Future Prospects for Epigenetics in Autism Spectrum Disorder

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Abstract
Despite decades of investigation into the genetics of autism spectrum disorder (ASD), a current consensus in the field persists that ASD risk is too heterogeneous to be diagnosed by a single set of genetic variants. As such, ASD research has broadened to include assessment of other molecular biomarkers implicated in the condition that may be reflective of environmental exposures or gene by environment interactions. Epigenetic variance, and specifically differential DNA methylation, have emerged as areas of particularly high interest to ASD, as the epigenetic markers from specific chromatin loci collectively can reflect influences of multiple genetic and environmental factors and can also result in differential gene expression patterns. This review examines recent studies of the ASD epigenome, detailing common gene pathways found to be differentially methylated in people with ASD, and considers how these discoveries may inform our understanding of ASD etiology. We also consider future applications of epigenetics in ASD research and clinical practice, focusing on substratification, biomarker development, and experimental preclinical models of ASD that test causality. In combination with other -omics approaches, epigenomics allows an improved conceptualization of the multifactorial nature of ASD, and opens future lines of inquiry for both basic research and clinical practice.

Key Points

Current knowledge of the autism spectrum disorder (ASD) epigenome implicates gene pathways that may contribute to condition development. Application of epigenomics to the fields of patient substratification, biomarker identification, and causality determination hold the potential to improve patient outcomes and understanding of heterogeneous ASD etiologies.

1 Introduction

Autism spectrum disorder (ASD) is a diagnosis that encompasses a group of neurodevelopmental disorders characterized by persistent impairments in social interaction and language, together with repetitive patterns of behavior and interests [1]. ASD has been consistently observed to have a male bias, with ASD being reported as 4.2 times more prevalent in male children than female children [2]. Prevalence of ASD has been increasing in the US since the Centers for Disease Control and Prevention (CDC) first began monitoring in 1996, with the most recent surveillance report observing ASD prevalence at 23.0/1000 children (1 in 44 children) [2]. However, due to increased awareness of ASD and changing criteria for ASD diagnosis over this time frame, debate persists over whether this increase in prevalence is the result of biological or sociological factors [3–5]. Nonetheless, the sheer magnitude of the rise in ASD prevalence in recent decades warrants consideration of biological explanations for this increase, including consideration of environmental risk [5–8].

Parsing how much of the increase in ASD prevalence is attributable to environmental risk is made difficult by ASD being both highly heterogenous in its etiology and dependent
on diagnosis through behavioral assessment. While ASD is defined by the presence of both social/language impairments and repetitive behaviors, these two parameters can be particularly difficult to assess in children under 3 years of age, leading to high potential for changing diagnosis even when adhering to ‘gold standard’ diagnostic tools such as the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) [9]. Furthermore, 70% of individuals with ASD also have psychiatric comorbidities (including social anxiety and/or attention deficit and hyperactivity disorders), whose own behavioral traits can mask ASD [9, 10].

Given the difficulties of behavioral diagnosis of ASD, studies of the molecular etiology of the condition have attracted interest for their potential to identify quantifiable biomarkers that can act as metrics of ASD severity. Studies of ASD heritability have firmly established a genetic component of ASD etiology, with a 58–90% concordance rate in monozygotic twins [11, 12]. However, heritability estimates as well as specific genetic risk factors for ASD vary dramatically between studies, with insufficient evidence to designate a singular set of ASD-specific genetic risk factors [13–16]. Epigenetics, literally meaning ‘on top of genetics’, is defined as alterations to DNA or chromatin that can alter gene expression without a change to the DNA sequence. DNA methylation, which collectively refers to methyl groups added at cytosine bases in DNA, is the most investigated epigenetic mark in human tissues and will be the focus of this review. As a multifactorial disorder, ASD development is often a result of the interplay between genetic and environmental factors, which is what DNA methylation marks are frequently reflecting. Thus, a complete understanding of the molecular etiology of ASD requires knowledge of both genetics and epigenetics associated with the condition.

The genetic components of ASD risk have been thoroughly covered in prior publications [16–20]. As such, this review will focus on recent advances in the comparatively young field of ASD epigenetics, with the specific goal of envisioning future prospects of improved diagnosis and targeted behavioral therapy.

### 2 Autism Spectrum Disorder Epigenetics

The insufficiency of solely considering genetic risk factors for the purpose of understanding ASD etiology has prompted a plethora of investigations into the environmental factors contributing to ASD development [21]. Various maternal medical conditions, including maternal prepregnancy body mass index (BMI) [22], diabetes [23], and hypertension [24], have been shown to increase the risk of ASD. Perinatal exposure to some common environmental pollutants, such as air pollution [25], and pesticides [26] have also shown an association with increased ASD risk. Some differences in maternal nutrients, including maternal iron supplement consumption [27] and maternal use of folic acid and multivitamin supplements [28], have also been shown to have potential protective effects against ASD development. These environmental exposures are often hypothesized to act at least partially through epigenetic mechanisms [29–33], although the tissue-specific and temporally influenced nature of epigenetic modifications can make repeated observation of epigenetic markers of ASD difficult. In spite of these inherent challenges, some progress has been made in identifying DNA methylation patterns from multiple tissue sources that distinguish ASD from typically developing controls (Fig. 1).

The neurodevelopmental origin of ASD makes post-mortem human tissue the most relevant tissue to provide direct epigenetic links between environmental exposures and altered genes in ASD. Insights gained from epigenetic changes associated with ASD in the brain therefore serve as the standard against which epigenetic data from other tissues are compared. Epigenomic analysis of post-mortem ASD brain DNA has focused largely on the cortex and cerebellum, as these regions of the brain have been implicated in prior examination of neural abnormalities associated with ASD [34–38] and are more commonly available from public brain banks. Among the most common methods for studying ASD epigenetics is the methylation array, which is manufactured to target regions of epigenetic interest in humans and uses hybridization-based fluorescent signaling to determine whether a given target nucleotide is methylated [39]. Despite the relative scarcity of available ASD brain tissue for investigations, methylation array-based analyses of epigenetic changes in the ASD brain have revealed a large number of differences at individual CpGs or differentially methylated regions (DMRs). These analyses rarely identify global differences in DNA methylation between ASD and control brains, regardless of the brain region surveyed.

Cerebral cortex has been the brain tissue of focus for multiple array-based ASD methylome analyses. For instance, Wong et al. extracted DNA from post-mortem prefrontal cortex and temporal cortex to perform region comethylation network analysis, revealing multiple comethylation modules significantly associated with ASD diagnosis [35]. Gene ontology (GO) pathway analysis of these comethylated modules revealed them to be enriched for pathways relating to immune functionality, synaptic signaling and regulation, and postsynaptic density, with these enrichments reflecting the known dual contributions of immune and neuronal dysregulation associated with ASD [40, 41]. Using an alternative approach of whole-genome bisulfite sequencing (WGBS), Vogel Ciernia et al. found similar gene pathway enrichments in ASD cortex, focusing on microglia as the source of the inflammatory signal [42]. Other
array-based methylomic studies of ASD cortex have made similar findings. Ladd-Acosta et al. reported three DMRs in ASD temporal cortex, with these DMRs being proximal to genes involved in neuronal and immune pathways as well as maintaining imprinting patterns [43]. Nardone et al. identified differentially methylated CpGs in ASD prefrontal cortex samples, with GO analysis revealing hypomethylated CpGs enriched for immune functions and hypermethylated CpGs enriched for neuron-neuron synaptic activity [44]. Further ASD methylomic analysis of the sorted nuclei from frontal cortex neurons revealed differential regions of comethylation that were enriched for synaptic, neuronal, GABAergic and immune processes [45].

Methylomic analyses of cerebellum has shown similar general findings, with differences in genic locations of ASD differences. Corley et al. performed DNA methylation analysis of the subventricular zone, a component of the cerebellum that serves as a source of neuronal stem cells, and reported differentially methylated loci (DML) in ASD samples [37]. The observed DML were underrepresented at promoter regions and overrepresented at gene bodies and intergenic sequences, with GO analysis of the ASD-related DMLs showing enrichment of genes involved in neuron proliferation, differentiation, and migration. A targeted investigation of the ASD-associated and neurodevelopmentally
linked EN2 gene by James et al. identified significant hypermethylation in the EN2 promoter region of ASD cerebellum samples relative to non-ASD samples [46]. In addition to ASD DMRs being identified in the cerebellum, differentially hydroxymethylated regions (DhMRs) have also been observed. Hydroxymethylated cytosines are the result of Tet protein interaction with methylated cytosines, and have been implicated to function in a manner unique from methylated cytosines in some biological functions [47]. Cheng et al. screened for DhMRs between ASD and non-ASD cerebellum and identified 797 DhMRs [48]. GO analysis of the 181 DhMRs that were identified in intragenic regions were found to be significantly enriched for ASD-implicated pathways such as ‘cell-cell communication’ and ‘heparan sulfate biosynthesis’, and cis functionality of the 599 intergenic DhMRs associated with ASD was predicted.

While studies of ASD-specific differential methylation in the brain are limited due to low sample availability, methylomic analysis of peripheral tissues does not suffer this same limitation. A wide range of investigations into DNA methylation patterns associated with ASD in non-neuronal tissue have been performed, with many of these studies using either ASD blood or maternal placenta as sources of DNA. While these peripheral tissue studies are less informative about consequential methylomic changes associated with ASD than studies on brain tissue, blood methylation levels at specific loci have been shown to be correlated with brain tissue methylation in prior studies [49, 50], and placental methylation levels have likewise been shown to associate with child ASD diagnosis [51–54].

Blood samples from individuals diagnosed with ASD versus matched controls offer an easily accessible sample type for interrogating the ASD epigenome, but questions have been raised about how well alterations in blood reflect the brain. ASD blood samples were demonstrated to have an impaired metabolic capacity for methylation [55], sparking further interest. Investigations of potential associations between blood DNA methylation and ASD have shown modest associations [56–58]. Of particular note is the meta-analysis of Andrews et al., where the authors conducted the largest methylome-wide association study on blood DNA ever performed, using samples from 1654 children [56]. No single CpG surveyed in this study was identified as being significantly associated with ASD, although seven CpGs passed the much lower threshold for suggestive association. A study from a fairly small subset of ASD-discordant monzygotic twin pairs showed some significant differentially methylated genes, with the significance of association decreasing when comparing ASD with non-ASD individuals more broadly [59]. Furthermore, the methylation chip used in this study screened for nearly 19x fewer CpG sites than the chips used in future studies, including Andrews et al. [60]. While blood DNA methylation profiles may well contain ASD-associated DMRs in small subsamples of highly genetically similar individuals, contemporary studies have shown them to be ill-suited to examining large and heterogenous groups.

In contrast to the somewhat limited utility of blood methylation studies from ASD patients, recent prospective investigations into the methylome of ASD placental, cord blood, and maternal blood samples have identified some consistent epigenetic changes that exist prior to the diagnosis of ASD. This is not particularly surprising given the importance of the placental in utero environment for fetal neurodevelopment [61, 62]. Mordaunt et al. performed WGBS of cord blood from a high-risk ASD cohort and identified 2299 DMRs across male and female samples, with ASD-associated DMRs being significantly enriched for methyl-sensitive developmental transcription factors that play a role in fetal neurodevelopment [63]. Sex-stratified DMRs associated with ASD were found to be highly enriched for X chromosome location, implying a potential epigenetic etiology to ASD’s male bias. Further implication of the role of the methylome in ASD development was reported by Zhu et al., where the authors performed WGBS of placenta samples and observed an ASD-associated 118 kb hypomethylated region at 22q13.33 [54]. This hypomethylated region was comprised of ASD-associated DMRs that were significantly enriched for multiple fetal brain enhancers and a novel gene named NHIP (neuronal hypoxia-inducible, placenta-associated). Bakulski et al. assessed the methylomes of both cord blood and placenta, finding nominally ASD-associated differentially methylated positions (DMPs) that correlated across both tissues [53]. Additionally, these DMPs were highly enriched for SFARI ASD risk genes. The authors also assessed the methylation of maternal whole blood DNA and found ASD DMPs that overlapped with those identified in placenta and cord blood. These results contribute to emerging evidence of conserved regions of cross-tissue methylomic profiles associated with neuropsychiatric disorders such as ASD [50, 51, 63, 64].

It is noteworthy that much of the existing methylomic analyses of ASD DNA (both in brain and peripheral tissues) are limited by the relatively narrow focus of the commercially available methylation array platforms. The vast majority of contemporary methylomic studies assess methylation with Illumina HumanMethylation450 or Infinium MethylationEPIC platforms, which provide coverage of only approximately 1.5–3% of all CpG sites in the human genome [60]. The targeted regions were determined by expert panels prior to whole methylome maps based on sequencing, introducing additional bias for particular regions of the genome with the least variation in methylation [65, 66]. As an example, > 80% of the ASD-associated DMRs identified in cord blood by WGBS did not overlap with a single probe represented on the Illumina HumanMethylation450 or Infinium
Future Prospects for Epigenetics in ASD

3 Application of ASD Epigenetics in Substratification

The heterogeneity of symptomatic presentation in ASD poses a challenge to both researchers and clinical practitioners. While all ASD cases are broadly diagnosed by the presence of impairments in social behavior, language, and repetitive behavioral patterns, the manifestations and severity of these symptoms can vary substantially between individuals. Furthermore, the potential for common comorbid psychopathologies produces great difficulty in attempting to generalize studies of ASD individuals to the broader population [67, 68]. In response to this problem, clinicians and scientists have often attempted to stratify ASD individuals into subgroups for the purpose of studying more distinct manifestations of ASD, such as those individuals with or without intellectual disability. The identification of reliable, consistent subtypes of ASD reflective of psychological and biological phenomena has transformative potential, as it would enable more highly targeted studies of specific pathways involved in the manifestation of subphenotypes. Such an approach may also highlight subtype-specific treatment outcomes from behavioral intervention therapy with ASD youth [69, 70].

Unfortunately, review of an assortment of subtype substratification studies reports that many identified ASD subtypes are insufficiently verified by more than two strategies [71]. Furthermore, many employed substratification methods have been found to be over-reliant on symptom scores and cognitive measures, with few adequately including sensory processing and biological variables [72].

The incorporation of ASD-associated methylation patterns into substratification criteria, in combination with other data such as transcriptomics or metabolomics, has the potential to substantially enhance the biological considerations of substratification criteria. This is especially true with regard to discerning subtypes of idiopathic ASD, defined as cases of ASD arising from unknown causes [73]. In contrast to occurrences of ASD where a specific cause can be identified (known as syndromic ASD), idiopathic ASD describes the approximately 85% of ASD cases arising from an unknown combination of genetic and environmental factors. Epigenetic analyses are uniquely well-suited to uncover the mechanics of gene-environment interactions, and have been highlighted in reviews of neurodevelopmental conditions such as ADHD and anxiety disorders as a likely origin of condition risk that cannot be directly explained by genetic variation [74, 75].

While few studies have attempted to incorporate epigenetic data into ASD substratification, preliminary investigations of the epigenetic profiles associated with phenotypic ASD subtypes offer support for an epigenetic component in substratification criteria. Lee and Hu compared methylation profiles across three phenotypically distinct subtypes (severe language impairment, intermediate, and mild) of ASD lymphoblastoid cell lines, and observed that a greater number of significant DMRs were detected when comparing control cell lines with a specific phenotype subtype than were detected when comparing control cell lines to all pooled ASD samples [76]. Furthermore, functional analysis of subtype-specific DMRs revealed those DMRs to be enriched for subtype-specific biological pathways. For example, severe language impairment ASD DMRs were shown to be highly enriched for neuritogenesis genes, while the intermediate ASD DMRs were heavily populated with neuroinflammation genes and the mild ASD DMRs included genes involved in sensory system development. Epigenetic investigation of ASD substratification holds the potential to uncover subtype-specific insights into ASD etiology that will enable researchers and clinicians alike to better understand the molecular dynamics of ASD’s various manifestations and etiologies (Fig. 2). In reducing the heterogeneity of ASD, these approaches may also empower large-scale ASD differential methylation analyses of peripheral tissue to uncover subtype-specific DMRs, even in the absence of more generalizable ASD DMRs [56, 58].

4 Epigenetic ASD Biomarkers

ASD’s heterogeneity of presentation can also contribute to difficulties in diagnosis and treatment, especially for younger children. The average age of clinical diagnosis has been found to be 4–5 years [77, 78], although it is fairly common for youth with ASD with poor access to health care services to remain undiagnosed until early adolescence [79]. This is particularly concerning given the body of evidence indicating early...
behavioral interventions can confer substantial improvements to ASD children’s social, vocal, and adaptive skills that translate into increased thriving later in life [80–82]. Such interventions have already been proven to reduce symptom severity, and sometimes even diagnosis rates, when administered at approximately 12 months of age [83, 84].

One strategy to lessen the consequences of delayed ASD diagnosis is to identify ASD biomarkers that are predictive of risk for ASD prior to diagnosis. Infants at a higher risk could be offered behavioral intervention, followed up with behaviorally based ASD diagnoses at 3–5 years of age, a time when diagnosis is more informative (Fig. 3). Extensive studies in this field have identified a broad spectrum of ASD biomarkers across various sample types, but have generally failed to identify a consistent set of genetic differences collectively associated with ASD diagnosis [85–87]. In light of this ‘missing heritability problem’ [88], epigenetic biomarkers of ASD have been investigated as alternative predictive features for ASD diagnosis. Studies focusing on biomarkers detectable before the typical ASD diagnosis window hold particularly high value for potential ASD intervention, and some early investigations have shown success in this field.

Reports of significant ASD-associated methylation biomarkers detected early in development from accessible peripheral tissues, including buccal swabs [89] and placenta [51, 54], could be implemented in ASD risk factor calculations, thereby giving clinicians additional levels of evidence when attempting to make an early ASD diagnosis. Furthermore, the development of methods for assessing ASD biomarkers prenatally through the assessment of cell-free fetal DNA (cffDNA) would provide a window of opportunity for in utero interventions. CffDNA is derived from the placenta, representing circulating fetal genetic material deposited in the maternal bloodstream throughout gestation [90, 91]. This potential value is highlighted in a preprint by Laufer et al. describing how longitudinal cffDNA DMRs in a macaque model of maternal obesity was associated with behavioral measure and brain methylation patterns of offspring [92]. Additionally, these cffDNA and brain DMRs associated with maternal obesity were found to significantly overlap with DMRs in human ASD brain and placenta, a result that aligns with established knowledge of maternal obesity as an ASD risk factor [22, 93]. Larger studies searching for prenatal cffDNA epigenetic biomarkers of ASD in humans may likewise identify consistent signals associated with the likelihood of ASD development. Working in combination with established postnatal ASD biomarkers, these new tools would empower earlier and more accurate ASD diagnosis, and in turn allow clinicians to begin administering behavioral intervention therapies in the developmental window where they are proven to be most effective.

5 Direct Investigation of the Epigenetic Mechanisms of ASD

While additional investigations of existing associations between epigenetics and ASD will provide actionable results that support clinical practices such as substratification and...
biodocerative biomarker development, they are fundamentally limited in the extent to which these correlative findings can uncover the direct mechanisms by which epigenetic alterations influence ASD phenotype. By their very nature, association studies of epigenetic alterations and ASD can only report differential epigenetic layers that associate with ASD phenotype. Even more direct interventions, such as knockout of chromatin-modifying enzymes [94, 95], are complicated due to the broad range of non-explicitly epigenetic functionality observed in such proteins [96–98]. Thus, these studies are capable of identifying loci whose epigenetics may play a role in ASD development but are incapable of identifying true causality [99].

However, the rapid proliferation of epigenetic editing technologies has begun to make casual studies between epigenetic alterations and phenotypes feasible [100–102]. Many laboratories have generated fusion proteins where a given deactivated DNA-targeting protein such as dCas9 is ligated to an effector domain capable of making chromatin or DNA methylation modifications. These fusion proteins are then introduced to a given organism, such as a mouse model or human cell line model, and researchers directly observe how the engineered modifications result in changes to organism phenotype [103]. Rather than being merely associative, these investigations using direct epigenetic editing of a locus make possible observations of causality (Fig. 4).

Applied to ASD, these methods can prove helpful in narrowing the scope of epigenetic investigations to loci shown to have direct phenotypic impacts in other models. While human epigenetic editing of loci implicated in ASD remains clinically difficult [104] and ethically dubious [105, 106], epigenetic mechanism experiments in non-human models of ASD can reveal the degree to which a given locus’s chromatin conformation directly contributes to the ASD phenotype. Considering the growing list of epigenetically divergent loci associated with ASD, filtering for proven high-impact epigenetic variants will enable researchers to focus their studies on regions whose epigenetic profile is likely to be most impactful in endeavors such as substratification and biomarker discovery.
For example, a plethora of preclinical mouse models of rare genetic syndromic forms of ASD have been generated that display novel social, communicative, and behavioral symptoms [107]. While investigations of ASD epigenetic mechanism in these models have been undertaken using enzymatic inhibition [108–110], an additional degree of mechanistic certainty of the role a particular epigenetic mark plays in ASD development could be acquired by making precise epigenetic edits to loci of interest and observing downstream effects on both the molecular and phenotypic level. Lu et al. describe an early example of dCas9-based epigenetic investigation, with the authors reporting targeted methylation of the MECP2 transcription start site in mice-induced deficits in social behavior [111]. These tools could also be employed in developed ASD mouse models to study the potential for phenotypic rescue. Furthermore, one could imagine a study applying the CRISPRoff tool designed by Nuñez et al. to selectively repress loci of interest in the Myt1l+/- mouse model of ASD developed by Chen et al. [101, 112]. The Myt1l+/- mouse model was shown to replicate ASD-related social impairments in males, and also had greater chromatin accessibility in some regions of cortex than wild-type mice. By using an epigenetic editor such as CRISPRoff to target some of these more accessible regions in the Myt1l+/- mouse model, and observing any changes in ASD-related trait severity, direct hypotheses as to whether the action of the MYTIL gene in mouse ASD-like pathways is explicitly epigenetic could be tested. Such conclusions could then inform future human studies.

Unfortunately, the heterogeneous origins of idiopathic ASD makes it a more challenging target for direct model generation and subsequent mechanistic investigations [113]. Nonetheless, some attempts to generate non-syndromic rodent ASD models have been mildly successful at reproducing ASD phenotypes [114–116]. These non-syndromic models exhibit a wide range of neurodevelopmental impacts caused by environmental exposures and accumulation of numerous mutations implicated in altered neurodevelopment. These multifactorial etiologies mirror the etiologies of many idiopathic cases of ASD in the US. While limited in their direct translation to humans, non-syndromic ASD rodent models could offer a valuable tool for refinement for epigenetic variants of interest in future studies.

6 Conclusions

ASD is one of the most common neurodevelopmental conditions in the US, and understanding its etiology requires epigenetic investigations at the interface of genetics and environment. The elucidation of differentially methylated gene pathways in ASD brain and peripheral tissues has substantially improved our knowledge of ASD epigenetics, and future studies will build upon this knowledge in basic research clinical application. While this review focuses on a small handful of potentially high-impact applications for epigenetics in the ASD field, others exist that are not detailed in this article. Incorporated together with other -omics informed technologies and predictive machine learning algorithms, epigenetics can facilitate the next-generation of ASD care, informing healthcare decisions from gestation to adulthood.

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Conflicts of interest Logan A. Williams declares no conflicts of interest. Janine M. LaSalle has submitted a patent application on ‘Methods and Compositions for Determining Risk of Autism Spectrum Disorders’.

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