Constitutional Epi/Genetic Conditions: Genetic, Epigenetic, and Environmental Factors

Laila C. Schenkel^{1,2} David Rodenhiser^{2,3,4,5,6} Victoria Siu^{2,4,5} Elizabeth McCready⁷
 Peter Ainsworth^{1,2,3,4,5,6} Bekim Sadikovic^{1,5,2}

¹Department of Pathology and Laboratory Medicine, Western University, London, Ontario, Canada

²Children's Health Research Institute, London, Ontario, Canada

³Department of Biochemistry, Western University, London, Ontario, Canada

⁴Department of Pediatrics, Western University, London, Ontario, Canada

⁵London Regional Cancer Program, London Health Sciences Centre, London, Ontario, Canada

⁶Department of Oncology, Western University, London, Ontario, Canada

⁷Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Address for correspondence Bekim Sadikovic, PhD, 800 Commissioner's Road E, B10-104, Victoria Hospital, London Health Sciences Centre, London, ON N6A 5W9, Canada (e-mail: Bekim.Sadikovic@lhsc.on.ca).

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Abstract

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There are more than 4,000 phenotypes for which the molecular basis is at least partly known. Though defects in primary DNA structure constitute a major cause of these disorders, epigenetic disruption is emerging as an important alternative mechanism in the etiology of a broad range of congenital and developmental conditions. These include epigenetic defects caused by either localized (in cis) genetic alterations or more distant (in trans) genetic events but can also include environmental effects. Emerging evidence suggests interplay between genetic and environmental factors in the epigenetic etiology of several constitutional “epi/genetic” conditions. This review summarizes our broadening understanding of how epigenetics contributes to pediatric disease by exploring different classes of epigenomic disorders. It further challenges the simplistic dogma of “DNA encodes RNA encodes protein” to best understand the spectrum of factors that can influence genetic traits in a pediatric population.

Introduction

The etiology of constitutional developmental disorders, including genetic syndromes, involves genetic and environmental factors that interact during embryonic development and in the newborn.¹ These interactions between genes and the environment can be facilitated by epigenetic processes, which reversibly regulate gene expression without changing the primary DNA sequence. Deregulation of normal epigenetic processes through either environmental effects or genetic variants can in turn affect the expression of specific genes and may result in clinical phenotypes. For example, prenatal exposure to environmental pollutants has been shown to induce epigenetic changes and developmental diseases.² In addition,

dietary changes in the general population have induced epigenetic changes and contributed to the growing incidence of metabolic syndrome.³ Changes in the DNA sequence, such as point mutations, insertions, deletions or trinucleotide repeat expansion, can affect the establishment of the local DNA methylation profiles. Additionally, noncoding RNAs (ncRNAs) have been shown to regulate DNA methylation and induce transcriptional gene silencing.^{4–6} Understanding the contribution of both genetic and environmental factors to the development of epi/genetic constitutional disorders is shedding light on some of the various pathological mechanisms of these diseases, with the promise of novel therapeutic and diagnostic applications used to manage these conditions.

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The association between epigenetic processes and normal development is complex with new knowledge being published at an astonishing rate. At the cellular level, epigenetic processes range from the reversible alteration of gene-specific DNA methylation to a more widespread modulation of chromatin structure and specific histone modifications.⁷⁻⁹ This review summarizes both classic examples by which epigenetic anomalies impact the human development, as well as recent associations between epigenetic disturbances and monogenic or complex diseases. We describe emerging evidence of the interplay between genetic and environmental factors in epigenetic etiology of constitutional “epi/genetic” conditions. We also provide an overview for

understanding how normal and altered epigenetic processes affect embryonic, fetal, and pediatric development, with particular emphasis on aberration that affects methylation patterns and can be assessed through routine and emerging diagnostic testing. This evolving understanding of the relationship between epigenetics and human health is exemplified by representative conditions associated with each of the following categories of epi/genetic conditions (– **Table 1**): imprinting disorders, trinucleotide expansion disorders, mutations in epigenetic regulatory elements including miRNAs, methylation defects in monogenic and complex epi/genetic conditions, and environmentally mediated epigenetic defects.

Table 1 Epigenetic mechanisms in constitutional conditions

Mechanism	Disorders	References
Imprinting defects	Angelman syndrome (AS); Prader-Willi syndrome (PWS); Beckwith-Wiedemann syndrome (BWS); Silver-Russell syndrome (SRS); Transient neonatal diabetes mellitus (TND1); Pseudohypoparathyroidism 1b; Wang syndrome; Temple syndrome.	Williams et al ³⁶ ; Cassidy and Driscoll ³⁷ ; Smith et al ¹⁴ ; Azzi et al ⁴¹ ; Mackay et al ³⁰ ; Kamiya et al ³¹ ; Jan de Beur et al ³² ; Wang et al ³³ ; Temple et al ³⁴
Trinucleotide expansion	Fragile X syndrome; Friedrich ataxia	Alisch et al ⁴⁵ ; Campuzano et al ⁴⁷
Mutation in epigenetic genes	Immunodeficiency, centromere instability and facial anomalies (ICF) syndrome; Hereditary sensory neuropathy with dementia and hearing loss (HSNDHL); Cerebellar ataxia, deafness, and narcolepsy (ADCAFN); Rubinstein-Taybi syndrome (RSTS); Coffin-Lowry syndrome (CLS); Floating-Harbor syndrome; Alpha-thalassemia/mental retardation syndrome (ATR-X); Rett syndrome Genitopatellar syndrome (GPS); Say-Barber-Biesecker-Young-Simpson syndrome (SBBYS); Brachydactyly mental retardation syndrome (BDMR); Kleefstra syndrome; Weaver syndrome; Kabuki syndrome; Claes-Jensen X-linked mental retardation syndrome; Siderius X-linked mental retardation syndrome (MRXSSD); Sotos syndrome	Jin et al ⁵⁶ ; Klein et al ⁶¹ ; Klein et al ⁶² ; Murata et al ⁶³ ; Biancalana et al ⁶⁶ ; Hood et al ⁶⁷ ; Gibbons et al ⁷⁰ ; Ghosh et al ⁷³ ; Simpson et al ⁷⁴ ; Clayton-Smith et al ⁷⁵ ; Williams et al ⁷⁶ ; Kleefstra et al ⁷⁹ ; Tatton-Brown et al ⁷⁸ ; Van Laarhoven et al ⁸¹ ; Claes et al ⁸⁴ ; Siderius et al ⁸³ ; Laumonnier et al ⁸² ; Tatton-Brown et al ⁷⁷ ;
Mutation in miRNA pathway	Amyotrophic lateral sclerosis 10; DiGeorge syndrome; Autosomal dominant multinodular Goiter	Zhang et al ⁹¹ ; Kozlova et al ⁹² ; Rio Frio et al ⁹³
DNA methylation in candidate genes	Susceptibility to dyslexia Obesity and type-1 diabetes Spinal muscular atrophy (SMA) Facioscapulohumeral muscular dystrophy 1 (FSHD1)	Tammimies et al ⁹³ ; Feinberg et al ¹⁰² ; Dick et al ¹¹³ ; Rakyan et al ¹¹⁴ ; Zheleznyakova et al ¹⁰³ ; Jones et al ¹⁰⁴ ;
Environmentally mediated epigenetic changes	Metabolic syndrome and obesity Autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) Chronic obstructive pulmonary disease (COPD) Idiopathic pulmonary fibrosis (IPF) Fetal alcohol syndrome (FAS)	Chen et al ¹²⁴ ; Finer et al ¹¹⁸ ; Chaste et al ¹²⁶ ; Nomura et al ¹²⁵ ; Soto-Ramirez et al ¹²⁹ ; Patil et al ¹³⁰ ; Qiu et al ¹³¹ ; Haycock ¹²¹

Epigenetic Mechanisms

DNA Methylation

The most widely studied epigenetic modification is DNA methylation, which is a covalent modification occurring primarily at cytosines within CpG dinucleotides. These CpGs are generally underrepresented in the genome but are disproportionately located in CpG “islands” at the 5′ – end regulatory regions or promoter regions of genes.¹⁰ CpG islands are usually unmethylated, providing a transcriptionally permissive chromatin state, whereas methylation of CpG islands in gene promoters leads to a condensation of chromatin and transcriptional repression. Methylation of CpG dinucleotides in intragenic fragments and imprinted regions can also have a similar impact on gene expression.^{11,12} For example, DNA methylation plays a role in regulation of paternal or maternal specific expression of genes at several imprinted loci.^{13,14} DNA methylation is also essential during silencing of one of the two X-chromosomes in females, ensuring gene dosage compensation^{13,15} and in repression of potentially unstable genomic elements such as transposable and retroviral elements.¹⁵ The maintenance of proper DNA methylation marks during development and tissue differentiation ensures appropriate regulation of gene expression and chromosome stability.

An intricate network of proteins that establish, maintain, and localize epigenetic modifications to chromatin are essential in ensuring that DNA methylation and other epigenetic changes can be both stably maintained in the genome yet remain flexible to respond to environmental stimuli. These proteins function generally as “writers” that establish the epigenetic marks, “readers” that interpret them, and “erasers” that remove the epigenetic marks.⁷ In addition, these active processes that tie together environmental signals to the genome are further integrated by the activities of chromatin remodeling proteins that can reposition nucleosomes (individual histone octamers associated with DNA), as well as establish insulator functions that define boundaries between epigenetic domains. These intricately regulated epigenetic regulators add additional control to the epigenetic state by serving as interpreters of the epigenetic state and through the synchronization of epigenetic signals across the genome.⁷

DNA methylation reactions are catalyzed by DNA methyltransferase (DNMT) enzymes that transfer the methyl group from S-adenosylmethionine (SAM) to cytosine.¹⁶ Five mammalian DNMTs have been identified, four of which are active isoforms – DNMT1, DNMT3a, DNMT3b, and the noncatalytic DNMT3L. The fifth DNMT, DNMT2, appears to methylate tRNA instead of DNA.¹⁷ DNMT1, DNMT3a and 3b are the major methyltransferases with distinct and well-characterized function.¹⁸ DNMT1 is categorized as a maintenance methyltransferase. It achieves its main function of copying DNA methylation patterns after replication through its close association with the DNA replication machinery and enhanced affinity for hemimethylated DNA. In contrast, DNMT3a and DNMT3b are essential *de novo* methyltransferases with no specific preference for unmethylated versus hemimethylated CpG sites. Recently, studies have emphasized the need for

continual cooperation between DNMTs to maintain DNA methylation patterns particularly in densely methylated regions.¹⁹ The complexity of the DNA methylation machinery is further evident in the interactions and crosstalk between these enzymes as well as other factors that permit region and/or locus-specific activity.²⁰ Studies have found that DNMT1, DNMT3a, and DNMT3b interact with histone deacetylases (HDACs) and chromatin regulatory proteins, such as methyl-CpG-binding protein 2 (MeCP2), supporting the linkage between DNA methylation, histone modification, and chromatin remodeling in the regulation of specific gene expression.^{21–23}

Histone Modifications

Alongwith modifications of DNA methylation, chromatin is further regulated by a complex and varied array of posttranscriptional modifications to histone proteins. Histones organize the DNA structure and regulate its accessibility to replication and transcriptional machinery, therefore providing an additional level of specific gene regulation. Histone protein modifications are coordinated by a variety of enzymes that catalyze histone methylation (HMT), acetylation (HAT), deacetylation (HDAC), phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation.⁸ The most studied histone modifications include acetylation and methylation. Histone acetylation is most commonly associated with open chromatin structure (euchromatin), promotion of gene expression and lack of DNA methylation, while deacetylation commonly involves compaction of chromatin (heterochromatin) and inhibition of gene transcription.^{24,25} Histone H3 methylation commonly occurs at multiple lysine residues, including K4, K9, K20, K27, K36, and K79, with varied effect on chromatin and transcriptional states. For example, K9, K20, and K27 methylation is associated with heterochromatin formation and inactive transcription. In contrast, K4, K36, and K79 methylation is associated with euchromatin formation and active transcription.²⁶

Noncoding RNAs

Another mechanism for DNA methylation change by genetic factors involves ncRNAs. ncRNAs are defined as functional RNA molecules, derived from intergenic or antisense transcription, which are not translated into a protein. Some of the best characterized ncRNAs include micro-RNA (miRNA), long noncoding RNA (lncRNA), and small interfering RNA (siRNA). miRNA are components of the RNA-induced silencing complex (RISC), which regulates gene expression by degradation of complementary protein-coding transcripts as well as by translation repression. Short interfering RNA (siRNA) and microRNA (miRNA) have been shown to regulate DNA methylation and induce transcriptional gene silencing.^{4–6} miRNAs can target DNA methyltransferases (DNMTs) and inhibit their translation, thus affecting global DNA methylation, or they may act at specific target gene(s) by affecting chromatin structure and/or DNA methylation.^{27,28} In addition, siRNA targeted to specific CpG island within the promoter of a specific gene can induce DNA methylation and histone H3 methylation, inhibiting gene transcription.⁵

Epigenetics-Associated Syndromes

Imprinting Disorders

DNA methylation plays a central role in both establishing and maintaining genomic imprinting. Genomic imprinting refers to parent-of-origin-specific gene expression and is an essential process in normal development.^{13,14} The majority of mammalian autosomal genes are expressed from both maternally and paternally inherited alleles. However, transcription from some genes is repressed from one of the parental chromosomes. The choice of which of the two inherited copies is repressed is not random, and the epigenetically mediated repression results in a reversible modification of gene activity depending on the sex of the transmitting parent. A key molecular mechanism involved in the establishment and maintenance of genomic imprinting is the allele-specific DNA methylation at imprinting control regions (ICR). The allele from one parent is unmethylated at the ICR and transcriptionally active whereas the allele from the other parent is methylated at the ICR and transcriptionally repressed. DNA methylation is established through the action of the *de novo* DNA methyltransferase 3a (DNMT3A) and the accessory protein DNMT3L in both germ lines before fertilization. Following fertilization, the parental-specific imprints must be maintained despite the extensive DNA demethylation taking place at this time; this activity being facilitated by DNMT1 and accessory proteins.²⁹

Imprinting defects can occur during establishment of genomic imprinting or may occur during cellular division and are responsible for a broad range of disorders, which can affect growth, development, and/or metabolism. Imprinting disorders may be caused by either epigenetic mutations or genetic mutations, affecting multiple genes under coordinated control. Eight congenital imprinting disorders have been described to date, including Angelman syndrome (AS), Prader-Willi syndrome (PWS), Beckwith-Wiedemann syndrome (BWS), Silver-Russell syndrome (SRS), transient neonatal diabetes mellitus,^{30,31} Pseudohypoparathyroidism 1b,³² Wang syndrome,³³ and Temple syndrome.³⁴ Recent evidence has also revealed multilocus methylation defects in patients with different clinically diagnosed imprinting disorders, potentially indicating the molecular overlap of these diseases.³⁵

AS and PWS have long represented the classical, well-described paradigm for imprinting disorders. The 15q11-q13 AS/PWS critical region contains multiple paternally expressed genes (including SNRPN) and a single maternally expressed gene (UBE3A). The most common cause of AS or PWS is a microdeletion of chromosome region 15q11.2-q13, with the phenotype dependent on parental origin of the deletion; however, multiple molecular mechanisms have been shown to affect imprinting at chromosome 15q11.2-q13, including uniparental disomy 15 (UPD 15; inheritance of the chromosome 15 homologs from the same parent), point mutations, chromosomal translocations involving chromosome 15 and imprinting defects. AS is characterized by severe intellectual disability, severe speech impairment, gait ataxia, tremulousness of the limbs, frequent laughter and excitability, and seizures.³⁶ AS cases are caused by (1) microdeletion of the

maternally inherited chromosome 15q11.2-q13 region, (2) paternal UPD 15, (3) mutation of the maternally inherited UBE3A gene, or (4) hypermethylation of the maternally inherited UBE3A gene.

PWS is characterized by severe hypotonia and feeding difficulties in infancy, followed by hyperphagia and gradual development of obesity, cognitive impairment, and hypogonadism.³⁷ It is most often caused by microdeletion of the paternally inherited chromosome 15q11.2-q13 or by maternal UPD 15. A strong candidate locus for a major role in the etiology of PWS is the small nuclear ribonucleoprotein N (SNRPN)/SNRPN Upstream Reading Frame (SNURF), which is located in the 15q11.2-q13 region and expressed exclusively by the paternally inherited allele. The 5'-end of SNRPN/SNURF locus contains an ICR essential for epigenetic regulation.³⁸ In addition to SNRPN/SNURF, loss of MAGEL2 gene expression can contribute to the features of PWS. MAGEL2 is also located in the PWS critical region, and mutations in this gene have been associated with PWS-like phenotype and autism spectrum disorder (ASD).³⁹ About 5% of AS and PWS are caused by imprinting defects. This can result from small deletions at the ICR, leading to errors in the mechanism for resetting and maintaining the genomic imprinting, or from solely an abnormal imprint (epimutation).^{38,40}

BWS and SRS are also well-known disorders resulting from imprinting defects, associated with overgrowth and growth restriction, respectively. Both syndromes are caused by dysregulation of imprinted genes associated with cell cycle and growth control that are clustered in the 11p15.5 chromosome region.^{14,41} Two important ICR have been identified in this region: the H19/IGF2 domain (ICR1), which is methylated in the paternal allele, and the KCNQ1OT1/CDKN1C domain (ICR2), which is methylated in the maternal allele. ICR1-mediated methylation extends to the *H19* promoter, silencing paternal *H19* expression, in contrast to *IGF2*, which is expressed only from the paternal allele.⁴² Genetic alterations that result in biparental *IGF2* expression cause BWS, an overgrowth syndrome characterized by the presence of macrosomia, macroglossia, and omphalocele. Most cases of BWS are sporadic and result from *de novo* epimutations at ICR within 11p15.5.⁴³ However, a range of molecular alterations from point mutations acting in *cis* to frameshift mutation acting in *trans*, as well as UPD11 are associated with familial BWS.⁴⁴ On the other hand, paternal loss of methylation at *H19* usually cause SRS, which is characterized by severe prenatal and postnatal growth compromise, developmental delay, and a variable combination of facial dysmorphic features. Molecular mechanisms for SRS are complex and involve structural chromosomal anomalies and maternal UPD11.¹⁴

Taken together, these examples demonstrate the importance of proper genomic imprinting and maintenance of DNA methylation at imprinted sites for the execution of the normally appropriate developmental cascade.

Trinucleotide Expansion Disorders

A second class of classical epigenetic diseases is caused by DNA trinucleotide repeat expansion associated with aberrant DNA methylation at specific sites. While trinucleotide repeat

disorders can result in pathology through various mechanisms, altered methylation has emerged as an important cause in at least some of these conditions. A well-defined example is the repeat expansion involving a cytosine-guanine-guanine (CGG) trinucleotide, which causes fragile X syndrome (FXS).⁴⁵ FXS is the most common inherited cause of intellectual disability in males, with clinical features including facial dysmorphism (long ears, long face, prominent jaw) and macroorchidism. It is caused by an expansion of CGG repeats from a normal range of 6–40 to more than 200 repeats (full mutation) within the 5' untranslated region of the *FMR1* (fragile X mental retardation 1) gene at Xq27.3. This expansion results in DNA hypermethylation of the CpG island at the *FMR1* gene promoter region and a consequent gene silencing. The fragile X mental retardation protein (FMRP) is an RNA binding protein that regulates protein synthesis during neuronal development, with several functional roles including the repression of mRNA translation through association with polyribosomes and via interactions between FMRP and microRNAs and Argonaute proteins that are part of the RNA-induced silencing (RISC) complex.⁴⁶

Trinucleotide repeat expansion is also seen in Friedrich ataxia (FRDA), an autosomal recessive neurodegenerative disorder caused by the expansion of GAA repeats within intron 1 of the *FXN* gene. Unaffected individuals have up to 43 GAA repeats, while affected individuals have 44–1700 GAA repeats. The GAA expansion is associated with a decrease in the expression of the mitochondrial protein frataxin,⁴⁷ resulting in the main pathological effects of this disease, and which are associated with loss of large sensory neurons and degenerative atrophy of spinal cord, contributing to symptoms of progressive ataxia, muscle weakness, and sensory deficit.⁴⁸ Recent studies have observed an increase in DNA methylation of specific CpG sites immediately upstream of the expanded GAA repeat sequence in FRDA patients.^{49,50} In the same manner as in FXS, DNA hypermethylation at these sites may be responsible for decreased frataxin expression in FRDA.^{49,51}

It has been proposed that certain trinucleotide repeat expansions located in noncoding regions of the gene, such as CGG and GAA expansions, induce abnormal DNA or DNA/RNA hybrid structures, such as hairpin formations, which in turn can result in DNA methylation and gene silencing.^{49,52} Evidence suggests that the tendency of DNA to form hybrid structures of this type is dependent on the repeat number and that DNA methylation is likely to be a direct consequence of hairpin formation.^{53,54} In the example of FXS, hairpins are suggested to provide the structural basis for the major molecular phenomena including site-specific fragility, repeat amplification, and hypermethylation of the CpG island adjacent to *FMR1*.⁵³

Mutations in Epigenetic Regulatory Genes and miRNA Pathways

The orchestrated regulation of epigenetic factors, including DNA methylation, histone modifiers, ncRNAs, and chromatin remodeling proteins, is essential for development and cellular differentiation. Extensive chromatin remodeling and genome-wide DNA methylation changes occur during early development. In addition, activation and maintenance

of specific epigenetic programs in somatic cells is essential for a normal developmental cascade. Genetic alterations in genes involved in epigenetic regulation influence the epigenome, and disruption of this process can be associated with several multifactorial and developmental diseases.⁵⁵

Mutations in the DNA methyltransferase *DNMT3B* result in genomic defects in DNA methylation patterns, causing immunodeficiency, centromere instability, and facial anomalies (ICF1, OMIM 242860) syndrome.⁵⁶ Most patients with ICF syndrome exhibit heterozygous missense mutations in the catalytic domain of the *DNMT3B* gene.⁵⁷ Loss of *DNMT3B* activity induces hypomethylation of satellite DNA-rich regions, which in turn alters gene expression due to abnormal scavenging of transcription factors, changes in nuclear architecture, and expression of ncRNAs.^{58–60} Similarly, mutations in the maintenance DNA methyltransferase *DNMT1* have been identified in two syndromes: hereditary sensory neuropathy with dementia and hearing loss (HSN1E, OMIM 614116) and autosomal dominant cerebellar ataxia, deafness, and narcolepsy (ADCADN, OMIM 604121),^{61,62} both of which are characterized by global genome-wide methylation defects.

Genetic alterations in genes involved in chromatin regulation also result in global epigenetic changes. Rubinstein-Taybi syndrome (RSTS1, OMIM 180849), Coffin-Lowry syndrome (CLS, OMIM 303640), Floating-Harbor syndrome (FLHS, OMIM 136140), and α -thalassemia/mental retardation syndrome (ATRX, OMIM 301040) are examples of diseases in which the molecular pathophysiology is related to the disruption of pathways involved in chromatin remodeling. RSTS1 is an autosomal dominant disorder characterized by intellectual disability, microcephaly, seizures, hypotonia, postnatal growth retardation, and heart defects. It is caused by mutations in the *CREBBP* (CREB-binding protein) gene located on chromosome 16p13.3.⁶³ *CREBBP* has been shown to control gene expression by inducing acetylation of histones H3 and H4 through HAT, and in turn decondensing chromatin and allowing gene transcription. It can also control gene expression by regulating the expression of HMT leading to hypertrimethylation, chromatin condensation, and in turn gene silencing.^{64,65} CLS is an X-linked condition characterized by cognitive impairment, microcephaly, short stature, soft fleshy hands, and characteristic facial features. CLS is caused by a mutation in the *RSK-2* gene, which also encodes a protein implicated in cell signaling through the CREB-binding protein.⁶⁶ Defects in the genes causing RSTS1 and CLS result in a generalized deregulation of DNA transcription, which in turn disorganizes protein production leading to dysfunctional neurodevelopment.¹³ In addition, mutations in the chromatin remodeling proteins *SRCAP* and *ATRX* cause FLHS and α -thalassemia X-linked intellectual disability syndrome (ATRX), respectively.^{67–70} Both FLHS and ATRX have been associated with multiple methylation defects. Interestingly, recent discoveries have identified an epi-signature for some of these conditions (manuscript in preparation). Such epi-signatures, which could potentially allow a relatively straightforward diagnosis of these conditions, can provide insights into the pathogenesis associated with mutations of genes involved in

epigenetics, highlighting the interplay between epigenetic signaling and gene function.

Another family of chromatin remodeling proteins possesses methylation binding domain (MBD) that interacts with methylated DNA to facilitate the recruitment of histone modifiers and chromatin remodeling complexes.⁷¹ Mutations in the *MeCP2* are involved in the pathogenesis of Rett syndrome (OMIM 312750). Rett syndrome is an X-linked neurodevelopmental disorder affecting primarily females whose symptoms include progressive neurological dysfunction with acquired microcephaly, loss of speech and purposeful hand use, ataxia, and stereotypic behaviors.⁷² *MeCP2* mutations causing Rett syndrome vary in their clinical relevance in that the location of mutations within specific functional domains of the protein has variable impacts on protein function and phenotype.⁷³

Mutations in genes involved in the histone modification, such as HAT and HDAC and HMT, are also associated with several genetic syndromes. For example, different mutations in the KAT6B acetyltransferase are associated with two rare syndromes, Genitopatellar syndrome (OMIM 606170) and Say-Barber-Biesecker-Young-Simpson syndrome (OMIM 605880).^{74,75} Heterozygous mutations in the HDAC4 deacetylase gene have been reported in Brachydactyly mental retardation syndrome (OMIM 600430), a complex disease with a wide spectrum of clinical symptoms.⁷⁶ In addition, mutations in the histone methyltransferases NSD1, EZH2, and EHMT1 cause Sotos syndrome (OMIM 117550),⁷⁷ Weaver syndrome (OMIM 277590),⁷⁸ and Kleeftstra syndrome (OMIM 610253),⁷⁹ respectively. Kabuki syndrome (OMIM 147920) is an autosomal dominant syndrome that may be caused by either mutation at histone methyltransferase MLL2 or by mutation at histone demethylase KDM6A.^{80,81} Finally, mutations at histone demethylase PHF8 and KDM5C cause Siderius X-linked mental retardation syndrome (OMIM 300263)^{82,83} and Claes-Jensen X-linked mental retardation syndrome (OMIM 300534), respectively.⁸⁴

In addition to the major epigenetic mechanisms of DNA methylation and histone modification, ncRNAs have been shown to play an important role in chromatin structure and gene expression.⁸⁵ MiRNA and siRNA have been reported to induce DNA methylation and histone modification, and in turn regulate gene expression at a transcriptional level.^{4,5,85,86} Consequently, dysfunction in the ncRNA machinery has been associated with many disorders, including cancer and neurodegenerative diseases.^{87–89} Furthermore, mutations in genes encoding proteins involved in miRNA pathway have been associated with multiple conditions including amyotrophic lateral sclerosis^{10,90,91} DiGeorge syndrome⁹² and autosomal dominant multinodular goiter.⁹³ Excellent reviews regarding the clinical and molecular characteristic of genetic syndromes of epigenetic machinery are available in the literature.^{94,95}

Methylation Defects in Monogenic and Complex Epi/Genetic Conditions

Mendelian monogenic disorders, like many that have been previously described, occur as a result of a mutation in a single gene. However, many monogenic disorders, with known

causative mutations, display little correlation between genotype and phenotype, suggesting therefore that other mechanisms of gene modification resulting in phenotypic expression must exist and that a mutation or polymorphism in a second gene or an epigenetic alteration affecting the function of other gene(s) can influence the phenotype caused by a monogenic mutation.⁴² On the other hand, complex multifactorial diseases are often caused by both genetic and environmental factors, which may affect specific gene–DNA methylation. The genetic basis underlining multifactorial diseases remain largely unknown, and lately the importance of epigenetic mechanisms has increasingly been appreciated. Two potentially overlapping hypotheses regarding genetic–epigenetic interaction have been posited in the context of monogenic and multifactorial diseases. First, whether genetic variants, such as point mutations, single-nucleotide polymorphisms (SNPs), and deletions/duplications, may alter the epigenetic patterns of a specific gene and could be used as a biomarker of the disease. Second, whether epigenetic, that is, more specifically DNA methylation, defects could play a significant role in the etiology of disease via alteration of gene expression.

Apart from the limited number of genetic variants in the genes encoding the epigenetic machinery, little is known about the influence of genetic mutations on the epigenetic marks in monogenic and multifactorial diseases. One straightforward mechanism that has been well described is the depletion of methylation sites by mutations or SNPs at CpG sites at gene promoters (see discussion below). If a mutation/SNP results in a specific loss of a methylation site, a modification in *cis* of the DNA methylation pattern is observed in a proximal DNA sequence.⁶ Chromosomal aberrations, such as deletions, duplications, inversions and/or translocations, can also induce inappropriate local rearrangements of the chromatin structure, affecting the normal expression of neighboring genes.⁹⁶ This is observed directly when genes encoding proteins involved in chromatin remodeling are mutated (for details, see section “Epigenetic Mechanisms”) but can also result indirectly from the effect of mutations that occur in proteins indirectly affecting chromatin structure. Finally, trinucleotide expansion mutations can cause structural modification in the DNA helix, which in turn induce DNA methylation of the mutated gene and result in gene silencing (for details, see section “Introduction”).

The association of SNPs with changes in DNA methylation has been widely reported, and it has also been suggested that DNA methylation may mediate the phenotypic effect of genetic variants in noncoding regions.⁹⁷ The *cis*-regulation of DNA methylation by SNPs has been found to alter the methylation mark in a single allele, also known as allele-specific DNA methylation (ASM). ASM at promoter CpG islands has been found to alter gene expression with the potential to affect phenotypic variation.⁹⁷ For example, a SNP situated in a CpG island within the regulatory region of *DYX1C1* (dyslexia susceptibility 1 candidate 1) has been shown to disrupt the CpG island, resulting in *DYX1C1* methylation and transcriptional inactivation.⁹⁸ In addition, studies have shown that SNPs and CpG islands are clustered in genomic regions with similar biologic pathways,^{99,100} creating population linkage between genetics and epigenetics.¹⁰¹

For example, the methylation of *PM20D1*, a gene previously associated with obesity and BMI, has been linked to SNPs in genes *SLC45A2*, *HERC2*, and *CTNNA2*; and both *PM20D1* methylation and these SNPs were identified as population stratification factors.^{101,102} Therefore, identification of DNA methylation changes associated with SNPs may provide evidence for a dynamic two-way interaction of genetic and epigenetic mechanisms involved in the regulation of gene expression and disease etiology.

The role of epigenetic processes in the etiology of a growing number of pediatric diseases has been described. First, evidence suggests that the pathogenesis of monogenic diseases may be modified by epigenetic factors. This has been well described in imprinting disorders, where the pathogenicity of a mutation in an imprinted gene depends on whether or not it is located in the expressed (nonmethylated) allele. In addition, DNA methylation at CpG islands has proven to represent an important epigenetic modification altering gene expression pattern, with changes in DNA methylation having apparent modifier functions in the context of some monogenic diseases. For example, in proximal spinal muscular atrophy (OMIM 253300 and OMIM 253550), which is an autosomal recessive neuromuscular disorder caused by mutations in the survival motor neuron gene 1 (*SMN1*), several differentially methylated candidate genes have been found associated with the disease severity.¹⁰³ Similarly, in facioscapulohumeral muscular dystrophy 1 (*FSHD1*; OMIM 158900), an autosomal dominant myopathy has been reported to be associated with epigenetic regulation. *FSHD1* results from contractions of the macrosatellite *D4Z4* repeat, which leads to the upregulation of *DUX4* gene. The pattern of DNA methylation at the *D4Z4* repeat correlates with disease manifestation, allowing the identification of *FSHD1* affected, *FSHD1* asymptomatic, and healthy controls.¹⁰⁴ Interestingly, in monogenic mental retardation disorders, human-specific clusters of CpG islands within candidate genes have been identified.¹⁰⁵ These CpG clusters represent regions of potential modulation by DNA methylation of contiguous genes, contributing to the etiology of mental retardation disorders by acting as a modifier of the phenotype.

In addition to monogenic diseases, complex multifactorial diseases can be associated with epigenetic factors and are the most common familial genetic diseases. They are caused by a multitude of genetic and environmental factors and, as discussed previously, both genetic and the environmental factors could impact on DNA methylation pattern. The role of DNA methylation in complex diseases is evidenced by recent studies of ASD. ASD is a neurodevelopmental disorder characterized by impairments in communication and reciprocal interaction and stereotyped patterns of interests and behaviors. There are numerous copy number variations that have been identified as risk factors for autism, including 7q, 15q, and 22q chromosome loci.¹⁰⁶ The terminal deletion at the chromosome 22q, known as 22q13.3 deletion syndrome, was studied in ASD patients, and a recurrent breakpoint within this region was found in the *SHANK3* gene.¹⁰⁶ *SHANK3* in the brain acts as a scaffold protein and plays a role in neuronal synapses. Deletions and point mutations in the *SHANK3* gene

have been associated with an increased risk for ASD.^{107–109} Recent studies have shown that DNA methylation is crucial for regulating *SHANK3* variant expression. *SHANK3* has five CpG islands in the promoter region and in body of the gene, which show a brain-specific methylation pattern.¹¹⁰ Analysis of the DNA methylation profile of the 5 CpG islands of *SHANK3* gene in postmortem brain tissue from ASD and control individuals revealed an increased overall DNA methylation in three intragenic CpGs.¹¹¹ The increase in DNA methylation in *SHANK3* was associated with altered isoform-specific expression of the *SHANK3* gene in 15% of ASD analyzed.¹¹¹ Thus, this evidence supports an association between expression of *SHANK3* gene and the involvement of epigenetic mechanisms in the susceptibility to ASD.¹¹²

Variant DNA methylation patterns have also shown to be involved in obesity and type 1-diabetes (T1D). A large-scale study of genome-wide DNA methylation found a strong association between body mass index (BMI) and methylation of *HIF3A* gene.¹¹³ *HIF3A* is a component of the hypoxia inducible transcription factor, which regulates oxygen tension in different physiological states and also plays a role in metabolism, energy expenditure, insulin sensitivity, and obesity. However, the association of methylation of *HIF3A* and BMI is not causal but occurs as a consequence of increased BMI.¹¹³ Methylation changes at genes previously related to obesity, including *PM20D1*, *MMP9*, *PRKG1*, and *RFC5*, were also associated with BMI.¹⁰² T1D has also been found to have an epigenetic component. An epigenome-wide association study found DNA methylation changes in genes known to be associated with T1D and immune function, such as *HLA-DQB1*, *NFKB1A*, *TNF* and *GAD2*.¹¹⁴ Differential DNA methylation at the insulin gene (*INS*) and at *FOXP3*, an important regulator of immune response, has also been associated with T1D, but it is not yet clear whether these epigenetic changes are the cause or consequence of diabetes.^{114–116} These findings suggest that DNA methylation signatures could be used as a disease marker, or for prognostic purposes, the same way as genomic SNPs have been associated with disease risk.

Environmentally Mediated Epigenetic Defects

Adverse environmental effects during growth and development can predispose infants toward childhood and adult diseases. It is well recognized that environmental stressors may include exposure of the fetus in utero, the neonate and/or the infant to alcohol, drugs, infection, malnutrition and maternal diabetes.^{117–121} Environmental exposure during gestation is an early externally mediated source of gene expression regulation that may confer risk to disease. Decades ago, documentation of the effect of the famines in the Netherlands and China demonstrated that poor maternal nutrition led to an increase in the adult incidence of several chronic diseases, including psychiatric, neurodevelopmental, metabolic, and heart diseases.¹²² More recently, with changes in current lifestyles, more pediatric diseases related to environmentally induced changes in epigenetic factors have been observed.¹²² For example, in utero exposure to maternal

obesity and gestational diabetes has been associated with an increased risk to develop metabolic disease and developmental delays in childhood. More specifically, exposure to maternal gestational diabetes (GDM) has been associated with future risk of childhood obesity and diabetes.¹¹⁸ More than 300 methylation variable positions in both placenta and cord blood of offspring born after GDM have been identified.¹¹⁸ These methylation changes include hypermethylation of the insulin signaling and metabolic genes during embryonic development, as well as hypermethylation of an imprinted gene, *GNAS*, previously associated with small-for-gestational age (SGA) presentation and with metabolic syndrome.^{123,124}

In addition to metabolic diseases (i.e., obesity and diabetes), ASD and attention deficit hyperactivity disorder (ADHD) have been associated with maternal metabolic conditions, such as obesity, hypertension, and diabetes.^{119,125} In these conditions, the adverse impact of environmental stressors has been reported to lead to developmental perturbation of epigenetic mechanisms regulating glial function, growth, and neural development.^{126,127} Among the several epigenetic mechanisms, studies have observed global changes in DNA methylation and changes in methylation at multiple imprinting regulatory regions, both in placental tissues as well as in the newborn, from pregnancies associated with obesity and diabetes.^{125,128} These findings suggest that DNA methylation and imprinting are important mechanisms in fetal growth and neurodevelopment and that disruption of this mechanism by environmental stressors can induce the development of pediatric neurodevelopmental and metabolic diseases.

Recent studies have also found an association between chronic obstructive lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD), and epigenetic programming during pregnancy and early development. Maternal obesity, inadequate maternal nutrition, fetal tobacco exposure, as well as infant diet and excessive weight gain have all been associated with chronic obstructive lung diseases.¹²⁰ The proposed underlying mechanism involves DNA methylation, which has been shown to regulate the expression of genes involved in the inflammatory immune response, such as *IL-4R* and *IL-13*, modulating the risk of asthma.^{129,130} Similarly, in COPD and idiopathic pulmonary fibrosis (IPF), a genome-wide methylation study has found differentially methylated regions in genes associated with immune and inflammatory system.¹³¹

Alcohol exposure during gestation is another example of environmentally mediated epigenetic variation. In utero alcohol exposure is associated with fetal alcohol syndrome (FAS), which is characterized by growth retardation, distinctive facial features, and brain damage.¹²¹ One study with neural stem cells demonstrated that alcohol retarded neuronal formation and induced a genome-wide diversification of DNA methylation.¹³² Studies in mice suggest that changes in DNA methylation may be mediated by acetaldehyde, which was found to decrease DNMT activity.¹³³ Alcohol was also found to interact with one-carbon metabolism, suggesting a link between ethanol and DNA methylation reactions.

Along with lifestyle-related environmental changes, the emergence of new technologies may also have unexpected

consequences on epigenetic patterning during development. Assisted reproduction technologies (ARTs), which involve manipulation and ex vivo culturing of early-stage embryos prior to implantation, have caught the attention of the medical community due to the potential importance of epigenetic effects on embryonic development. Manipulation of the embryo occurs during the same stages as imprinting patterns are being established. In one study, DNA methylation at the differentially methylated regions of the *IGF2/H19* and *IGF2R* loci was found to be altered in individuals conceived using ART.¹³⁴ Furthermore, ART has been associated with an increased incidence of the imprinting disorders, AS¹³⁵ and BWS syndrome.^{136,137} In addition, several mouse and rat studies comparing in vivo and in vitro fertilization have found variation in DNA methylation at multiple loci (for review, see reference¹³⁷), suggesting an association between ART and imprinting disorders. Screening for imprinting disorders in children conceived using ART would enable early detection of these rare syndromes.

Testing for Epigenomic Disorders in a Pediatric Population

Until recently, laboratory testing for epi/genetic conditions has been largely restricted to DNA mutation testing, micro-deletion analysis, and methylation-sensitive technologies of single gene loci. While DNA and chromosomal testing modalities are useful for the molecular diagnosis of direct, heritable changes to DNA sequences, specialized testing is required to detect epigenetic aberrations of DNA or chromatin modifications. In the clinical setting, epigenetic testing has been primarily restricted to DNA methylation analysis of either imprinted loci or loci known to be hypermethylated in specific disorders, such as the CGG repeat in FXS. Classical DNA methylation testing has been frequently done using either methylation-sensitive restriction enzymes or bisulfite sequencing. Both of these assays rely on differential modification of targeted DNA sequences depending on the methylation status of CpG dinucleotides at these loci. While these techniques are generally sensitive for detecting aberrant methylation patterns at the examined locus, it requires prior knowledge of which region to target and has limited application for detecting aberrant methylation elsewhere in the genome. More recently, genomic methylation arrays have become commercially available that enable simultaneous analysis of methylation patterns across the entire genome. Given the broad range of conditions associated with epigenetic changes, genome-wide DNA methylation screening has the potential to become a part of the routine diagnostic work-up for patients with wide range of congenital and/or developmental phenotypes. For example, genome-wide DNA methylation testing using the microarray-based technology has been successfully used by our laboratory to screen for common imprinting disorders (manuscripts in preparation), FXS (manuscript in review), and multiple genetic conditions with specific epigenomic signatures (manuscripts in preparation). Importantly, this comprehensive epigenomic screening can now be performed effectively at costs comparable to a single imprinting disorder test. In addition to increased

diagnostic yield and decreased cost for screening of known epigenetic disorders, epigenomic screening of patients with well-defined clinical phenotypes has a potential to enable discovery of novel epigenetic disease associations. While current genomic diagnostic approaches, including cytogenetic microarray and exome sequencing, focus on identification of pathogenic DNA defects that result in deregulation of gene expression and gene dosage, the effect of promoter methylation defects on gene expression, and consequent disease associations, particularly in pediatric disorders, remains largely unexplored.

Conclusion

Epigenetic gene modulation plays a significant role in the development of pediatric diseases through a range of mechanisms, including imprinting defects and environmentally induced change in gene-specific DNA methylation, as well as genetically induced alterations in the epigenetic machinery. DNA methylation has emerged as a key regulator of gene function and genomic stability during early stages of human development and cellular differentiation. As such, methylation defects have long been known to play a role in human diseases including imprinting disorders, trinucleotide expansion disorders, disorders caused by genetic defects of epigenetic regulatory genes, environmentally mediated complex disorders, and acquired (nonconstitutional) conditions including cancer.

Although there are still technological challenges associated with implementation of comprehensive epigenomic screening technologies in the routine clinical practice, particularly limited ability to interpret findings for genes with no current clinical associations, this is similar to the challenges faced with early adaption of cytogenetic microarray testing and exome sequencing. Some of the first adaptors of cytogenetic microarray technology in the early 2000 have used it as a replacement of the existing FISH assays that were available at the time. It was not until a decade later, largely due to the retrospective assessment of clinical-genomic associations discovered in the clinical service laboratories, that the full potential of cytogenetic microarray screening technology was unlocked. Similar scenario is currently unfolding in respect to clinical exome sequencing, and we anticipate that clinical genome-wide DNA methylation screening will evolve similarly.

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