomal interactions, and the chromatin structure.^{60,61} These altered histones contain specialized structural folds in the *N*-terminal tail domain, which changes the interaction with DNA-binding proteins such as transcription factors and other

binding proteins. Figure 3 explains the formation of histones. In Fig. 4, we present a detailed structure of histones. Histone-DNA and histone-histone interactions occur in the globular domain.

Covalent modifications include acetylation, ubiquitination, the addition of small ubiquitin-like modifier (SUMO; SUMOylation), glycosylation, hydroxylation, phosphorylation, sulfation, acetylation, citrullination, crotonylation, malonylation, and ADP-ribosylation.⁶¹ These affect gene expression by altering interactions of the positively charged *N*-termini of histones with negatively charged DNA and creating binding sites for modified histone residues.⁶²

Histone Acetylation

Histone acetylation occurs at conserved lysine residues on the *N*-terminal tails.⁵⁸ Most underlying modifications are seen in H3 and H4, not in H2A and H2B. Key positions for acetylation are lysine K9 and K14 on histone H3; and K5, K8, K12, and K16 on histone H4.^{63,64} The degree of histone acetylation is directly proportional to transcriptional activity. The acetylation and deacetylation of histones are a dynamic process managed by two enzyme systems, the histone acetyltransferases (HATs) and histone deacetylases (HDACs).⁶³

The HATs promote the transfer of acetyl moieties from acetyl coenzyme A to lysine residues of histone proteins. The HATs and HDACs are two counteracting enzyme families that control the acetylation state of protein lysine residues in the *N*-terminal extensions of the core histones. The activity of HDACs is controlled by targeted recruitment, protein-protein interactions, and post-translational modifications. The HDACs control cell cycle progression, survival, and differentiation; these enzymes were first noted in malignant transformation. Therefore, HDAC inhibitors were developed as antineoplastic drugs.

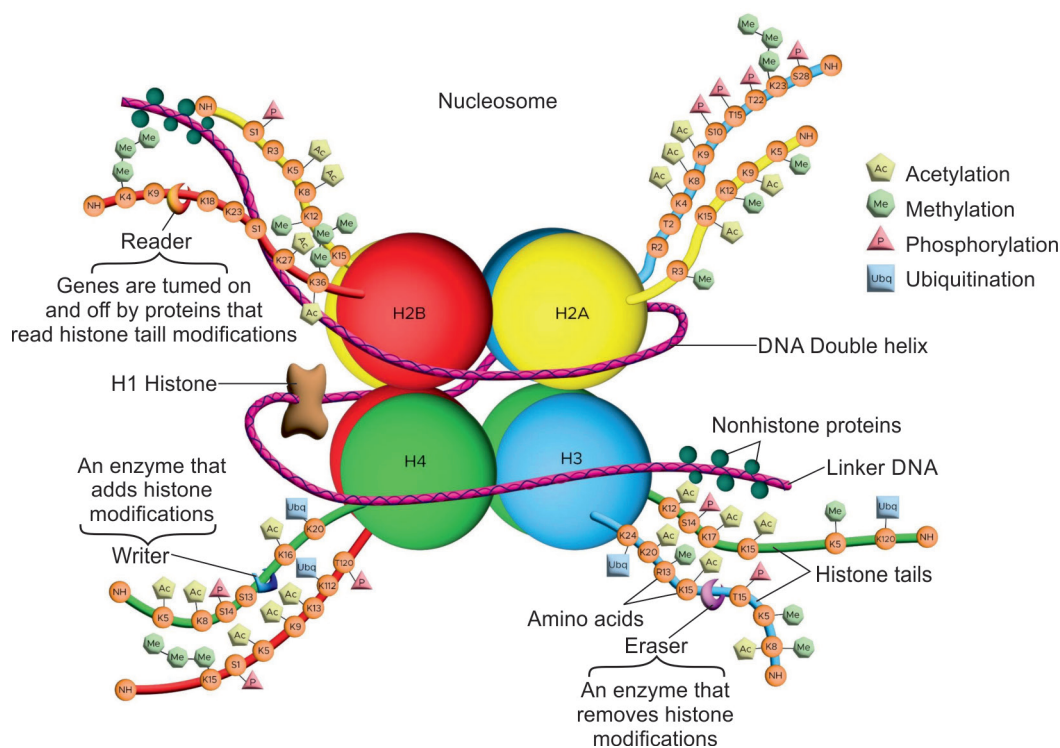


Fig. 4: Detailed structure of histones

Table 3: Classification of histone deacetylase inhibitors

Class	Compound
Hydroxamate	Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydroxamide (CBHA), LAQ-824
Cyclic peptide	Depsipeptide, apicidin, and the CHAPs
Aliphatic acid	Valproic acid (VA), phenylbutyrate (PB)
Benzamide	MS-275, CI-994
Electrophilic ketone	Trifluoromethyl ketones, alpha-ketoamides.

The HDACs suppress transcription; inhibiting these enzymes promotes histone hyperacetylation transcriptional activation of some genes.⁶³ Several histone deacetylase inhibitors (HDACIs), small specific/global inhibitors of HDACs with distinct structural characteristics, are known: hydroxamates, cyclic peptides, aliphatic acids, benzamides, and electrophilic ketones (Table 3).⁶⁵

Histone Methylation

Histone methylation, an irreversible epigenetic mark, can both activate and repress gene expression.⁶⁶ Methylation of H3/K9 and H4/K20 are associated with heterochromatin formation; H3/K9 and K27 with transcriptional silencing; and K4, K36, and K79 of histone H3 associated with gene activity.^{66,67} Methylation of histones and DNA can occur together during chromatin remodeling.^{68,69}

Prions

Prions are infectious conformational states of some proteins, which transfer their misfolded state to other proteins, creating new, diverse heritable traits without any changes in the nucleic acid sequence.⁷⁰ These may be involved in the pathogenicity of some neurodegenerative diseases.

Chromatin Remodeling

Altered spatial organization and chromatin interactions can contribute to some human diseases, such as Rubinstein–Taybi syndrome (RSTS).⁷⁰ These conditions are being recognized as Mendelian disorders of the epigenetic machinery and are being classified as “chromatinopathies.”⁷¹ The RSTS is caused by *de novo* mutations in epigenetics-associated genes such as the cAMP response element-binding protein (CREBBP). This protein encoded by this gene is referred to as CBP. The EP300 gene encodes the p300 protein, a CBP homolog. The CBP has an intrinsic histone acetyltransferase activity required for CREB-mediated gene expression. Protein acetyltransferase activity in CBP destabilizes promoter-bound nucleosomes, leading to transcriptional activation. Overall, chromatin structure is dynamic and precisely controls cellular processes such as gene expression.⁷² Global chromatin remodeling plays a major part in gene expression and epigenetic memory.⁷³

Chromatin acts as a dynamic signal platform through active histone modifications. Chromatin structure is modified either by breaking the interactions between nucleosomes or by recruitment of protein factors to the unraveled nucleosomes.⁷⁴ Histone-modifying enzymes include acetyltransferases, methyltransferases, serine/threonine kinases, ubiquitin ligases, and proline isomerases. The encoded covalent modifications affect the physical remodeling of chromatin structure or recruitment of signaling complexes that drive/repress transcription.⁷⁵ Histone modifications and chromatin remodeling are critical for gene expression during the memory processes and learning.

Nucleosome Positioning

Nucleosomes contain a histone core inside a DNA bundle (Fig. 1). The position and modifications of nucleosomes are key to altered genome regulation in developmental defects and cancer.⁷⁶

Nucleosome-remodeling ATPases maintain the chromatin in a dynamic state responsive to environmental, metabolic, and developmental cues. These enzymes undergo conformational changes that promote binding to and hydrolysis of ATP and interaction with DNA and histones. The resulting histone–DNA interactions in target nucleosomes may lead to the complete or partial disassembly of nucleosomes, the exchange of histones for variants, the assembly of nucleosomes, and/or the movement of histone octamers on DNA. Remodeling can maintain DNA sequences close to interacting proteins. It can also promote histone modifications or RNA metabolism, which is important for stable epigenetics.⁷⁷

In chromatin, the DNA sequence is occluded by histones and non-histone chromatin components. Access to DNA is restricted due to the compact chromatin organization. The proteins cannot easily associate with DNA strands on the nucleosomal histone surface. Also, the nucleosomal DNA shows major tertiary structural curves around the histone octamer. There are also post-translational modifications in histones that further fold the nucleosomal fibers into “higher order” structures which are even less accessible. Nucleosome “remodeling” enzymes allow DNA access to be regulated by changing histone–DNA interactions as a means of disrupting, assembling, or moving nucleosomes and liberating segments of DNA by complete or partial disassembly, alteration of the composition of nucleosomes and by affecting the folding of nucleosomal fibers.^{78–82}

Generally, nucleosomes are stable structures due to the cumulative effect of many weak histone–DNA interactions. Remodeling reactions require biochemical coupling in histones to ATP hydrolysis and are reversible. These include disruption of histone–DNA interactions, the sliding of a histone octamer on or off a particular DNA sequence, or alteration of the composition of histone variants. Remodeling factors are multisubunit complexes; these enzymes, despite the dynamic nature of the chromatin transitions, are involved in the assembly and propagation of stable epigenetic states. The typical roles for nucleosome remodeling include regulatory elements that affect expression programs of specific genes, nucleosome assembly, and exchange of histone variants. Serial deletions that alter the 3-dimensional interactions between heterochromatic nucleosome depleted regions (NDRs) have helped understand the relationship between inter-NDR distance and defects in nucleosome positioning. Poor nucleosome positioning can also alter heterochromatin stability.⁸³

Sex-specific Epigenetic Mechanisms

Genomic Imprinting

Genomic imprinting involves mono-allelic expression depending on the parental origin; the gamete-specific epigenetic modifications result in differential expression of the two parental alleles in somatic cells. DNA gets tagged in a sex-dependent manner, resulting in differential gene expression in accordance with the parent of origin. Imprinting occurs during gametogenesis and is maintained by DNA replication in the somatic cells.

Imprinting control regions (ICRs) are composed of repetitive DNA sequences nearby or in imprinted genes; removal of an ICR results in loss of imprinting. Epigenetic modifiers of gene expression, such as DNA methylation, histone modifications, non-RNA factors, and higher-order chromatin formations within ICRs can help maintain the imprinted state. The ICRs can be important components of nucleation sites for gene silencing/

activation. These are also associated with enhancers and boundary elements to restrict imprinted regulation to specific domains.

Most imprinted genes share common ICRs in clusters that direct parent-specific regulation of multiple genes. The ICRs contain differentially methylated regions (DMRs) that achieve parent-specific DNA methylation tags either in the germline or in somatic cells. Histone acetylation creates an accessible chromatin conformation. In contrast, histone deacetylation can initiate histone methylation. Altered chromatin conformation can promote silencing and the formation of heterochromatin.⁸⁴ Histone methylation can activate/repress transcription depending upon methylation of specific lysine: histone 3-lysine 9 (H3K9), histone 4-lysine 20 (H4K20), and histone 3-lysine 27 (H3K27) are silencing modifications, whereas histone 3-lysine 4 (H3K4) methylation typically activates chromatin. Histone modifications in imprinted regions can promote the formation of higher-order chromatin. Transcriptional inactivation of an imprinted allele involves the formation of heterochromatin.

RNA interference is a post-transcriptional silencing mechanism in which double-stranded RNA (dsRNA) promotes the formation of complementary RNA transcripts by forming RNA silencing complexes (RISCs). The RNA-I silencing pathway helps recruit DNA methyltransferases and other factors to promote higher-order chromatin structures.⁸⁵ Non-coding RNA and RNA-I are also regulators of genomic imprinting. This might involve histone acetylation/methylation and/or DNA methylation. Differential allele-specific DNA methylation is localized to regions termed differentially methylated regions. Differential methylation of parental alleles can be involved in this process. These domains often contain many imprinted genes, such as *Igf2/H19*, *Cdkn1c/Kcnq1*, and *Zac*, that play important roles in growth and postnatal development. Many imprinting gene loci are related to neonatal diseases.

The insulin-like growth factor (IGF) system, including IGF-1 and IGF-2, is one of the most important endocrine and paracrine growth factor systems regulating fetal and placental growth. The IGF-2 and H19 genes are important imprinted genes.^{86,87} The IGF-2 assays are standard for determining the presence of genomic imprinting.⁸⁸ The H19 is maternally and IGF2 is paternally expressed.^{89,90} The H19 has one ICR located upstream of the H19 gene and is also paternally methylated.^{91–93} Once established, paternal-specific methylation is identified and maintained in the somatic cells.^{94,95}

Diagnostic Methods

Different methods have been developed to identify and assess the scale of epigenetic alterations, focusing on DNA methylation and histone modification detection. Bisulfite, enzyme sensitivity, and antibody specificity-based techniques are used for DNA methylation, whereas histone modifications are detected by antibody recognition.⁹⁶ To detect DNA methylation, traditional techniques such as polymerase chain reactions (PCRs) or cloning methods are not applicable because methyl groups are not copied during PCR amplification.⁹⁷ Pretreatment methods can be used to study the original methylated DNA strands to discriminate methylation from non-methylation regions.^{98,99}

Chromatin immunoprecipitation (ChIP) is a reliable way of detecting histone modifications and altered chromatin structures.¹⁰⁰ The ChIP utilizes specific antibodies to recognize specific histone modifications and/or epigenetic modulators along with particular DNA fragments, which allows for assigning

Table 4: Laboratory methods to decipher epigenetic signatures⁹⁶

Technique	Types	Description
Restriction digestion-based techniques	MS-AFLP	Methylation-sensitive amplitude fragment length polymorphism
	DMH	Differential methylation hybridization
	CHARM	Comprehensive-high throughput arrays for relative methylation
	MMASS	Microarray-based methylation assessment of single samples
	HELP	HpaII tiny fragment enrichment by ligation-mediated PCR
	MS-MLPA	Methylation-specific multiplex ligation-dependent probe amplification
	LUMA	Luminometric methylation assay
	RLGS	Restriction landmark genomic scanning
	MCA	Methylated CpG island amplification
	Bisulfite treatment-based techniques	MSP
MSP-ISH		Methylation-specific PCR <i>in situ</i> hybridization
BSP		Bisulfite sequencing PCR
MS-RTPCR		Methylation-sensitive real-time PCR
HRM		High-resolution melting
Bisulfited DNA pyrosequencing		
SNuPE		Methylation-sensitive single nucleotide primer extension
MALDI-TOF		Matrix-assisted laser desorption ionization time-of-flight
COBRA		Combined bisulfite restriction analysis
Affinity-based technology		MeDIP
	MBD protein affinity approach	Methyl binding domain protein affinity approach
	5hmC detection	5-Hydroxymethylcytosine detection
Histone modification analysis	ChIP assay	Chromatin immunoprecipitation assay
	Modified-ChIP-based methods	Carrier ChIP (CChIP), quick and quantitative ChIP (Q2 ChIP), MicroChIP (μ ChIP), Fast ChIP, and Matrix ChIP.
	Site-specific analysis of histone modifications	
	DNAse I hypersensitivity analysis of chromatin structure	

locus-specific functions of histone modifications or transcriptional factor complexes.¹⁰¹ Sequencing-based ChIP methods such as ChIP-chip or ChIP-seq can analyze protein–DNA binding events and histone modifications at different loci for non-specific modification patterns.^{102,103} Table 4 provides an overview of these techniques for detecting epigenetic effects.

Uses in Neonates

Environmental factors typically induce epigenetic effects during the first 1000 days after conception. Extrinsic factors such as allergens, viruses, pollutants, diet, and/or microbiome can activate atypical metabolic reprogramming, metabolic dysfunctions, and defective immune responses, such as in allergic disease.¹³ Flowchart 1 summarizes the major epigenetic exposures in the perinatal period.

miRNAs in Neonatal Sepsis

During infections, epigenetic changes may occur, leading to reprogramming of gene expression. Post-transcriptional regulation by short non-coding RNAs (microRNAs) may have a role in the pathophysiology of neonatal sepsis and may be used as potential

biomarkers.¹⁰⁴ MicroRNAs of 22-nucleotide length regulate immune functions and inflammatory response.

Necrotizing Enterocolitis (NEC)

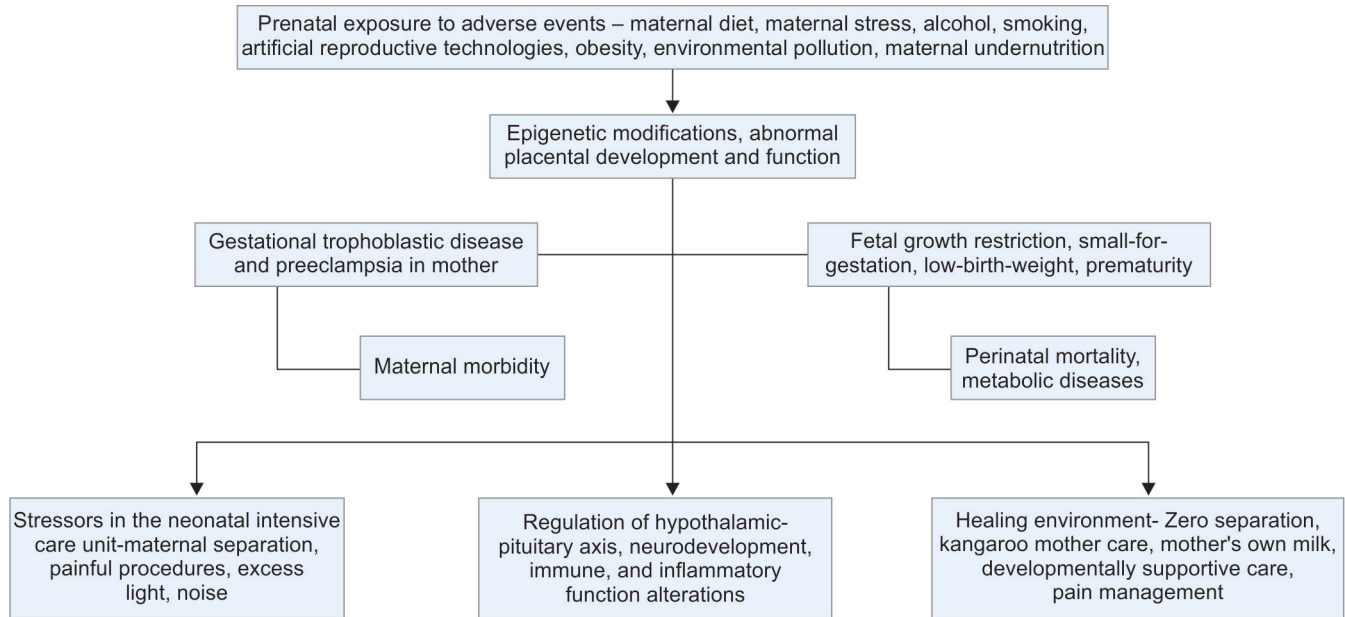
In neonatal pathologies, epigenetic changes are used to identify targets for therapeutic intervention and predict outcomes.¹²

Persistent Pulmonary Hypertension of the Newborn

Epigenetics regulates this endothelial cell-specific expression of eNOS, including DNA methylation, histone acetylation and methylation, and RNA interference.⁵⁸ The PPHN significantly increases DNA CpG methylation in the eNOS promoter and decreases ten eleven translocase demethylases (TET) activity in pulmonary artery endothelial cells (PAEC). The PPHN decreased Sp1 occupancy and density of the active mark, lysine 12 acetylation of histone 4, and increased density of the repression mark, lysine 9 trimethylation of histone 3 around Sp1 binding sites in endothelial nitric oxide synthase (eNOS) promoter. These results suggest that epigenetic modifications are primed to decrease Sp1-binding at the eNOS gene promoter in PPHN. Reduced expression of eNOS, a key mediator of perinatal transition, characterizes PPHN.



Flowchart 1: Epigenetic exposures in the perinatal period



Congenital Disorders

Epigenetic modifications have a role in congenital disorders (such as Silver–Russell and Beckwith–Wiedemann syndrome), transient neonatal diabetes mellitus (TNDM), intrauterine growth retardation (IUGR), and PPHN.⁵⁸ Epigenetics is also involved in genomic imprinting and X-chromosome inactivation in humans, and the failure of this mechanism causes a subset of congenital syndromes.¹⁰⁵ The SNRPN gene, located in chromosome 15q12, is an imprinted gene where the maternal allele is inactivated and the paternal allele is expressed. The UBE3A gene is another imprinted gene where the paternal allele is inactivated, and the maternal allele is expressed. Failure to express the SNRPN gene due to deletion of paternal chromosome 15 or abnormal methylation of the paternal allele results in Prader–Willi syndrome. Failure of expression of the UBE3A gene due to either deletion of maternal chromosome 15 or abnormal methylation of the maternal allele results in Angelman syndrome.^{106,107} Around 100 imprinted genes clustered in certain genome regions have been identified. In genomic imprinting, the inactivated allele is methylated in its promoter region, whereas the expressing allele is not methylated; whereas in some disorders, both alleles are methylated, leading to a complete lack of gene expression.

Lyonization of the X-chromosome occurs in females, but in some rare diseases, both X-chromosomes are active, leading to severe developmental delay. DNA is methylated by specific enzymes, such as DNA methyltransferases (DNMTs). In a disease with immunodeficiency [immunodeficiency, centromere instability (ICF) syndrome], mutant DNMT3B fails to methylate DNA in certain genomic regions. Genes are suppressed by epigenetic mechanisms through proteins, such as the methyl CpG-binding proteins. In Rett syndrome, a mutant methyl-CpG binding protein 2 (MeCP2) fails to repress its target genes.

Epigenetics and Neonatal Nutrition

Epigenetic changes have long-lasting effects on gene expression and are induced by the early development environment. Epigenetic

DNA imprinting activity is the most active from preconception to early infancy and modifies the risk for non-communicable disorders later in life, such as cardiovascular diseases, metabolic diseases, and diseases of the reproductive system.¹⁰⁸

Genetics, Epigenetics, and Transcriptomics of Preterm Birth

Maternal toxic exposure to heavy metals, air pollution, and pesticides has been correlated with reduced placental methylation, which may cause genomic instability and mutations.¹⁰⁹ Epigenome-wide association meta-analysis studies (EWAS) have shown that many prenatal exposures associated with spontaneous preterm birth also change DNA methylation in cord blood. The EWAS has shown reproducible associations between blood DNA methylation in newborns and maternal folate levels, exposure to smoking during pregnancy, air pollutants, and exposure to heavy metals.^{110–113}

Premature uterine contractions have been associated with pathogenic variants of the sarcomere gene TTN and transcriptomic variations of sarcomeric premature uterine contraction genes regulated by epigenetic factors, including methylation and long non-coding RNAs.¹¹⁴ Maternal age is an independent risk factor for preterm birth. Inter-individual differences between chronological and biological age have also been noted, which might be related to genetic background and environmental exposures. Estimation of biological age using genome-wide DNA methylation has found an association between a mother's biological age and gestational age at delivery.¹¹⁵

Epigenetics and the Placenta

Placental growth and function, pregnancy maintenance, and parturition are under epigenetic control.¹¹⁶ Prostaglandin biosynthesis enzyme (prostaglandin H synthase-2) can be modified by alteration of histone acetylation status and DNA methylation status in the human placenta.¹¹⁷ These inflammatory mediators affect uteroplacental hemodynamics and are critical in the

mechanisms of labor. The regulatory mechanisms extend back in pregnancy till implantation.¹¹⁸

Candidate Genes

Many epigenetically modifiable genes may promote programmed changes in growth and insulin sensitivity in small-for-date and/or premature infants. Changes in methylation at the imprinted IGF-2 receptor gene could lead to decreased IGF-II activity at birth.^{119,120} Overexpression of RASGRF1, which regulates postnatal growth and is known to be imprinted in rodents, could explain the taller stature and higher IGF-I levels in IVF children. Under-expression of RASGRF1, which is known to be imprinted in rodents, could explain poor growth in premature and small-for-date infants.¹²¹ There appears to be a critical window in early life in which under-nutrition might cause aberrant methylation of LEP and in turn, persistent insulin resistance with later obesity and hypertension. The GRB10 is another imprinted gene that acts to inhibit insulin and IGF-I receptor signaling and its hypomethylation during the fetal or early neonatal periods can lead to insulin resistance, poor growth, and abnormalities in the GH/IGF-I axis as observed in SGA and premature children.^{116,122}

Possible Therapeutic Implications

Since epigenetic alterations are reversible, modifying epigenetic marks contributes to disease development may provide an approach to designing new therapies.⁶⁵ Gene hypermethylation

and histone hypoacetylation are attractive targets for treating epigenetic diseases because these changes could be reversible. Despite limitations such as non-specific activation of genes and transposable elements in normal cells, corrected epigenetic modifications may revert to their previous state because of the reversible nature of DNA methylation and histone modification patterns. However, this may be prevented with continued treatment.

Epigenetic dysregulation leads to the initiation and progression of some cancers. Global DNA hypomethylation has been noted in altered growth. Promoter CpG hypomethylation can promote gene expression. Promoter CpG hypomethylation can increase gene expression. DNA methylation refers to the post-synthetic methylation of cytosine bases at position 5 of the pyrimidine ring by a DNA methyltransferase (DNMT), which catalyzes the transfer of the methyl group from S-adenosylmethionine (SAM) to cytosine to form 5-methylcytosine and S-adenosylhomocysteine (SAH). Diets deficient in methyl donor precursors (folate, methionine, and choline) may play a role in DNA hypomethylation. The DNA hypomethylation has been detected in those receiving low dietary folate and can be reversed by folate repletion. Histone acetylation and deacetylation status may be altered by inhibiting HATs or HDACs. Gene silencing is associated with histone deacetylation, which is catalyzed in human cells by at least three classes of HDACs (class I, II, and III).

Table 5 summarizes the definitions associated with epigenetics.

Table 5: Definitions associated with epigenetics¹²³

Epigenetics	Non-genetic heritable (mitotically and/or meiotically) genomic modifications involved in gene expression regulation but do not entail a change in DNA sequence. ^{5,6}
Gene	The fundamental physical and functional unit of heredity, which carries information from one generation to the next.
Chromosome	A linear end-to-end arrangement of genes and other DNA, sometimes with associated protein and RNA.
Chromatin	Chromatin refers to a mixture of DNA and proteins that form the chromosomes. ^{124,125} Histones package the massive amount of DNA in a genome into a highly compact form that can fit in the cell nucleus.
Euchromatin	Euchromatin is defined as the area of the chromosome which is rich in genes that actively participate in the transcription process. ¹²⁶
Heterochromatin	Heterochromatin is a tightly packed form of DNA in the nucleus that is so compactly organized that it is inaccessible to the proteins involved in gene expression. Heterochromatin is highly condensed, gene-poor, and transcriptionally silent, whereas euchromatin is less condensed, gene-rich, and more accessible to transcription (Fig. 2). ¹²⁶
Nucleosome	A nucleosome is the basic repeating subunit of chromatin. It has a nucleosome 'core' linker DNA, and a linker histone. ¹²⁷ It comprises a segment of DNA wrapped around eight histone proteins, known as histone octamer. The octamer is made up of two copies of each of the histone proteins: H2A, H2B, H3, and H4.
Histone	Histones are basic proteins found in chromosomes that help pack and organize DNA helix in chromatin fiber in the nucleus (Fig. 4).
Transcription	The synthesis of RNA from a DNA template.
Methylation	The process by which methyl groups are added to the DNA, and contributes to cellular reprogramming, tissue differentiation, and normal development. ^{23,24}
CpG island	CpG islands (CGIs) are short, interspersed DNA sequences that deviate significantly from the average genomic pattern by being GC-rich, CpG-rich, and predominantly non-methylated and are the sites of transcription initiation. ²⁶
Polycomb proteins	<i>Polycomb</i> -group (PcG) genes encode chromatin proteins involved in stable and heritable transcriptional silencing. PcG proteins participate in distinct multimeric complexes that deposit or bind to specific histone modifications (e.g., H3K27me3 and H2AK119ub1) to prevent gene activation and maintain repressed chromatin domains. PcG proteins can silence gene expression globally, particularly during development and differentiation. ²⁸
RNA silencing	RNA silencing or RNA interference is an evolutionarily conserved gene inactivation system by which gene expression is negatively regulated by non-coding RNAs such as microRNAs.
small interfering RNAs (siRNA)	A class of double-stranded RNA, 20–24 base pairs in length, which interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation. ¹²⁸

(Contd...)



Table 5: (Contd...)

microRNA s (miRNA)	MicroRNAs are small, single-stranded, non-coding RNA molecules containing 21–23 nucleotides involved in RNA silencing and post-transcriptional regulation of gene expression. ¹²⁹
piRNA	piRNAs are a group of small ncRNAs with approximately 21–35 nucleotides in length. The piRNA is known to associate with PIWI proteins and form piRNA-induced silencing complexes during germline development. ⁴⁹
lncRNA	Long non-coding RNAs are RNA transcripts of more than 200 nucleotides that are not translated into protein. They are involved in epigenetic regulation, chromatin remodeling, and protein metabolism control. The term 'lncRNAs' encompasses RNA polymerase I (Pol I), Pol II, and Pol III transcribed RNAs, and RNAs from processed introns. ¹³⁰
Genomic imprinting	It is the process by which only one copy of a gene (maternal or paternal) in an individual is expressed while the other copy is suppressed. ¹³¹
ChIP technique	An antibody-based technology that selectively enriches specific DNA-binding proteins and their DNA targets. ¹³²
Developmental Origins of Adult Disease Hypothesis or Barker's theory	According to the DOHaD hypothesis, epigenetic adaptations are made to the fetal/neonatal DNA in response to environmental influences. ¹ The hypothesis further explains that the body responds to the environment and makes genetic changes (predictive adaptations) in anticipation of a presumed future environment. If the predictive adaptations are incorrect, then the metabolic state of the individual is altered to a degree whereby the risk of chronic disease in adulthood is increased; research also shows that the increased risk might be transgenerational. ¹³³
EWAS	Epigenome-wide association studies (EWASes) investigate the association between a phenotype and epigenetic variants, most commonly DNA methylation. ^{134,135}

FUTURE DIRECTIONS

Epigenetic alterations are crucial in the first 1000 days of life. Modifying epigenetic marks can play a role in developing new therapies. There is a need to unravel the complete repertoire of mechanisms of epigenetic modifications. Recent research is also delving into epigenetics as a causative mechanism in the pathogenesis of many neonatal disorders. Epigenetic remodeling of the genome plays a role in the phenotypic expression of various health and disease states in the newborn. Future efforts should be directed toward the possible therapeutic implications of epigenetics.

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