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Review

Modeling rare pediatric neurogenetic disorders with IPSCs

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Abstract: Intensive research has been performed to identify the pathological mechanisms of many pediatric neurogenetic disorders and to identify potential therapeutic targets. Although research into many pediatric neurological disorders has provided tremendous insight into the mechanisms of disease, effective treatments remain elusive. A significant impediment to progress has been a lack of thorough disease models. Transgenic/knockout animal models have been very valuable in determining the mechanisms of many neurogenetic disorders; however, these models cannot always mimic human-specific pathology and can be inadequate in representing human pathogenesis. This can be especially true for diseases of the nervous system. Alternatively, human patient-derived nervous tissue can be dangerous to acquire and difficult to propagate. The development of patient-derived induced pluripotent stem cells (IPSCs) has given researchers a fresh means of modeling these disorders with renewable human cells that can be used to generate neurons and glia. IPSCs are somatic cells that are reprogrammed back to a pluripotent stage, which can provide an unlimited source of human cells possessing patient-specific genetic mutations. Their potential to be differentiated into any cell type enables them to be a flexible platform to investigate neurogenetic disease. Of course, efficient methods for differentiating IPSCs into homogeneous populations of somatic cells must be established to provide the “disease-in-a-dish” systems. We will discuss the current methods for generating IPSC-derived neural cells to model pediatric neurogenetic disorders, as well as provide examples of the disorders that have been studied that include several neurodevelopmental and neurodegenerative disorders (Rett syndrome, spinal muscular atrophy, hereditary spastic paraplegias, and leukodystrophies). In addition, we provide examples on how patient-specific neural cells can be used in therapeutic development with high-throughput drug screening platforms or with correction via genome editing.

Keywords: pediatric neurogenetic disorders; human induced pluripotent stem cells; disease modeling

1. Introduction

Pediatric neurogenetic disorders are due to diverse range of genetic mutations that can lead to a variety of neurodevelopmental and neurodegenerative disorders, many of which are associated with limited therapeutic options. Oftentimes, these changes compromise the normal development and function of the central nervous system (CNS). As a result, this leads to a variety of phenotypes that are a combination of the timing of dysfunction and the region of the nervous system that is affected (e.g. cerebellar dysfunction in Freidreich ataxia) [1]. Other diseases may involve more diffuse involvement during CNS development and lead to neurodevelopmental disorders (e.g. Rett syndrome) [2]; however, it is important to note that symptoms not only differ from disorder to disorder, but also from one patient to another, even when the same gene is involved. Therefore, it is necessary to identify the specific mechanisms of neurological dysfunction in order to develop novel treatments to protect neurons against the pathological changes.

The direct study of disease-associated human cells (e.g. neurons or glia) is limited by the difficulty in obtaining affected tissues. Invasive procedures (e.g. brain, spinal cord or retinal biopsies) are not practical to complete, and even when performed, generated materials are difficult to propagate for extended studies. Postmortem tissue analyses have also been used, but often can only provide insight into end-stage pathology, and therefore lack the ability to study the disorder during its progression. As an alternative approach, non-neural patient-derived cells (fibroblasts or transformed cell lines) have generated valuable information in some detailed mechanistic studies; [3] unfortunately, the biology of these cells may not always properly conform to that of the affected neurons or glia and so it may be unclear if the data generated are directly comparable to the pathology seen in the nervous system. Another method to model human neurogenetic disorders has been with transgenic/knockout animal models. These models have been extremely valuable in determining the molecular mechanisms of many disorders [4], although there are several disadvantages associated with using them. These include the requirement for a significant investment of time for their development, and the possibility that the model might not accurately express the expected human pathology or behavior [5]. Other possible issues are that the clinical and pathological changes seen may be milder or more severe than the human disorder, as a result of the presence or absence of species-specific compensatory mechanisms. Of course, the representative species must also possess the appropriate orthologous genes, without which there can be no model. Lastly, the complexity of the human CNS may result in an animal model that is not able to accurately represent the human behavioral or cognitive disability. Overall, these modeling systems have been extremely valuable, especially when combined together, although many cannot individually answer all the possible questions associated with a particular human disease.

Embryonic stem cells (ESCs) are pluripotent stem cells (PSCs) that are capable of growing indefinitely and can differentiate into various cell types. Initially thought to be a revolutionizing new development for modeling disease and therapeutic interventions in the CNS, ESCs have not been a useful model for a number of technical and ethical reasons. Generating disease- or patient-specific ESCs has been the biggest limitation in their disease-modeling ability (although recent gene editing

techniques may assist with this now). Ethical concerns about using human embryos as the primary source of ESCs has also limited their potential value. Furthermore, the use of ESCs for therapeutic transplantation was limited by potential immunological response to ESC-derived allografts [6].

Fortunately, the development of induced pluripotent stem cells (IPSCs) provided a means to circumvent these technical disadvantages, while avoiding the ethical issues associated with ESCs [7]. IPSCs were developed by overexpressing the pluripotency-related genes in somatic cells (e.g. fibroblasts), which generated cells that exhibit the renewability and the capacity to differentiate into a number of different cell types [8,9]. In neurogenetic disorders, patient-specific IPSC-derived neural cells have enabled researchers to evaluate the morphology and function of affected neurons and glia without the need for further genetic modification [10] (Figure 1). As a result, patient-derived IPSCs have become valuable new cellular models for studying the mechanisms behind many neurogenetic disorders and have assisted in the development of therapies in disorders that require human-specific models [5]. These directed differentiation strategies have provided a means to generate various neural cell types in large enough quantities to study disease-associated cellular dysfunction *in vitro* (as well as cellular function in wild-type cells) [7,11] (Figure 1). To do this, a detailed understanding of embryonic development was required for the production of these complex differentiation methods. This included a detailed understanding of the factors involved in directing cell fate during embryonic development, and required the identification of reliable molecular markers to detect the specific cell types that were being targeted for evaluation [12]. These are ongoing processes, but have been productive to date. Subsequently, the directed differentiation of IPSCs has provided us with phenotypic information regarding affected neural cells, which has aided our identification of biomarkers and/or molecular targets of therapy that are important for therapeutic interventions (e.g. drug screening and/or cell transplant therapy) (Figure 1). Finally, IPSC-derived brain organoids are a recent development for the study neurogenetic disorders with an immense potential for providing three-dimensional models of neural development [13,14].

In addition to the development of directed differentiation protocols, several recently developed methods for genetic targeting and manipulation have also dramatically enhanced the application of IPSCs. Technologies like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 have provided the ability to repair or induce genetic mutations in affected or unaffected cells, respectively (Figure 1). These valuable methods have provided a means by which isogenic IPSC lines can be made for studies wherein the mutation-of-interest is the only variable involved in the study, allowing researchers to have unprecedented experimental control with identical genetic backgrounds within their control and disease-affected lines [15,16]. These methods may also provide an important direct application for correcting affected tissues in patients with hematological or other disorders that are amendable to an *ex vivo* therapeutic approach.

In this review, we will discuss the production of IPSCs and the assorted differentiation strategies for neurons or glia, as well as provide examples of their application in the study of pediatric neurogenetic disease. We will also review recent advances in genetic engineering strategies and prominent examples of their application in disease modeling and the development of cell-based therapies.

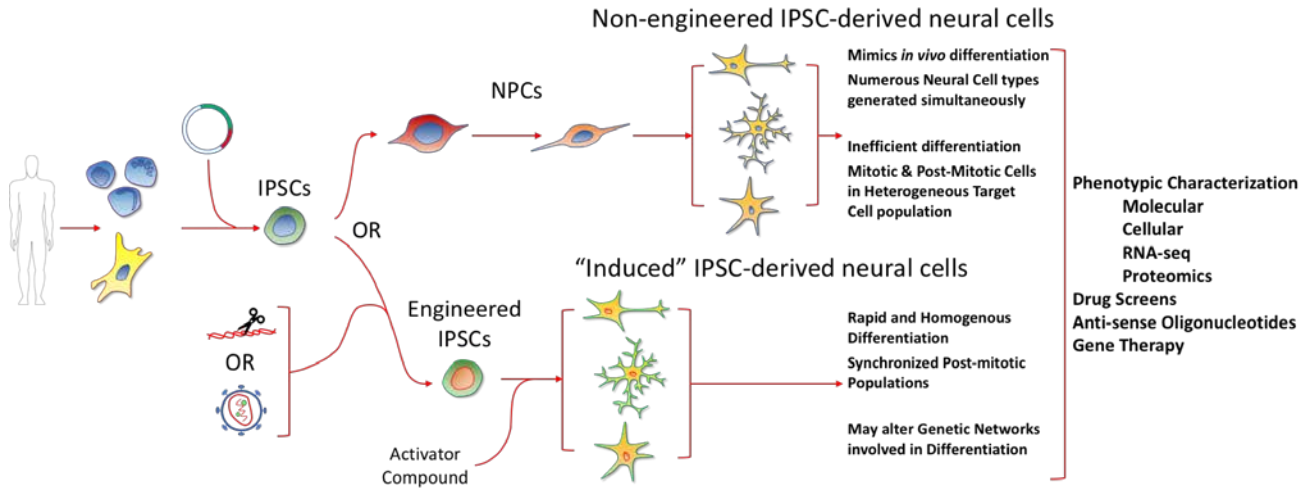

Figure 1. *In vitro* disease modeling of neurological disorders using human IPSCs. Skin fibroblasts or white blood cells from patients can be reprogrammed into IPSCs by expression of the pluripotent transcription factors such as OCT4, SOX2, KLF4 and c-MYC. Conventional methods of differentiation typically involves supplying specific morphogens to generate NPCs, followed by maturation into either neurons, astrocytes or oligodendrocytes. Alternatively, inducible neural transgene cassettes can be inserted into the host IPSC genome by gene-editing tools or retroviruses that can be activated to overexpress the differentiation factors to directly differentiate into specific neural cell types with more efficiency than conventional methods. Generated neural cells from either method will provide as valuable tools for studying disease mechanisms and testing therapeutic interventions.

2. Generation of patient-derived IPSCs for disease modeling

The very first human pluripotent stem cells (PSCs) identified were ESCs [17]. These cells originate from the inner cell mass of blastocysts and can proliferate indefinitely, as well as being able to differentiate into many different types of somatic cells [5]. Although this was a revolutionary development for modeling human diseases and potential therapeutic cell applications, the initially-predicted potential associated with ESCs has not been achieved as a result of several technical and ethical reasons: i) concerns about using human embryos as the primary source of ESCs; ii) the limited availability of disease- or patient-specific ESCs to model disease; and iii) the possibility of immunological responses to the allogeneic ESC-derived tissues after transplant into hosts [5,11].

The development of IPSCs circumvented many of these issues. The generation and production were a significant advancement that was based on studies that suggested the cell differentiation process was not a permanent and unidirectional event, but rather a reversible one [18,19]. Subsequently, a handful of factors were identified that were capable of maintaining pluripotency. Further work indicated that adult mouse or human fibroblasts could be transformed or induced into PSCs with expression of just four factors: octamer-binding transcription factor 4 (OCT4), sex-determining region Y box-2 (SOX2), Kruppel-like factor 4 (KLF4) and c-Myc. Additional work

suggested other combinations of similar factors were also capable of transforming fibroblasts into IPSCs (e.g. OCT4, SOX2, NANOG and LIN28 [20]; or OCT4, SOX2, KLF4 and L-Myc [21]). This initial work was a transformative advance; but the use of retroviral vectors that permanently integrated transgenic DNA into host genomes raised concerns for its experimental and clinical use because: i) of the chance for insertional mutagenesis in the host genome could cause unpredictable genetic changes; ii) the possibility of malignant transformation after transplantation into host organisms; and iii) the potential for overexpression or prolonged expression of these compounds to produce artifacts in the cellular phenotype being evaluated. Taking these issues into consideration, alternative methods were developed to reprogram somatic cells that utilized the positive feedback loop these transcription factors have on their endogenous promoters [22]. Therefore, transient expression of these reprogramming proteins could self-activate expression, while avoiding genomic integration. Transient expression methods included use of: i) episomal plasmid vectors [21]; ii) non-integrating adenoviruses [23,24]; iii) Sendai virus [25]; and iv) the direct delivery of proteins or modified RNAs of the reprogramming factors [26,27]. While using these 'safer' approaches, it was also found that the reversion to pluripotency was not restricted to fibroblasts, but was also capable with hepatocytes, keratinocytes, hematopoietic cells, and a variety of other cells that could be readily accessible from affected individuals [27].

2.1. Differentiation of IPSCs into neural progenitor cells

When differentiating IPSCs into neural cells, researchers must initially provide the specific molecular cues in order to generate specific early progenitor cells that can subsequently be differentiated into the neural cells of interest. Defined as neural progenitor cells (NPCs), these NPCs are commonly identified by protein markers that include SOX1, paired-box protein 6 (PAX6), and Nestin, along with many others [28]. Currently, there are two principal methods available to produce IPSC-derived NPCs that are committed to a neuroectodermal lineage with limited self-renewal: i) the formation of cell aggregates in a free-floating state called embryoid bodies (EBs); or ii) the use of monolayer cell cultures treated with specific inducer/inhibitors [29]. The formation of EBs mimic the embryonic neurodevelopmental process wherein neural differentiation occurs spontaneously, but further refinement can be achieved by inhibiting bone morphogenic protein signaling (with fibroblast growth factors (FGFs) or noggin or alternatively, LDN193189 as a noggin replacement) [30]. An alternative approach for a more controllable process for neuroectodermal specification (i.e. consistent number of IPSCs generated per dish) uses monolayer cell cultures with antagonists to Wnt, Nodal and transforming growth factor β signalings (e.g. dickkopf-1, lefty-1 and SB431542, respectively) that inhibit the Smad signaling pathway and are therefore known as dual-Smad inhibition protocols [30–32]. These methods have consistently produced NPCs that can be further differentiated into neural or glial cells and have been used to model several neurogenetic disorders, which will be described later [33–36].

2.2. Differentiation of IPSCs into cortical neurons

Excitatory cortical neurons are created in the dorsal forebrain *in vivo* and give rise to cells of the developing cerebral cortex [37,38]. During cortical development, actively dividing NPCs differentiate and migrate from the periventricular region along radial glia and form the cerebral

cortex in an “inside-out” manner, where the first differentiated cells become deeper-layer cortical neurons and the later-differentiating cells becoming upper-layer neurons. A few key transcription factors are involved in cortical neurogenesis and can be used as markers for the presence of cortical neurons (e.g. T-box brain 1 (TBR1), Fez-family zinc finger 2 (FEZF2) and COUP-TF-interacting protein 2 (CTIP2), special AT-rich sequence-binding protein 2 (SATB2)) [12,38]. Remarkably, *in vitro* derivation of cortical neurons using specific inducing factors (e.g. retinoic acid (RA), purmorphamine, brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF)), followed the same a layer-specific sequential order of generation seen during embryonic development [12,39]. A well-defined end target-point for these IPSC-derived cortical neuron differentiation protocols was the production of upper layer-cortical neurons that expressed SATB2. *In vivo*, $SATB2^+$ neurons are typically located in cortical layers II-IV and send axonal projections to the contralateral hemisphere [40–43]. Of note, when IPSC-derived cortical neurons were transplanted into mice, they were found to be capable of integrating into the cortical circuitry as functional neurons [44,45]; subsequent refinement of the process of generating $SATB2^+$ cells with the live-staining of cells with antibodies against forebrain surface embryonic antigen 1 (FORSE-1) and isolation of FORSE-1^+ cells with fluorescence-cell activated sorting (FACS) increased the final yield of upper-layer cortical neurons for further transplant studies [46,47]. Together these methods were capable of producing mature functional cortical neurons that could be used to investigate disorders that primarily affect the cerebral cortex.

2.3. Differentiation of IPSCs into motor neurons

Motor neurons have a primary role in transmitting signals from the brain to the spinal cord (via upper motor neurons) and then from the spinal cord to individual muscle groups (via lower motor neurons) for motor function. Because of this and their association with several neurodegenerative disorders, motor neurons have been an attractive target for IPSC-derived disease modeling [48]. The development of a thorough gene expression profile in these cells has allowed researchers to identify the key markers for motor neurogenesis that include: i) homeobox 9 (HB9) (also known as motor neuron and pancreas homeobox 1 (MNX1)); ii) SMI-32; and iii) LIM homeobox 3 (LIM3)) [49,50]. For example, α-motor neurons (AMNs) are a motor neuron subtype that can be generated from IPSCs. AMNs innervate skeletal muscle fibers and secrete acetylcholine which initiates muscle contraction. The currently available protocols for IPSC-derived AMN production commonly used a combination of posteriorizing factors (e.g. sonic hedgehog, RA, BDNF and GDNF) that induce expression of choline acetyltransferase (ChAT), a transferase enzyme that synthesizes neurotransmitter acetylcholine [51,52]. In order to further enrich cultures for AMN production, an HB9 promoter-linked fluorescent reporter protein was introduced to cells so that fluorescent cells could be isolated with FACS and then further differentiated into post-mitotic AMNs that expressed SMI-32, ChAT and vesicular acetylcholine transporter (VAChT) [53,54]. Further *in vitro* studies with mature motor neurons showed normal electrophysiological activity [51,55], while *in vivo* studies grafting motor neurons into transected mouse tibial nerves showed the engrafted motor neurons projected axons into denervated gastrocnemius muscle fibers and formed functional neuromuscular junctions [56,57]. These grafts subsequently attenuated denervation atrophy and restored contractile force. The IPSC-derived motor neurons enabled the researchers to investigate how motor neurons associate with other types of cells (e.g. astrocytes, oligodendrocytes and muscle cells), while also

providing a valuable tool for modeling genetic motor neuron disease (e.g. spinal muscular atrophy), as well as other diseases of the neuromuscular junction (e.g. Lambert-Eaton myasthenic syndrome).

2.4. Differentiation of IPSCs into glial cells

Glial cells are neural cells that provide structural, metabolic, and trophic support to neurons and have important roles in neurodevelopment (e.g. trophic and nutrient support of neurons, myelination, regulation of axon caliber). The two major categories of glia are astrocytes and oligodendroglia, with both cell types being involved in several neurogenetic disorders [11]. Therefore, IPSC-models of glial disorders have been important for understanding the roles these cells play in disease.

Similar to neuronal differentiation, the differentiation of glia from IPSCs requires the initial step of neuroectodermal regionalization that is followed by an extended period of exposure to specific morphogens involved in glial fate (leukemia inhibitor factor, ciliary neurotrophic factor and cardiotrophin-1) to induce the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway [58–60]. After 12–15 weeks of exposure, immature astrocytes began to emerge and expressed nuclear factor 1-A (NF1A), S100 calcium-binding protein β (S100β) and CD44 [61]. Further maturation of these early astrocytes required the removal of mitogenic factors leading to terminal differentiation of the cells and expression of glial fibrillary acidic protein (GFAP), glutamate transporter 1 (GLT-1) and aquaporin 4 (AQP4) [60,62]. When these IPSC-derived astrocytes were transplanted into the brains of immunodeficient recombination activating gene 1 $(Rag1)^{-/-}$ mice previously exposed to hypoxic ischemia to cause white matter injury, the IPSC-derived astrocytes showed signs of myelogenesis and had improved behavioral performance [63].

Oligodendroglia are another neural cell-type that have been produced from IPSC-derived NPCs. The exposure of NPCs to specific inducing factors (e.g. FGFs, RA and purmorphamine) generated oligodendrocyte progenitor cells (OPCs) that were identified by their expression of specific protein markers: oligodendrocyte transcription factor 2 (OLIG2), NK2 homeobox 2 (NKX2.2), SOX10, A2B5 and neuron-glial antigen 2 (NG2) [34,64,65]. These OPCs were further differentiated with thyroid hormone-3, platelet-derived growth factor-AA (PDGF-AA), neurotrophin-3 and insulin-like growth factors to become immature oligodendrocytes that express O4 and PDGFα-receptors (PDGFαR). The final step in their maturation was the result of a reduction in the concentration of these factors, which generated post-mitotic oligodendrocytes expressing galactocerebrosides and O1 [66], as well as proteolipid protein (PLP) and myelin basic protein (MBP) [65]. This work was validated in experiments where IPSC-derived OPCs transplanted into the brains of myelin-deficient *shiverer* mice were capable of rescuing the dysmyelinated white matter present in these mice [34].

As demonstrated above, *in vitro* generation of IPSC-derived astrocytes and oligodendrocytes have provided a valuable source of cells for therapeutic engraftment into the CNS of subjects with white matter disease. Unfortunately, these interventions will need to overcome several obstacles for its application to be more useful that include: i) the generation of mature glial cells from IPSCs is time-consuming (>3 months to complete) [11]; ii) the measurement of cellular function is typically only achieved following *ex vivo* transplantation, therefore it is difficult to assess their functionality prior to being transplanted [34,67,68]; and iii) there needs to be further evaluation of the safety of IPSCs and IPSC-derived cells with regards to potential *in vivo* immunogenicity and tumorigenicity.

3. Directed programming of IPSCs into neural cells

The previously mentioned differentiation protocols have been the most conventional methods to produce IPSC-derived neural cells. To date, they have provided a valuable means for the *in vitro* evaluation of neurogenetic disorders that involve astrocytes, oligodendroglia, cortical or motor neurons. New protocols involving microglia and blood-brain barrier endothelia have also been in development [69,70]. Even with this progress, there have been some intrinsic issues with these methods that may complicate and confound their use in disease modeling, which include: i) inadequate temporal synchronization of differentiation between cells from one well to another and from one batch to another; ii) the substantial amount of time required to reach the peak period of targeted differentiation; and iii) less than total percentage of targeted neurons or glial cells generated by the protocols. To address these issues, researchers have begun to look for more direct and regulatable means to differentiate IPSCs.

3.1. Induced neuronal differentiation from IPSCs

Recent work has used drug-inducible gene expression systems to induce expression of transcription factors that are involved in neuroectodermal specification and differentiation of IPSCs. The doxycycline (DOX)-inducible system has been frequently used for this purpose. For neuronal differentiation, these factors have included proteins such as neuronal differentiation 1 (NeuroD1), neurogenin-1 (NGN1) or NGN2. In early studies that used lentiviral delivery and DOX-treatment, IPSCs inducibly-expressing NeuroD1, NGN1 or NGN2 were converted into neuronal cells in less than 1 week and mature cortical neurons within ~2 weeks [71,72]. This method was then modified to use plasmid vectors (I^3N system) containing components for TALEN-mediated targeted-integration of a DOX-inducible NGN2 transgene into the adeno-associated virus integration site 1 (AAVS1) safe-harbor locus [73]. After isogenic isolation of cells that had stably integrated the vectors, I^3N-IPSCs exposed to DOX became post-mitotic neurons within days of treatment and expressed neuronal markers (e.g. βIII tubulin). After 4 weeks of further maturation, these neurons also expressed microtubule-associated protein 2 (MAP2) and neuronal nuclei antigen (NeuN) [73]. When they were co-cultured with glial cells, they formed mature synapses that contained juxtaposed pre- (e.g. bassoon and vesicular glutamate transporter (VGLT1)) and post-synaptic markers (e.g. homer and glutamate receptor 2/3) that responded to current injection by firing action potentials [73]. A similar approach was used to rapidly differentiate IPSCs into $HB9^+$ and $SMI32^+$ motor neurons by activating a stably integrated DOX-inducible transgene expressing a combination of Islet-1, LIM3 and NGN2 transcription factors [74]. The above examples of using regulable induction of specific transcription factors for rapid and homogeneous differentiation has tremendous potential for translating the platform to neuronal disease modeling. It should be noted that these cells may be inadequate in some examples of complex neuronal disease modeling, as they do not follow typical differentiation patterns, but may be ideal for modeling disorders with metabolic or biochemical dysfunction as they produce cultures of IPSC-derived neurons that are homogenous and synchronized in their development.

3.2. Induced glial cell differentiation from IPSCs

Transgenic expression strategies have also been employed for rapid and efficient differentiation of IPSC-derived glial cells. These methods also use inducible systems, but express gliogenic transcription factors for this purpose. For example, astrocyte differentiation was achieved using IPSCs containing a DOX-inducible system that inducibly-expressed NF1A and/or SOX9 [75]. Upon DOX treatment, a homogeneous population of functional and transplantable astrocytes were produced within 4–7 weeks [75]. Alternatively, IPSC-derived oligodendrocytes were produced with a lentiviral-based DOX-inducible system that expressed specific transcription factors (SOX10, OLIG2 and NKX6.2). This method dramatically enhanced oligodendroglial differentiation with up to 70 percent of cultured cells becoming $O4^+$ oligodendrocytes within 28 days of induction. Furthermore, these oligodendrocytes could be engrafted and myelinate the CNS of $Mbp^{shi/shi}$ $Rag^{-/-}$ mice [76]. Another group used lentiviral vectors to deliver a DOX-inducible system expressing SOX10 to NPCs, which could efficiently generate $O4^+$ and MBP^+ oligodendrocytes within 22 days of treatment that could myelinate neurons *in vivo* after transplantation into the mouse CNS [77]. All of these studies indicate that these technological advances have a real potential for use in patients with neurogenetic disease.

4. IPSCs as effective tools to model pediatric neurogenetic disorders

IPSC models of neurogenetic disease have provided valuable insight into the pathology of many of these disorders. In general, pediatric neurogenetic disorders exist as two broad categories: chronic static neurodevelopmental disorders or as progressive neurodegenerative disease. Inherited neurodevelopmental disorders often arise prior to the completion of neurodevelopment and are associated with dysmyelination and/or brain malformations that can cause developmental delays, cognitive dysfunction, and other neurologic symptoms [78]. Alternatively, there are a number of neurogenetic disorders that arise after the development of the nervous system with the child having normal development until a progressive neurodegenerative process sets in with symptoms of cognitive and/or motor regression. Both chronic neurodevelopmental and progressive neurodegenerative disorders may be associated with symptoms that can include seizures, spasticity, movement disorders, inattentiveness, incontinence, and neuropsychiatric issues.

Patient-derived IPSCs have been used to generate neural cells to model the cellular and molecular phenotypes of both of these types of neurogenetic disorders. The challenges involved with these sorts of disorders are intrinsic to what type of disease is involved. Neurodevelopmental disorders may have subtle abnormalities in their neural cells, with these abnormalities primarily affecting the network of neurons *in vivo*, which is not easily recapitulated *in vitro*. Alternatively, neurodegenerative disorders may exhibit more fulminant cellular phenotypes that may limit the survival or basic functions of cells *in vitro*. In the following section, we will highlight examples of disease modeling with IPSCs in these types of disorders and provide further insight into their overall use.

4.1. IPSC models of neuronal disease

Rett syndrome (RTT) is a X-linked dominant disorder that predominantly affects females in early childhood [79]. Females who are affected by RTT typically experience normal early development followed by developmental plateauing or regression between 6 and 18 months of age associated with cessation of head and brain growth and the development of autistic behaviors (e.g. stereotypies, hand wringing and anxiety), respiratory abnormalities, seizures, apraxia, and gait ataxia [80,81]. The disorder is caused by *de novo* mutations in the gene encoding methyl-CpG-binding protein 2 (MECP2), which is ubiquitously expressed in mammals with a high abundance in the CNS [79,82,83]. MECP2 acts as a genome-wide transcriptional regulator that can bind to methylated CpG dinucleotides of target genes and recruit corepressors (SinA3) and histone deactylases to the DNA locus for transcriptional repression [84–87]; as well as act as a transcriptional enhancer that interacts with the transcription factor CREB at the promoter of activated genes [81]. In relation to this, the mutation in MECP2 causes dysfunctional interactions with neuronal developmental genes (e.g. BDNF and distal-less homeobox 5) that leads to abnormal CNS development [83].

Several different types of *MECP2* mutations (e.g. missense and C-terminal frameshift mutations) have been studied with RTT patient-derived IPSCs [81]. Using wild type (WT)- and RTT-IPSCs, the mutant IPSCs exhibited normal sequential differentiation into EBs, NPCs, and immature neurons with no detectable alterations in the proliferation of RTT-IPSCs, -NPCs, or in the survival of early-stage neurons [88,89]. The numbers of generated GABAergic and glutamatergic neurons that were derived from WT- and RTT-IPSCs were similar, although a reduction in glutamatergic synapse numbers was observed in the RTT-neurons [88]. Interestingly, different phenotypic features became evident in RTT-IPSCs during late neuronal maturation that were consistent with *in vivo* RTT-mouse studies, which showed smaller neuronal somas, fewer dendritic spines, and a reduced number of neurite arborizations [88–92]. RTT-neurons also showed abnormal calcium signaling and electrophysiological activity, which had a significant decrease in the frequency and amplitude of spontaneous excitatory and inhibitory postsynaptic currents [88,90]. RTT-IPSCs were also used to investigate how the WT- or RTT-neurons interacted with their WT- or RTT-astrocytes counterparts [83,93]. Co-culture of RTT-astrocytes with WT-neurons revealed that the RTT-astrocytes negatively influenced the morphology and function of WT-neurons. Interestingly, similar negative effects were observed in WT-neurons cultured with conditioned media from mutant RTT-astrocytes, suggesting the possible involvement of soluble factors in the pathology [94]. To support this interpretation further, co-culture of WT-astrocytes with RTT-neurons had a beneficial effect on RTT-neurons [94]. This data suggests that astrocytes also play a significant role in Rett syndrome and as MECP2 is ubiquitously expressed, there may be other affected cells in the CNS that may include oligodendrocytes or microglia that need to be further investigated.

4.2. IPSC models of motor neuron disease

Spinal muscular atrophy (SMA) is an autosomal recessive disorder with mutations in the survival motor neuron (*SMN1*) gene. The disorder presents with progressive muscle weakness and atrophy due to the degeneration and loss of spinal motor neurons [95–97]. SMN1 is ubiquitously expressed within and without the mammalian nervous system and has roles in the splicing of

pre-mRNA into mRNA and biogenesis of small nuclear ribonucleoproteins [96,98,99]. It is also thought to be involved in axonal growth, axonal mRNA transport, and neuromuscular junction formation [100,101]; however, the precise mechanism by which decreased SMN dysfunction causes the motor neuronal death is not yet fully understood.

To understand SMA pathogenesis, patient-derived IPSCs were generated and differentiated into post-mitotic motor neurons. SMA-motor neurons had several phenotypic differences compared to WT-motor neurons during differentiation that included: i) SMA-motor neurons were smaller in size and had slower growth of neuronal processes; ii) they had diminished number of synapses and had poor arborization in distal axons and motor nerve terminals; iii) they had increased expression of the pro-apoptotic markers; caspase-3, caspase-8, and Fas ligand; and iv) there was a significant decline in motor neuron numbers over time [97,102,103]. Further data indicated that when modeling the neuromuscular junction by co-culturing SMA-motor neurons with the murine myoblast cell line C2C12, SMA-motor neurons showed significantly less clustering of acetylcholine receptors along the myotubes (smaller and fewer in numbers) [97,103]. IPSC-derived SMA-astrocytes were also abnormal with altered morphology that showed enlarged cell bodies with shorter and thicker cell processes compared to WT-astrocytes [104]. Furthermore, SMA-astrocytes had several of their functional roles impaired, which included altered intracellular calcium signaling mechanisms that in turn affected proper cellular communication, along with lower levels of GDNF expression for inhibition of apoptosis in motor neurons. Together, these results suggest that SMA-astrocytes could also be contributing to the disease process [104,105]. Similar to MECP2, SMA is ubiquitously expressed and so may affect other cell types in the nervous system (e.g. oligodendroglia). It would also be of interest to determine how SMA-neurons and -glial cells interact within a co-culturing system (e.g. SMA-neurons with WT-oligodendrocytes or WT-neurons with SMA-oligodendrocytes).

4.3. IPS cell models of glial disorders

Leukodystrophies are a group of disorders that primarily affect glial cells and lead to disruption of white matter development and/or function. Disorders affecting the developing CNS can result in hypomyelination or dysmyelination and cause neurodevelopmental issues that can include motor and cognitive delays that can be associated with other neurological findings (e.g. epilepsy, spasticity and ataxia). The disorders are typically associated with chronic static neurodevelopmental syndromes. Alternatively, demyelination is the result of disorders affecting a more mature CNS and involves a neurodegenerative process associated with progressive symptoms that can include spasticity, gait abnormalities, incontinence, and developmental regression [106]. Clinical dysfunction is the result of progressive accumulation of toxic compounds after normal white matter development.

Pelizaeus-Merzbacher disease (PMD) and spastic paraplegia type 2 (SPG2) are examples of X-linked hypomyelinating leukodystrophies that are caused by altered fatty acid metabolism. The disorders are both caused by mutations in *PLP1* gene, which is involved in myelin formation. *PLP1* encodes two protein isoforms, PLP1 and DM20 [107–109]; SPG2 only has altered expression of DM20, while PMD involves alterations of both DM20 and PLP1. PMD can be caused by missense mutations of *PLP1*, which were thought to cause large amounts of misfolded PLP1 and DM20 (and other proteins) that become trapped within the endoplasmic reticulum (ER), as well as decreasing the total amount of PLP1 and DM20 that localize to the plasma membrane to form myelin [107,110]. Previous models of the disorder were inadequate to test this hypothesis; therefore, PMD-IPSCs were

created from subjects with PMD-causing missense mutations [110]. These PMD-IPSCs were differentiated into oligodendrocytes and were shown to accumulate toxic misfolded PLP1 protein products within the ER, inducing ER stress, and leading to apoptosis of affected oligodendrocytes. Electron microscopy also showed these IPSC-derived PMD-oligodendroglia had altered myelination. This work characterized the disorder in more depth than previous models and indicated that IPSC-derived PMD-oligodendroglia underwent less complete maturation and had limited survival [110].

In contrast to PMD, metachromatic leukodystrophy (MLD) is a progressive demyelinating disorder caused by lysosomal dysfunction. This recessive lysosomal storage disorder is due to mutations in the arylsulfatase A (*ARSA*) gene [111], which catalyzes sulfatides in lysosomes and is involved in the development and function of myelin-producing cells as well as in the organization and maintenance of myelin structure [112,113]. Excess sulfatides are toxic to oligodendrocytes and Schwann cells leading to demyelination and dysfunction of the central and peripheral nervous systems [114]. MLD-IPSCs were produced and differentiated into oligodendrocytes, which mimicked the MLD phenotype *in vitro* by causing: i) an increased number and size of lysosomes per cell in both MLD-NPCs and oligodendrocytes; ii) increased sulfatides that co-localized to lysosomes; iii) a lower number of oligodendrocytes along with a substantial lack of MBP^+ cells; and iv) increased oxidative stress, leading to apoptosis [115]. Lentiviral delivery of a functional *ARSA* gene into MLD-IPSCs rescued the pathological cellular phenotypes mentioned above *in vitro* and, when transplanted into the brains of MLD mice, were found to normalized CNS sulfatide levels [116].

Alexander Disease (AxD) is an example of a leukodystrophy with astrocytes as the primary-affected cells. AxD is due to mutations in the *GFAP* gene, which is the major intermediate filament protein that provides support and strength to astrocytes [117,118]. GFAP mutations lead to accumulation of structurally altered GFAP in the cytoplasm (Rosenthal fibers), which impairs cellular function producing devastating effects on astrocytes, including cell apoptosis and demyelination [119]. AxD-IPSCs were produced and differentiated into astrocytes that possessed a dense aggregation of filaments that resembled early Rosenthal fiber structures containing GFAP and small heat shock proteins αB-crystallin [119,120]. Other studies showed that co-culturing AxD-astrocytes with WT-OPCs reduced the number of OPCs as compared to when they were co-cultured with WT-astrocytes, suggesting AxD-astrocytes inhibited OPC proliferation. In addition, it was found that the number and area of MBP^+ oligodendrocytes were substantially reduced when WT-OPCs were co-cultured with AxD-astrocytes in contrast to when they were co-cultured with WT-astrocytes [121].

4.4. IPSC models of mixed neuronal, glial, and retinal disorders

Spastic paraplegia type 11 (SPG11) is a complicated form of recessive hereditary spastic paraplegia that is caused by mutations in the *SPG11* gene encoding spatacsin. The disorder causes progressive spasticity, cognitive impairment, retinal degeneration, and juvenile parkinsonism [122]. SPG11 is also associated with progressive thinning of the corpus callosum, bilateral periventricular white matter lesions, and atrophy of the frontoparietal cortex [123,124]. The spatacsin protein has been shown to colocalize with several markers in the endosomal-lysosomal compartment and associates with the adaptor-protein-5, a protein involved in endosomal trafficking [125,126].

SPG11 patient-derived IPSCs reinforced the role of spatacsin in the regulation of lysosomal and vesicular trafficking, while also raising questions about its role in axonal transport [127,128].

SPG11-IPSCs were differentiated into cortical neurons but found to have several abnormalities in axonal growth as well as a reduction in neurite complexity, which seemed to be associated with downregulation of several axonal-related genes and the accumulation of membranous bodies within axonal processes [127]. These latter abnormalities were likely the result of neuronal lysosomal or vesicular dysfunction, similar to what had been seen in SPG11 null mice and human patient-derived fibroblasts [124,129]. Further work showed a significant decrease in the proliferation of SPG11-NPCs leading to a lower yield of neurons [128]. Correlating with this observation, the global transcriptome profiling of SPG11-NPCs displayed dysfunctional genes that are involved in cortical development, which included genes involved in callosal development [128]. Furthermore, transcriptional expression of several genes involved in autophagic and endolysosomal pathways were altered in comparison to WT-NPCs [128]. Whether the axonal abnormalities were downstream effects of lysosomal storage or due to direct effects of SPG11 dysfunction on anterograde transport are important questions needing answers. Of note, the disorder's involvement of glial and retinal cells in its pathology requires further evaluation of these cell types in relation to whether their pathology is related to lysosomal storage or perhaps another mechanism which can be investigated with SPG11 patient-derived IPSCs differentiated into retinal or glial cells.

4.5. Brain organoids and modeling of neurogenetic disorders

A newer development in the modeling of neurogenetic disorders with IPSCs is the use of brain organoids [130,131]. Brain organoids are three-dimensional cellular networks derived from millions of IPSCs that self-assemble into tissues containing neurons and glia in a process that loosely mimic the development and structure of the human brain [132]. The brain organoids have tremendous potential to be used as models for neurodevelopmental disorders that involve the generation and migration of specific cell types to structures similar to the cerebral cortex [13,14]. The three-dimensional neural structures not only possess somewhat-similar cellular organization as the developing brain, but also have similar epigenetic and transcriptional profiles. Disorders of microcephaly, macrocephaly, and autism have already been modeled with this technology and has provided unique data [133–135]. IPSC-organoid modeling is an emerging field that provides a vast potential for more realistic models of human disease; however, its more widespread use will be dependent on the organoid system's ability to be further refined in order to produce more mature and complex neural structures. This would have a tremendous potential to better understand disorders of human cortical organization and migration that are not available with IPSC-monolayers, as well as provide another means to investigate therapeutic interventions.

5. Genome editing for modeling and treatment of neurogenetic diseases

Recently, sophisticated genome editing technologies have emerged that have had a significant impact on IPSC disease modeling. These editing technologies include ZFNs, TALENs and RNA-guided CRISPR-Cas9 nuclease systems. ZFNs were developed by combining the DNA binding domain of a FokI restriction enzyme with alternative DNA binding zinc fingers that could be assembled in different combinations to target specific genomic sequence loci with unique DNA double-stranded breaks (DSB) [136–139]. Of note, ZFNs had several limitations that included: i) a high probability of off-target binding/modifications; ii) a high cost of implementing the system; and

iii) an increased toxicity to host cells [7]. Subsequently, TALEN technology was developed using the fusion of a TAL effector DNA-binding domain from *Xanthomonas* bacteria and a FokI DNA cleavage domain that could also be engineered to target unique DNA sequences and induce DSBs [140,141]. Limitations of using this technology in IPSCs included the small number of targets where TALE DNA binding sites could be utilized [7]. In contrast to these first two editing methods, the CRISPR-Cas9 nuclease uses a short (~20 nucleotide) guide RNA to generate targeted nicks or cuts at specific DNA sequences [142,143]. DNA breaks are then repaired by the error-prone process of non-homologous end joining or homology-directed repair (HDR). The HDR mechanism has the advantage of using repair-templates from the corresponding locus of the sister chromosome or from an exogenous repair-template containing the mutation or repair of interest. Although off-target effects have been a major concern, whole-genome sequencing has been applied to monitor the level of potential off-site mutational loads [144]. To date, whole-genome sequencing analysis has shown that TALENs and CRISPR-Cas9 were highly specific in human IPSCs with little-to-no off-target mutations observed in the analyzed clones [145]. Furthermore, CRISPR-Cas9 was able to specifically target either the mutant or the WT allele in patient-derived IPSCs with little disruption at the other allele even when they differed by as little as a single nucleotide [146]. As newer strategies continue to be developed to increase CRISPR's specificity, this technology is becoming the predominant gene-editing tool [147–149].

These technologies may be the next critical step in the development of stem cell-based gene therapy by enabling *ex vivo* gene correction of patient-derived IPSCs with eventual cell transplantation or replacement therapy. These methods could use a subject's own (isogenic) cells to dramatically decrease transplant rejection. Promising results from *in vitro* studies demonstrated that adverse phenotypes associated with several disorders were able to be rescued after correction by genome editing. For example, in an IPSC model of frontotemporal dementia, patient-derived IPSCs carrying a granulin $(GRN)^{IVS1+5\ G>C}$ mutation showed significantly decreased corticogenesis compared with WT controls. Correction of this mutation using a ZFN and homologous recombination template restored progranulin expression and the corrected patient-derived IPSCs were found to be capable of normal cortical neuron differentiation [150]. For SMA-IPSC studies, single stranded DNA oligonucleotides were used to convert the *SMN2* gene (an *SMN1* gene paralogue) into an *SMN1*-like gene after their introduction into undifferentiated SMA-IPSCs. These edited isogenic IPSC lines were subsequently capable of producing increased amounts of the functional full-length SMN protein. The approach not only resulted in prevention of motor neuron degeneration, but also in increased formation of neuromuscular junctions in co-culture assays of motor neurons and myotubes [96,103]. Furthermore, gene array analysis on these gene-corrected cells revealed rescued expression of differentially-expressed genes involved in RNA metabolism, axonal guidance, and motor neuron development. In addition, the corrected SMA-motor neurons showed improved engraftment after intraspinal transplantation of a mouse model of SMA and caused prolonged survival of these animals when compared to transplantation of non-modified SMA-motor neurons [96,103]. This data highlights the regenerative potential of gene-corrected patient-specific IPSC-derived neurons in the context of cell therapy for neurodegenerative diseases.

Alternatively, genome-editing technologies also offer a straightforward method to model diseases by creating specific disease-causing mutations in WT-IPSCs from unaffected subjects. This application allows for the direct comparison of WT and mutant cell lines that originated from the same unaffected parental cell lines. These mutated IPSCs may minimize variables often present in

control lines such as gender, ethnicity, or genetic background. Conversely, the approach may show that there are additional genetic factors (modifier variants present in other genes) that may be required for a disease phenotype to be seen at the cellular or organismal level. In any case, the technology may allow research to address the level of influence of different genetic backgrounds may have on specific disorder or even specific mutations.

6. Use of IPSCs for the therapeutic drug screening and delivery

Patient-derived IPSCs offer an unrestricted supply of disease-relevant cells to enable repeated experiments to study the response of the disease phenotype on a large scale. They can be excellent tools for therapeutic development, drug target identification, and screening for toxicity or differentiation compounds. For example, the phenotypes observed in IPSC-derived SMA-motor neurons (e.g. the decrease in the number of neuromuscular junctions) could be partially reversed with valproic acid, tobramycin, morpholino, or oligonucleotides treatments [96,103,151–153]. In an IPSC model of RTT, a distinct connection between MECP2 deficiency and growth factors like BDNF and IGF-1 was established and led to studies of their potential therapeutic value [154,155]. It was previously known that MECP2 knockout mice had normal BDNF expression during their early asymptomatic stage, but that BDNF declined during the onset of RTT-like behavioral and neuropathological phenotypes [156–158]. IGF-1 expression levels also underwent a similar decline. A complex pathway was subsequently identified wherein MECP2 positively-regulated BDNF expression, which played a role in IGF-1 regulation via a microRNA intermediate. Specifically, the decrease in BDNF expression resulted in a decrease in the amount of a microRNA processing factor called lin28a. Loss of lin28a led to overproduction of a microRNA called let7f, which subsequently inhibited IGF-1 production [155]. With this idea in mind, RTT-neurons (derived from several subjects with different mutations) were treated with IGF-1 or GPE (a peptide containing the first three amino acids of IGF-1), which reversed their mutant phenotype by normalizing neuronal morphology and increasing glutamatergic synapse number. These results suggested that growth factor treatment could improve symptoms due to RTT neuronal dysfunction and are an excellent example of how IPSC modeling can lead to potential therapeutic interventions [88,159]. Unfortunately, delivery of these compounds to the CNS has been limited by their poor ability to penetrate through the blood-brain barrier. One approach to solve this problem would be to transplant autologous IPSC-derived NPCs expressing these therapeutic neurotrophins into the CNS. Indeed, previous studies in other disorders (e.g. cerebral ischemia and Parkinson's disease) have demonstrated that transplantation of NPCs overexpressing BDNF or GDNF improved neurological function 12 weeks after injuries [160,161]. This work has been promising, but the long-term effects of constitutive expression of these neurotrophins in the brain may have toxic side effects and so the use of drug-inducible systems (e.g. DOX or mifepristone) to regulate neurotrophin expression may be a way to minimize unexpected complications and if necessary, to even shut down expression [72,73,162].

7. Conclusion

The convergence of several major technological advances in the field of stem cell biology has rapidly transformed our ability to use these cells to model neurogenetic disorders. The advances include technologies enhancing the derivation of patient-specific IPSCs, methods to increase the

efficiency and speed of differentiation protocols for a variety of neural cell types, and the application of genome-editing technologies for correcting or introducing mutations into IPSCs. This work has ushered in a new era for using stem cells to study pediatric neurogenetic disorders, as well as providing new means for the development of potential therapies. The cellular models in association with animal models for these disorders will provide a range of methods to further understand the disorders and develop therapeutic interventions. Altogether, this points to a new renaissance in human disease modeling and cell-based therapies which could dramatically affect our understanding and treatment of neurogenetic disorders.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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