Development and disease in a dish: the epigenetics of neurodevelopmental disorders

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Human neurodevelopmental disorders (NDDs) involve mutations in hundreds of individual genes, with over-representation in genes encoding proteins that alter chromatin structure to modulate gene expression. Here, we highlight efforts to model these NDDs through *in vitro* differentiation of patient-specific induced pluripotent stem cells into neurons. We discuss how epigenetic regulation controls normal cortical development, how mutations in several classes of epigenetic regulators contribute to NDDs, and approaches for modeling cortical development and function using both directed differentiation and formation of cerebral organoids. We explore successful applications of these models to study both syndromic and nonsyndromic NDDs and to define convergent mechanisms, addressing both the potential and challenges of using this approach to define cellular and molecular mechanisms that underlie NDDs.

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The human cerebral cortex is a complex and highly organized structure with stereotyped neuronal circuitry, which integrates responses to myriad stimuli through balanced electrophysiological activity throughout life. Formation of the cortex during embryonic and fetal development involves ordered processes that allocate pluripotent early embryonic cells to the neural plate, specify telencephalic regional identity at its rostral end, generate progenitors with regionally restricted identity, drive their differentiation into distinct neuronal cell types, control their proper integration into circuits, and modulate neuronal function through later life [1,2].

The complexity of corticogenesis and the extended time frame involved renders it highly sensitive to disruption, which contributes to neurodevelopmental disorders (NDDs) including autism spectrum disorder and intellectual disability (ASD/ID) syndromes, schizophrenia, bipolar disorder, and inherited epilepsies [3]. Recent genomic sequencing efforts have cataloged many human genetic variants that contribute to the pathogenesis of these NDDs [4–10]. Although some syndromic NDDs involve mutations in a single gene, genetic susceptibility to most NDDs appears more complex; a heritable component can be defined, but hundreds of individual genes are implicated, with each potentially pathogenic mutation occurring in only a small fraction of cases. Further underscoring the complex etiology of these disorders, it remains unclear how relative contributions of pathogenic mutations interact with other aspects of an individual patient's genetic background, and with nongenetic or environmental contributors, to cause these NDDs [11–14].

Examining classes of likely or confirmed highly penetrant pathogenic gene mutations that contribute to NDD risk has been informative. Some susceptibility genes encode proteins involved in neuronal function, such as ion channels and signal transduction pathways involved in synaptic function or neurotransmitters and their receptors. Mutations in these genes would be predicted to alter the function of neuronal circuits, contributing to NDDs. However, perhaps unexpectedly, many confirmed or suspected mutations implicated in NDD causation are in genes encoding proteins that regulate chromatin structure [4,5,15,16]. Such 'epigenetic' regulatory proteins and complexes act on the nucleosomal histone octamer and genomic DNA architecture of chromatin to modulate gene expression.

For example, in ASD/ID and epilepsy, recent unbiased genome-wide studies have revealed contributory mutations in many genes that encode proteins that remodel chromatin, control histone methylation and demethylation, and recognize methylated DNA, as well as in histone variants that can alter chromatin accessibility [5,17]. Potentially pathogenic variants in genes that regulate chromatin during early development are highly over-represented in ASD probands but not their unaffected siblings, with some estimates suggesting that half of the genes with a high confidence link to ASD are involved in epigenetic regulation [4]. These findings implicate epigenetic regulation of neuronal development and/or function as a central contributor to nervous system dysregulation in NDDs. However, in most cases, neither the role of these epigenetic regulatory proteins in normal neurodevelopment nor how their disruption contributes to NDD etiology is well understood. This review focuses on how these questions are being addressed by using *in vitro* differentiation of human neurons derived from patient-specific induced pluripotent stem cells (iPSCs), which provide an experimentally tractable model for defining cellular and molecular mechanisms that underlie NDDs.

Formation & patterning of the mammalian telencephalon

The mammalian cortex is built by stepwise developmental processes that begin with formation of the neural plate during the third week of human embryogenesis [18,19]. Many aspects of corticogenesis have been extensively studied in mouse models and appear to be conserved in primates, including humans. Therefore, while the structure and patterning of the rodent and primate cortex differs in several respects, we have briefly summarized central, conserved aspects of corticogenesis below, to provide a context for describing how these processes are presently modeled with human iPSC-derived neurons and organoids *in vitro*. For an in depth description of human cortical development, we refer the reader to excellent reviews on this topic [2,18].

Neurodevelopment begins in early embryogenesis when pluripotent embryonic cells acquire distinct fates through differential exposure to growth factor signaling. Signaling through the TGF- β pathway induces non-neural, mesendodermal fates, while antagonism of this growth factor signaling enables induction of the neural plate. As development proceeds, the neural plate acquires regional identity along its rostral-caudal axis, delineating territories that form the brain and spinal cord. The neural plate also develops into the neural tube, through morphogenetic changes whereby a central groove forms, surrounded by two neural folds, which elevate and fuse at the dorsal midline. Dorsal BMP and Wnt signaling and ventral SHH signaling centers control the expression of transcription factors that confer dorsal-ventral regional neural identity along the extent of the neural tube. Within the forebrain, this regionalization generates a dorsal telencephalic progenitor territory that will develop into the cerebral cortex, which expresses genes encoding transcription factors such as *EMX1/2*, *PAX6*, and *NEUROG1/2*, and ventral telencephalic progenitor domains enriched for expression of different transcription factors such as *DLX1/2*, *NKX2-1*, *ISL1*, *SP8*, and *ASCL1* (Figure 1) [18,20,21].

Following the establishment of regional neural progenitor domains within the developing telencephalon, neurogenesis drives their differentiation into distinct neuronal cell types and subsequent assembly into circuits. Prior to cortical neurogenesis in the dorsal telencephalon, neural stem cells (NSCs) proliferate in a simple neuroepithelium at the rostral end of the neural tube through symmetric cell divisions. These divisions generate a layer of cells called the ventricular zone (VZ), proximal to the underlying ventricle. Neurogenesis is initiated around gestational week 5 in humans [1,18], as VZ NSCs downregulate epithelial markers and transition into asymmetrically dividing neural progenitor cells (NPCs) called radial glia (RG) [18,22]. RG can divide such that one daughter cell remains in the VZ to maintain the RG population, while the other differentiates, generating neuronal and glial cortical progenitors. RG progenitors remain attached to both the ventricular and pial surfaces of the developing cortex, serving as a scaffold for migration of differentiated cortical neurons. Differentiating RG migrate into a dorsally located sub-ventricular zone, forming progenitors with more restricted potential and giving rise to postmitotic neurons, which form the six layers of the cortical plate in an inside-out manner [1]. In the ventral telencephalon, neural progenitors likewise acquire regional identities, forming the ganglionic eminences, from which GABAergic interneurons derive (Figure 1). Activities of ventrally enriched transcription factors drive specification and differentiation of these future inhibitory interneurons, which then undergo a long-range tangential migration to the cortex and establish circuits with cortical excitatory neurons to modulate their activity [23].

Normal progression of neurodevelopment through these complex events results in the formation of a cortex of the proper size and with stereotypical patterning, in which circuits formed by cortical excitatory neurons and inhibitory interneurons can properly modulate functional output, maintaining a finely tuned balance between neuronal excitation and inhibition. Corticogenesis in humans involves an extended developmental period encompassing

Figure 1. Modeling neurodevelopmental disorders with patient-specific iPSC-derived neurons. (A) Schematic depicting several stages of human neural development. Crown to rump length of the embryo is approximately 1.5 mm at 3 weeks, 5 mm at 5 weeks, and 140 mm at 15 weeks of fetal development. Inset at right schematizes a coronal section through the 15-week telencephalon along the plane shown in red, highlighting dorsoventral patterning of the telencephalon into the cortex and ganglionic eminences. Layers of the developing cortex are abbreviated as follows: VZ = ventricular zone; SVZ = subventricular zone; CP, SP, IZ = cortical plate, subplate, intermediate zone. Cortical radial glial neural progenitors in the ventricular zone express the marker *PAX6* and differentiate into glutamatergic excitatory neurons. The MGE and LGE are demarcated by expression of *NKX2-1* or *ISL1/SP8* and differentiate predominantly into cortical interneurons and striatal projection neurons, respectively. **(B)** *In vitro* neuronal differentiation of patient-derived iPSCs. Fibroblasts are obtained from both patients with NDDs and related or unrelated healthy individuals as controls and are reprogrammed to iPSCs expressing the pluripotency marker *OCT4/POU5F1*. These can undergo directed differentiation in 2D culture to generate progenitors and differentiated neurons that resemble types found in the developing cortex, including glutamatergic cortical excitatory neurons and GABAergic cortical interneurons, and can also undergo directed differentiation in 3D culture to form cerebral organoids. **(C)** Using *in vitro* differentiated neurons to assess the effects of mutations that cause NDDs. A wide range of parameters can be assessed and phenotyping can be combined with genetic manipulations, genomic analysis methods, and high throughput chemical and molecular screening approaches. Scale bars for the iPSC, cerebral organoid, and neuron immunofluorescence images = 50 μ m. iPSC: Induced pluripotent stem cell; LGE: Lateral ganglionic eminence; MGE: Medial ganglionic eminence; NDD: Neurodevelopmental disorder; Tel: Telencephalon.

much of gestation, with continued refinement and maturation of cortical structure and function through childhood and adolescence [1]. Accordingly, many aspects of these developmental programs are highly sensitive to disruption, which alters normal cortical function and contributes to NDDs. These disruptions can involve alterations in the number of neurons that contribute to particular brain structures, alteration of the time windows during which NSC expansion, specification, and differentiation occur to form particular neuronal types and brain structures, and often interrelated later alterations in cortical size, patterning, neuronal composition, and circuit establishment and function, any of which can affect the balance between neuronal excitation and inhibition and can contribute singly or in combination to the emergence of NDDs [24].

Epigenetic regulation of neural development & contributions of its dysregulation to NDDs

As described above, many disease-causing variants in NDDs have, perhaps surprisingly, been found in genes that encode epigenetic regulators. During cortical excitatory and inhibitory neuron development, the interplay between transcription factors and the epigenetic machinery has a central role in establishing and maintaining regulatory networks that drive neuronal specification and differentiation programs. Accordingly, many gene mutations that perturb these regulatory programs appear to alter corticogenesis in some manner, contributing to NDDs. The term epigenetics generally refers to changes in gene expression and function that do not involve changes in the DNA sequence [25]. This typically involves post-translational modification of histone proteins or methylation of DNA, but can also include the activities of noncoding regulatory RNAs. Several classes of proteins are key regulators of these processes of epigenetic modification, including chromatin remodelers and histone and DNA methylases and demethylases. Below, we outline some roles of these modifiers in neural development, to provide a context for studying how their perturbation may contribute to NDDs. As an example, subsets of genes encoding epigenetic regulatory proteins in these classes that are mutated in ASD are shown in Figure 2, with some discussed below.

Chromatin remodeling

Chromatin is comprised of DNA wrapped around a histone octamer to form nucleosomes. These can be more loosely packed, as in euchromatin, where the DNA is more accessible to the transcriptional machinery, or tightly packed, as in heterochromatin, where DNA is more inaccessible. Modulation of chromatin structure by remodeling proteins and complexes modulates gene expression at many stages of neural development. For example, ATP-dependent chromatin remodelers move nucleosomes along the DNA or exchange nucleosome variants [26]. One such family of chromatin remodeling complexes is the mammalian SWI/SNF-like BAF complexes. The *SMARCA2* gene (also called *BRM*), encodes the core ATPase subunit of this complex and is mutated to cause a syndromic NDD (Figure 2). BAF complexes play several key roles in mammalian neurodevelopment. For example, in neural progenitors, they interact with REST, a repressive transcription factor, to inhibit neural genes and maintain progenitor identity, while an alternate neural BAF complex is involved in later aspects of neurodevelopment, including dendritic morphogenesis and neural circuit wiring [26]. Another ATP-dependent chromatin remodeler that is involved in neural development and is among the most frequently mutated genes in ASD cases is *CHD8* (Figure 2). CHD8 binds to trimethylated histone H3 lysine 4 (H3K4me3) at active promoters and may also repress Wnt/ β -catenin target genes by recruiting the linker histone H1. Interestingly, putative CHD8-regulated genes include many other ASD risk genes, suggesting that many NDD contributory genes act within shared regulatory networks [27].

Histone modifications

Within the histone octamer, which canonically consists of two copies each of histone subunits H2A, H2B, H3 and H4, post-translational modifications of the N-terminal tails of several histones can alter chromatin structure. These modifications include acetylation, methylation, ubiquitination, and phosphorylation of histone tail residues (Figure 2). Several classes of proteins are involved in this process, including those that add or remove histone modifications (sometimes referred to as ‘writers’ and ‘erasers’), and those that interpret these modifications and mediate changes in gene expression (readers). For example, histone methyltransferases methylate several histone residues, while histone demethylases remove these modifications. Although methylation of several histone residues, such as trimethylation of histone H3 at lysine 9 or 27 (H3K9me3/H3K27me3), facilitates transcriptional repression, other methylation events, such as trimethylation of histone H3 at lysine 4 (H3K4me3), are associated with active transcription. This modification appears to facilitate recruitment of protein complexes that promote transcription [5,26]. In general, histone acetylation is associated with active gene transcription, as it counteracts the

	Symbol	Activity	SFARI score
Chromatin remodeling	HMGN1	Chromatin associated	3
	CHD2	Chromatin remodeling	2
	CHD8	Chromatin remodeling	S
	CHD7	Chromatin remodeling	S
	SMARCA2	Chromatin remodeling	S
	ATRX	Chromatin remodeling	4
	CECR2	Chromatin remodeling	NR
	SMARCC2	Chromatin remodeling	3
Histone post-translational modification/variants	EP300	Histone acetyltransferase	4
	KAT2B	Histone acetyltransferase	2
	KAT6A	Histone acetyltransferase	3
	HDAC4	Histone deacetylase	S
	HDAC6	Histone deacetylase	NR
	JMJD1C	Histone demethylase	4
	KDM6B	Histone demethylase	3
	PHF2	Histone demethylase	3
	KMT5B	Histone methyltransferase	1
	SETDB1	Histone methyltransferase	4
	KMT2E	Histone methyltransferase	3
	ASH1L	Histone methyltransferase	1
DNA methylation and methyl binding domain proteins	DNMT3A	DNA methylation-methyl binding	3
	MBD1	DNA methylation-methyl binding	4
	MBD3	Subunit in NuRD complex	4
	MBD4	DNA methylation-methyl binding	4
		Interacts with deubiquitinating	
	MBD5	Polycomb repressive complex	3
	MECP2	DNA methylation-methyl binding	2

Figure 2. Examples of genes encoding epigenetic regulatory proteins that are mutated in autism spectrum disorder. Examples shown include proteins that remodel chromatin, post-translationally modify histones, or recognize methylated DNA. Cartoons at left schematize the types of chromatin modification involved and are described in greater detail in the text. At right, score assignments in the SFARI Gene database reflect the strength of evidence linking mutations in each gene to autism: S = Syndromic, 1 = High confidence, 2 = Strong candidate, 3 = Suggestive evidence, 4 = minimal evidence, NR = no rating yet available in SFARI. Me: methylation (histone or DNA); P: Phosphorylation; Ub: Ubiquitination; Ac: Acetylation.

positive charge of histones and reduces their interaction with negatively charged DNA phosphate groups to increase accessibility [28].

During development, different combinations of histone modifications are used to regulate gene expression. In particular, stem cells and cells of the early embryo are thought to adopt a 'bivalent' histone modification state at genes involved in developmental processes. This involves the presence of both repressive H3K27me3 and activating H3K4me3 modifications on adjacent nucleosomes. Bivalent genes are generally not expressed but adopt a 'poised' state and can undergo rapid changes in gene expression in response to developmental signaling. This enables loss of one modification and retention of the other to readily promote activation or repression of gene subsets appropriate to the acquisition of particular cell fates [29]. For example, genes encoding many transcription factors with roles in neural development, such as the *NGNs*, *PAX6*, *NESTIN*, *SOX1*, *SOX3*, *NKX2.2*, and *ASCL1*, are bivalent in pluripotent early embryonic cells and *in vitro* cultured embryonic stem cells (ESCs), but become active during neural cell specification. This involves the loss of H3K27me3 and retention of H3K4me3, while genes controlling pluripotency or alternate cell fates instead retain H3K27me3 and lose H3K4me3 to facilitate silencing of these transcriptional programs [30]. Although Polycomb repressive complex 2 catalyzes the H3K27me3 histone post-translational modification to repress gene expression, the H3K27-specific demethylase KDM6B is upregulated during the fate transition from ESCs to neural progenitors and removes the H3K27me3 modification from neural-specific genes such as *PAX6* and *NESTIN* [30]. Mutations in *KDM6B* are associated with ASD,

suggesting that disrupting these changes in histone methylation contributes to NDDs (Figure 2). In association with the resolution of the bivalent state, histone acetylation can promote and maintain active gene expression. Several histone acetyltransferases are likewise implicated in ASD, including *EP300*, *KAT2B*, and *KAT6A* (Figure 2). As these examples illustrate, mutations in genes encoding proteins that control the histone modification state during neural development could result in misregulation of this developmental program, contributing to nervous system abnormalities associated with NDDs.

DNA methylation/demethylation

Another layer of epigenetic regulation involves DNA methylation, usually at the fifth position of cytosine (5mC) in CpG dinucleotides; however, in some contexts, such as ESCs and brain tissue, non-CpG methylation can also occur [31]. Although most promoter CpG islands (regions with high CpG frequency) are unmethylated, high levels of CpG methylation in gene promoters are typically associated with repression of gene expression. Promoter methylation can confer chromatin inaccessibility to transcription factors, and can also recruit methyl-binding proteins and chromatin-modifying proteins that further inhibit promoter accessibility [5,32]. During neurodevelopment, DNA methylation correlates with long-term repression of both pluripotency- and non-neural fate-related genes in differentiated neurons. Resolution of the bivalent histone modification state is also highly correlated with an increase in CpG methylation, as non-neural genes become more permanently silenced [30]. DNA methylation is carried out by DNA methyltransferases, with DNMT3A and DNMT3B establishing *de novo* methylation, whereas DNMT1 maintains the methylation state during DNA replication. It is thought that DNMT3B plays an earlier role in neurogenesis, while DNMT3A likely plays a role in both the prenatal regulation of neurogenesis and in postnatal neural maturation [25]. The importance of *DNMT3A* in neural development is evidenced by the fact that mutations in this gene have been associated with ASD (Figure 2). Mutations in *DNMT3B* cause immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome. Although these patients only have mild cognitive defects, DNMT3B deficiency during iPSC-based neural differentiation *in vitro* alters the timing of differentiation and maturation, also supporting the role of DNMT3B in neural development [33].

Methyl-binding proteins are responsible for connecting DNA methylation patterns to changes in gene expression by binding methylated DNA along with protein complexes that repress transcription. *MECP2* encodes a methyl CpG binding protein, and mutations in this gene are associated with Rett Syndrome (RTT), a NDD (Figure 2). *MECP2* appears to have a variety of functions and binding locations, most commonly binding methylated DNA to act in transcriptional repression, with other roles involving altering chromatin structure, mediating alternative splicing of RNA, and perhaps promoting gene activation [28,34,35]. It is highly expressed in the brain and plays a role in neuronal maturation, with *BDNF* being one well-known target [32]. Other Methyl-CpG binding domain (MBD) proteins have also been implicated in ASD, including *MBD1*, *MBD3*, *MBD4*, and *MBD5* (Figure 2). For example, *MBD1* is highly expressed in the brain and plays an important role in neural development; this includes binding directly to the promoter of *FGF2*, a gene encoding a growth factor that promotes proliferation of NSCs [36]. These examples highlight the importance of epigenetic regulation during neural development and underscore how perturbations in several types of epigenetic regulators can contribute to NDDs.

Stem cell models of NDDs involving epigenetic dysregulation

Since Takahashi and Yamanaka generated the first iPSCs from human fibroblasts in 2007, patient-derived iPSCs have held great promise for modeling human disease [37]. This approach is particularly appealing for studying NDDs, as fetal tissue is inaccessible during development and neural development is substantially more complex and prolonged in humans than in the mouse and other model organisms [38,39], such that animal models often do not recapitulate human disease phenotypes [40]. In addition, for many complex NDDs, both potentially pathogenic mutations and the genetic background can interact to contribute to the phenotype and these distinct contributions often cannot readily be defined or mimicked in animal models [11–13]. Therefore, developing human, patient-specific models for NDDs is desirable to model key cellular and molecular features of the underlying biology.

In vitro approaches to modeling human disease have intrinsic limitations, perhaps especially in the case of NDDs, which often appear to have a complex etiology involving multiple abnormalities of fetal brain development at both a structural and cellular level, and consequently complex effects on neuronal circuit formation and function [24]. However, several NDDs, including ASD/ID, schizophrenia, bipolar disorder, and epilepsy, appear to frequently involve an imbalance between the excitatory activity of glutamatergic projection neurons and inhibitory GABAergic interneurons in the cerebral cortex [24,41]. Therefore, some of these defects can be modeled by using both patient-

derived and control iPSCs as starting materials to generate these neuronal cell types and to define cellular, molecular, and functional changes that correlate with and may contribute to disease. iPSCs are readily and efficiently obtained by reprogramming a number of somatic cell types from patients, including skin fibroblasts [37], renal epithelial cells [42], blood [43], and dental pulp [44], via expression of four transcription factors (OCT4, SOX2, KLF4 and c-MYC) or related strategies [37]. Both excitatory glutamatergic projection neurons and inhibitory GABAergic interneurons can be efficiently generated from iPSCs *in vitro* (Figure 1). To produce cortical excitatory neurons, a ‘dual SMAD inhibition’ protocol is commonly used that involves the addition of inhibitors of both BMP signaling through SMAD1/5/8 and TGF- β signaling through SMAD2/3 to induce formation of NPCs of dorsal telencephalic character [45], which are subsequently induced to undergo differentiation in 2D monolayer culture. Recent protocols have also been developed to derive inhibitory GABAergic interneurons from human iPSCs, which involve the use of a SHH agonist and Wnt signaling antagonist in addition to dual SMAD inhibition. This promotes the formation of NPCs with a ventral telencephalic character, which are subsequently differentiated into GABAergic cortical interneurons [46,47].

As these protocols are capable of efficiently producing large numbers of neurons which resemble cortical excitatory and inhibitory neurons, they can be used to probe many of the molecular, cellular, and functional abnormalities that correlate with or contribute to disease (Figure 1). However, 2D monolayer culture of specific neuronal cell types does not recapitulate many aspects of *in vivo* cortical development, which involves cell–cell interactions in the context of a complex 3D structure [48,49]. Given these limitations, many recent efforts have focused on the production of cerebral organoids, which can mimic many aspects of cortical development and organization, including the production and interaction of tissues with some features of dorsal and ventral telencephalic tissues of the cortex [50–52] (Figure 1). These include the formation of multilayered, stratified organoids that express the forebrain markers *PAX6* and *FOXG1*, wherein neuroepithelial rosettes differentiate into neurons that express markers of all six cortical layers [50,51]. Until recently, microglia, which play an important role in CNS development and are involved in synaptic pruning have been absent from these organoids, due to their embryonic origins in the yolk sac. However, a recent study has demonstrated that iPSC-derived microglia-like cells are capable of integrating into organoids, an exciting prospect for studying disorders such as schizophrenia, which has been associated with abnormal synaptic pruning [53].

A common challenge for both 2D and 3D differentiation protocols is that it can take several months to produce mature neuronal cells that can be assessed functionally (e.g., for electrophysiological activity) [52,54]. However, as the following examples indicate, many of the aberrant cellular phenotypes associated with human NDDs become evident even prior to neuronal maturation and function. The examples below also illustrate an emerging theme: even among NDDs involving mutations in many different genes, some of the aberrant cellular phenotypes appear to converge upon similar pathways involved in early neural development, which may implicate alterations of common core pathways that drive early neurodevelopment as a driver of many later abnormalities in brain circuitry and function that accompany these disorders. In addition to the examples below, Figure 1 provides an overview of some experimental approaches and phenotyping strategies that can be used to model alterations of neural development and function that contribute to NDDs in iPSC-based models.

Modeling NDDs in iPSC-derived neurons: examples

Genes that encode epigenetic regulatory activities have been implicated in both nonsyndromic and syndromic ASD and other NDDs, some of which have been studied using iPSC-based models. Syndromic NDDs have been most extensively studied, since the link between causative genes and these disorders are well established. As introduced above, one such gene associated with syndromic autism encodes the chromodomain helicase DNA-binding protein 8 (*CHD8*), a member of the CHD family of ATP-dependent chromatin-remodeling proteins. As one of the most commonly mutated genes in ASD, several studies have used iPSC-based modeling to define how *CHD8* mutations alter neurodevelopment to contribute to ASDs [55,56]. Wang *et al.* used both 2D- and 3D-based differentiation approaches to demonstrate that a significant number of ASD and schizophrenia candidate genes were differentially expressed in *CHD8* homozygous knockout-derived cells, compared with heterozygous knockout and unaffected controls [55,56]. Notably, *CHD8* knockout lines exhibited upregulation of *TCF4*, a transcription factor that mediates Wnt pathway signaling responses and has been associated with bipolar disorder. The top differentially expressed gene in this study was the long noncoding RNA *DLX6-AS1*, which regulates expression of *DLX* transcription factors, important regulators of GABAergic interneuron development [55,56]. Interestingly, in a study that derived organoids from patients with idiopathic autism, *DLX6-AS1* was also the top differentially expressed gene after 11 days of

terminal differentiation and continued to be among the top 10 differentially expressed genes after 31 days of terminal differentiation [54]. *CHD8* loss has been proposed to contribute to these disorders via dysregulation of direct and indirect downstream targets, including the aforementioned *TCF4* and *DLX* transcription factors, as well as Wnt signaling through β -catenin [57]. As other members of the *CHD* family have also been implicated in NDDs, including *CHD1*, *CHD2*, *CHD3*, *CHD5*, and *CHD7*, these will be interesting genes for future study using iPSC-based models to determine their role in normal and altered neurodevelopment [26].

A very well characterized syndromic NDD is RTT, which is caused by heterozygous loss of function mutations in methyl-CpG-binding protein-2 (*MECP2*). Using a 2D differentiation protocol, Marchetto *et al.* showed that RTT patient iPSC-derived neurons exhibited defects in synapse number, spine density, soma size, calcium signaling, and electrophysiological properties by comparison to controls, across multiple patient samples [58]. Interestingly, these investigators also found that iPSC-derived neurons derived from patients with *MECP2* duplication syndrome demonstrated a contrasting cellular phenotype, exhibiting increased dendritic arborization and synaptogenesis and more synchronized burst events, as determined by multielectrode arrays [59]. The authors suggest that these phenotypes are related to the reported role of *MECP2* in modulating multiple pathways involved in dendritic plasticity [59]. Other studies using RTT-derived iPSCs have shown a reduction in soma size in neurons derived from a clonal iPSC line in which the X chromosome carrying the mutant *MECP2* allele was active, compared with a line in which the X chromosome with the nonmutant allele was active [60,61]. Using a similar strategy, Kim *et al.* found that RTT iPSC-derived neurons have a reduction in neuronal maturation, as determined by immunostaining for the neuronal marker TUBB3/TuJ1, the NPC marker PAX6, and sodium channel markers [62]. Another study showed an increase in astrocytic differentiation at the expense of neuronal differentiation in a RTT-derived iPSC line and demonstrated that this results from inability of *MECP2* to bind the *GFAP* promoter and inhibit astrocyte differentiation [63]. These groups all conclude that their phenotypes recapitulate some of the nervous system alterations seen in RTT patients, who exhibit decreased neuron and brain size, reduced synaptogenesis, and deficiencies in dendritic arborization and spine number [39].

To our knowledge, nonsyndromic NDDs involving mutation of genes that encode epigenetic regulators have not yet been modeled in iPSCs. However, iPSC-based NDD modeling of mutations in other gene classes provides insight regarding potentially shared phenotypes. As suggested by Ernst, a common convergence point for many gene mutations that cause NDDs may involve altered regulation of cell proliferation and differentiation of neural stem and progenitor cells [64]. Several studies using iPSCs derived from patients with idiopathic NDDs support this idea. Mariani *et al.* used a 3D organoid differentiation scheme, starting with iPSCs derived from ASD patients with increased head circumference [54]. The authors found a decrease in cell cycle time in patient-derived iPSCs and early NPCs, and a corresponding increase in the rate of neuronal maturation and in synaptic overgrowth. They also saw evidence for increased formation of inhibitory synapses, with no change in formation of excitatory synapses, as marked by VGAT and VGLUT1, respectively. In this study, transcriptional analysis by RNA-seq demonstrated that the most significantly perturbed groups of genes were associated with transcriptional regulation of cell proliferation and cell fate, neuronal differentiation, neurite outgrowth, and synaptic transmission [54]. Using a 2D culture system, Marchetto *et al.* also found differences in the proliferation and differentiation of NPCs derived from iPSCs from multiple idiopathic ASD patients with macrocephaly [65]. The authors showed that β -catenin transcriptional activity was reduced in patient-derived NPCs. The authors also found differentiation defects in the cells, with a reduction in excitatory NPCs and an increase in markers for inhibitory neuron progenitors. The mature ASD-derived neurons had a reduction in spontaneous activity, leading to a deficiency in network connectivity [65]. Aksoy *et al.* similarly defined NPC proliferation and differentiation defects using iPSCs derived from a patient affected by a global developmental delay, speech delay, and language impairments with a disruption in the gene *GTDC1* [66]. Interestingly, this defect involved decreased NPC proliferation, followed by lowered efficiency of maturation into MAP2-positive neurons, a slight decrease in soma size, and a reduction in axon thickness in patient-derived cells versus controls. Although they found no differences in NPC proliferation, Griesi-Oliveira *et al.* also documented reduced neurite length and spine density in iPSC-derived neurons derived from an ASD patient with a mutation in *TRPC6*, indicating a differentiation deficit [67]. Although these studies do not represent the full array of iPSC-based studies of nonsyndromic NDDs, they illustrate some phenotypes that might be shared across a range of mutations that contribute to NDDs, including those involving mutations in genes that encode epigenetic regulators.

Challenges & considerations for iPSC-based disease models

Using neurons derived from iPSCs to model disease presents several challenges, particularly for modeling aspects of the complex phenotypes seen in NDDs. First, even in cases where evidence strongly supports a causative role for a mutation, many of these disorders are likely to involve polygenic contributions to disease penetrance. In particular, the genetic background and other fundamental characteristics, such as the sex of the patient, may have strong modifier effects on disease etiology and penetrance [68]. Therefore, it is important to select control iPSCs carefully, with the best control potentially being an isogenic iPSC line with CRISPR/Cas9-based correction of a particular mutation, to define its contribution to the phenotype. However, it is also useful in parallel to study the same mutation across different genetic backgrounds, looking for alterations in penetrance of any phenotype linked to a potentially pathogenic mutation. In addition, it is important to ensure that a given phenotype can be observed between different iPSC clones from the same patient, since iPSCs can display clonal variability [69]. Another challenge involves identifying shared cell-based phenotypes across different patients, both within and across mutations and disorders. These would be advantageous both for understanding shared mechanisms underlying the disease biology and to define potential targets for development of therapeutics.

Another issue that is particularly relevant to this review is how well iPSC-based differentiation models recapitulate the epigenetic changes that occur during development of any neuronal cell type *in vivo*. This is an especially important consideration for mutations of X-linked genes, such as *MECP2*, where differences in X chromosome inactivation state in iPSC-derived NPCs versus in brain tissues *in vivo* could dramatically influence cell-based phenotypes [60,70]. It is also important to consider the epigenetic state of the starting iPSC cell lines. Although iPSCs and human ESCs (hESCs) exhibit similar gene expression profiles and overall epigenetic state, several groups have identified differences between these two human pluripotent cell types. Notably, in iPSCs, megabase-scale regions of chromatin near centromeres and telomeres appear to be particularly resistant to reprogramming, resulting in aberrant non-CG methylation. This leads to other epigenetic changes, such as differences in both CG methylation and in histone modification state changes that are sensitive to DNA methylation status, such as H3K9me3, resulting in differences in gene expression between hESCs and iPSCs [71]. It is also important to note that both iPSCs and so-called ‘primed’ hESCs, which remain the most widely used *in vitro* models, generally exhibit genome-wide hypermethylation compared with the ICM cells of the blastocyst. However, efforts are being made to define culture conditions that maintain hESCs in a more ‘naive’ state, which may more closely resemble the methylation state of ICM cells [72]. Finally, iPSCs have been shown to have a bias in differentiation potential, depending on the cell type from which they were derived [73].

Despite these concerns, human stem cell-based neural differentiation models have been shown to recapitulate many epigenetic changes expected to accompany neuronal differentiation *in vivo*, and resembling that which occurs during neurodevelopment in animal models, including a general shift from hypomethylated DNA regions in ESCs to more hypermethylated DNA, as the cells undergo neuronal differentiation [74]. Therefore, while iPSC-derived *in vitro* models cannot display the full range of defects that contribute to NDD causation *in vivo*, extant examples support their high utility for modeling cellular and molecular abnormalities of neuronal development and function likely to contribute to disease etiology. Epigenetic regulation is a central aspect of neural development and function, and mutations in these genes play a major role in NDDs. As iPSC-based models can readily be scaled to enable high throughput genomic, epigenomic, and chemical screening approaches, they may offer a particularly attractive model for defining cellular and molecular abnormalities that underlie NDDs and developing approaches to modify these phenotypes to alleviate aspects of the neuronal dysfunction that contribute to the severity of disease.

Future perspective

As additional iPSC-based studies of NDDs emerge, using iPSCs derived from additional patients with both syndromic and nonsyndromic disorders, this work will increase our understanding of shared cellular and molecular mechanisms that contribute to NDDs, as well as specific contributory mechanisms stemming from each gene mutation. Both 2D-based methods, which enable differentiation of specific neuronal cell types, and organoid-based methods, which mimic the 3D interactions of multiple cell types in the brain, will prove useful in these studies. Stem cell-based modeling of human cortical development will also enhance our understanding of transcriptional and epigenetic regulation of normal development and how perturbation of these networks may contribute to some NDDs. The ability to reprogram somatic cells from a variety of different sources will increase the availability of patient-derived samples, particularly for children with NDDs such as autism, where skin punch biopsies may be prohibitive. It will be particularly beneficial to build relationships between physicians and researchers and resources

for data sharing, both within and between institutions, to facilitate the availability of patient-derived cells for further study. Although the full potential of iPSC-based models has not yet been realized, iPSC-derived human neurons provide an experimentally tractable, scalable, and genetically and genomically manipulable model with high utility for defining the cellular and molecular mechanisms that underlie NDDs.

Executive summary

Background

- Human cortical development is complex and protracted, increasing its sensitivity to perturbation.
- In particular, imbalances between excitatory and inhibitory neuronal activities in the cortex can contribute to neurodevelopmental disorders (NDDs), with autism and epilepsy as notable examples.
- Genome-wide studies have demonstrated mutations in genes that encode epigenetic regulatory proteins as major contributors to NDDs, with studies focusing on autism highlighted here.

Formation & patterning of the mammalian telencephalon

- Involves stepwise developmental process controlled by differential exposure to growth factor signaling and transcription factor expression, as well as epigenetic regulation.
- Normal progression of neural development results in a cortex of the proper size with a balance of excitation from glutamatergic excitatory neurons and inhibition from GABAergic interneurons.

Epigenetic regulation of neural development & contributions of its dysregulation to NDDs

- Three major classes of epigenetic regulation are discussed: chromatin remodeling, histone modifications, and DNA methylation and demethylation.
- Each of these classes of epigenetic regulators plays important roles in normal cortical development.
- [Figure 2](#) provides examples of different types of epigenetic regulators that have been associated with autism spectrum disorder (based on the SFARI database).

Stem cell models of NDDs involving epigenetic dysregulation

- Studies have used both 2D-directed differentiation and 3D organoid models to differentiate patient-derived and control induced pluripotent stem cells (iPSCs).

Modeling NDDs in iPSC-derived neurons: examples

- iPSC-based studies largely focus on syndromic NDDs, including Rett Syndrome and CHD8 mutations.
- To date, few iPSC-based models have been generated to study effects of mutations in genes that encode epigenetic regulators, particularly in nonsyndromic cases of NDDs.
- Among those mutations that have been modeled, a common theme involves alterations in the proliferation and differentiation of neural stem cells.

Challenges & considerations for iPSC-based disease models

- Controls must be selected with care, because genetic background greatly influences penetrance of NDDs.
- When gene is X-linked, particular consideration must be made regarding X chromosome inactivation status during reprogramming and differentiation.
- It remains to be determined how well epigenetic changes that accompany iPSC differentiation *in vitro* mimic the *in vivo* biology.

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