

Balancing serendipity and reproducibility: Pluripotent stem cells as experimental systems for intellectual and developmental disorders

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Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) and their differentiation into neural lineages is a revolutionary experimental system for studying neurological disorders, including intellectual and developmental disabilities (IDDs). However, issues related to variability and reproducibility have hindered translating preclinical findings into drug discovery. Here, we identify areas for improvement by conducting a comprehensive review of 58 research articles that utilized iPSC-derived neural cells to investigate genetically defined IDDs. Based upon these findings, we propose recommendations for best practices that can be adopted by research scientists as well as journal editors.

Introduction

In the United States, ~3%–4% of children are diagnosed with intellectual and developmental disabilities (IDDs) annually (Zablotsky et al., 2017). Many IDDs are associated with genetic conditions, including Down syndrome, fragile X syndrome, Rett syndrome, tuberous sclerosis, Dravet syndrome, Williams syndrome, Angelman syndrome, and copy-number variants (CNVs), including 22q13.3, 15q11-q13.1, and 16p11.2. Human pluripotent stem cells (hPSCs) provide an essential tool to understand alterations that occur during this inaccessible period of prenatal brain development. Specifically, induced pluripotent stem cells (iPSCs) derived from patient somatic cells can recapitulate *in vitro* developmental steps that may be disrupted during brain development *in vivo*. iPSC-derived neuronal and glial cells retain genetic contributors to IDDs present in the patient from whom they were derived, reveal altered development and/or function that may contribute to IDDs. iPSC-derived cells also provide platforms for chemical and molecular screening to identify novel therapeutic targets for IDD-related neurodevelopmental abnormalities, potentially leading to new treatments.

To assess progress in IDD research, we identified 249 PubMed articles investigating IDDs published from 2010 to 2018 (Figure 1; Table S1) using the listed search terms (supplemental information). Papers were manually screened to include only primary literature involving

both generation of patient-derived iPSCs and neuronal cell differentiation, which narrowed the final list to 58 papers. Each paper was evaluated based upon information supplied in the manuscript and the supplemental information and given an overall “quality score” based upon several categories (Table S1). We did not include numbers of lines or of patients studied as criteria for scoring, because these parameters are often study design dependent and thus cannot readily be evaluated in a quantitative, objective manner. Each publication was evaluated on a scale of 0–23, with 0 as the lowest and 23 the highest possible score (Table S1; supplemental information). Evaluation categories were based on scientific guidelines for lab resources published by Stem Cell Research and the STAR Methods (structured, transparent, accessible reporting) published by Cell Press. The analyses performed here are by no means exhaustive and did not incorporate articles that solely generated iPSCs from IDD patients or solely differentiated and characterized neural cells to study IDD.

Among the 58 papers reviewed, scores ranged from 8 to 20 (median = 16; mean = 15.43, Figure 1A). The distribution is relatively symmetric with a negative skew (Figure S1A), as expected because we selected standard, common scoring practices. To better compare overall publication quality over time, we divided the 58 primary articles into 2 subgroups—articles published between 2010 and 2014 (n = 28) and between 2015 and 2018 (n = 30). This analysis identified a downward trend in publication quality over time that did not reach statistical significance (Figure 2A). Interestingly, this trend is primarily driven by a significant decline in reported information related to iPSC derivation and QC assessment (Figures 1B and 2B). However, recent papers often utilize more patients and/or more iPSC lines, as indicated by the color and size of each data point (Figure 1). While this may influence the QC performed/reported on each line, the trend toward using more patients and iPSC lines to study IDD is generally encouraging. We did not observe a significant score change over time for the category of experimental

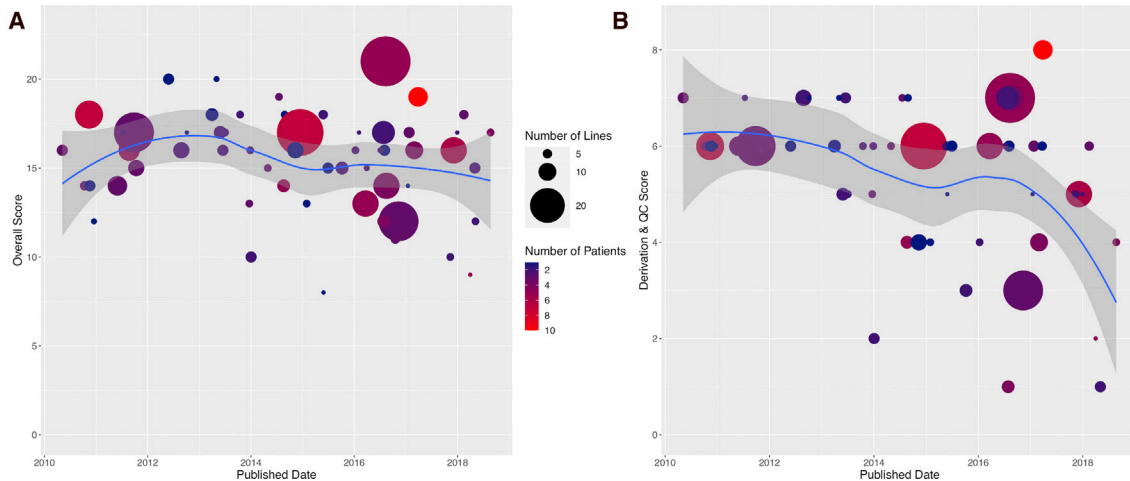


Figure 1. Publication quality over time

Review and scoring of 58 primary articles published between 2010 and 2018 (x axis) utilizing iPSCs to investigate genetically defined IDD. (A) Each publication was scored on a scale of 0–23 (y axis) and arranged in chronological order (x axis). (B) iPSC derivation and quality controls were scored on a scale of 0–8 (y axis) and arranged in chronological order (x axis). For graphs for the other two categories, see [Figure S1](#). Numbers of iPSC lines and patients modeled is indicated by the size and color of the circles, respectively. A smooth regression line (blue) and confidence band (gray shade) are shown using `geom_smooth` function through `ggplot2` in R.

design and background information, nor for the neural differentiation and QC categories ([Figures S1B and S1C](#)).

Numerous factors could contribute to a decline in iPSC derivation and QC assays reported: (1) as iPSC technology becomes more established, less emphasis is placed on best practices for culture and systematic QC; (2) increased numbers of iPSC lines and clones create challenges in balancing the large amount of work and resources needed to conduct both QC and hypothesis-driven experiments; (3) laboratories often use previously published protocols, but fail to detail modifications; and (4) space for experimental detail and QC data may be limited by journals. We posit that these factors contribute to decreased publication quality and diminished cross-site reproducibility ([Volpato et al., 2018](#)). It is unclear to what extent biological differences, technical differences driven by differentiation methods, inconsistent QC and culturing practices, choices of controls, or all of the above, contribute to discrepancies in data from iPSC studies of IDDs. Furthermore, although compelling evidence for altered glial development in IDDs has been reported recently ([Cresto et al., 2019](#); [Wong, 2019](#)), this analysis focuses on neural progenitor cells (NPCs) and/or cortical neurons, as these remain the focus of most current IDD-related studies. Literature related to glial differentiation of iPSCs and IDD studies in iPSC-derived glia is presented in [Table S2](#). Here, we highlight key biological and technical variables that may contribute to decreased cross-site reproducibility of iPSC-based IDD studies. Importantly, we provide recommendations to

improve transparency and reproducibility, so that findings have greater impact for patients and families affected by IDDs ([Table 1](#)).

Biological considerations for studying IDDs in patient-derived iPSCs

iPSC-derived neural cells provide a powerful human experimental system to study how IDD-contributory genetic mutations alter neurodevelopment, yet challenges exist. iPSC-derived neural cells are best suited to analyzing cellular and molecular mechanisms that contribute to IDDs and are less useful for network and systems level inquiry, given the lack of synaptic maturation and the absence of some cell types found *in vivo*. Investigating phenotypes that can be recapitulated with iPSC-derived neurons (e.g., oxidative stress) is hampered by biological variability, as discussed below. Nonetheless, iPSC models can effectively be used to study both syndromic IDDs attributed to single gene mutations ([Deneault et al., 2019](#); [Deshpande et al., 2017](#); [Griesi-Oliveira et al., 2015](#); [Wang et al., 2017](#)) and subjects with IDDs of unknown etiology but with shared phenotypic characteristics, such as macrocephaly ([Marchetto et al., 2017](#); [Mariani et al., 2015](#); [Schafer et al., 2019](#)).

Genetic heterogeneity of human subjects

Unlike animal experimental systems, which are generally inbred, genetic variation between iPSC lines is a challenge linked to the inherent genetic variability among humans. Strategies can minimize the effect of genetic background in iPSCs, which may lessen variability at a cellular and

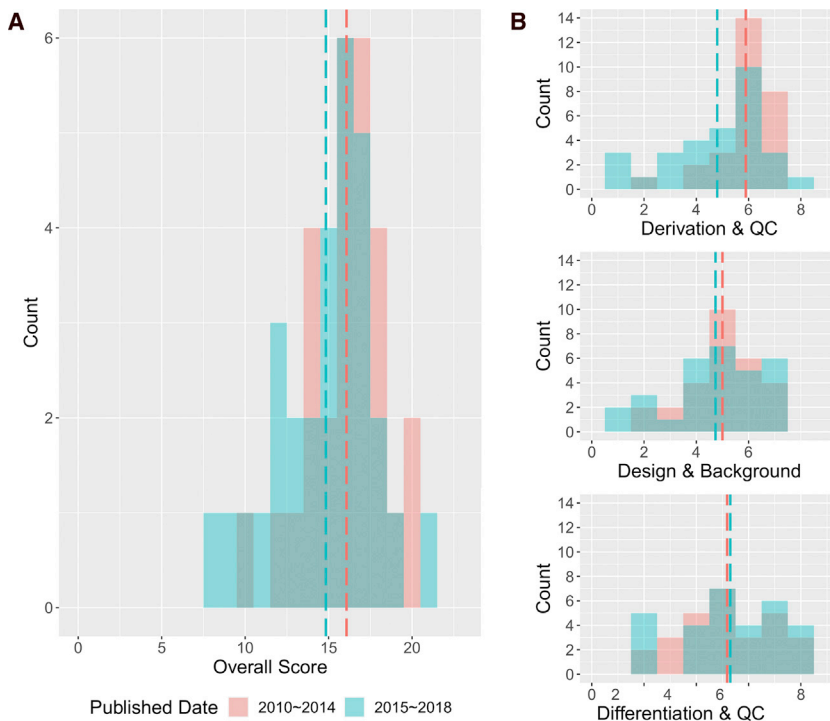


Figure 2. Score distribution analysis of publication quality

(A) Overall score distribution (x axis) of 58 primary articles published from 2010 to 2014 (n = 28, red) or 2015 to 2018 (n = 30, turquoise). Dashed lines in corresponding colors indicate mean scores of 16.07 and 14.83, respectively (p = 0.08087).

(B) Score distributions (x axis) and comparison between the two groups of articles for each main category evaluated: iPSC derivation and QC (top, p = 0.00958), experimental design, background information (center, p = 0.53114), and neuronal differentiation and quality control (bottom, p = 0.80436). p values determined by two-sample t testing.

molecular level. First, the number of individuals from whom samples are obtained must be considered. Power analysis to calculate the needed sample size for an expected effect size is challenging, due to difficulties in defining both the level of heterogeneity and effect size of molecular and cellular phenotypes (Hoekstra et al., 2017). For example, a sample size of five gives a power ratio of 0.5 if heterogeneity is low. However, as heterogeneity increases, so does the ideal sample size (e.g., >10 individuals per group). Unfortunately, simply increasing sample size is not feasible for many studies, given the enormous time and cost associated with this work, as well as limited patient availability for rare IDD. Second, selecting control individuals is critical. Many iPSC-based studies choose controls based solely on lack of diagnosis and age and/or sex match. Yet, this simple design does not account for underlying genetic heterogeneity that may contribute to phenotypes. Controls that include non-affected family members with partially shared genetic backgrounds can lessen effects of this underlying genetic variation.

Challenges in distinguishing biologically relevant cellular phenotypes that contribute to IDD-related traits versus those resulting from individual variability between patients' genetic backgrounds can also be addressed by using isogenic control cells. For monogenic disorders, isogenic controls can be generated through CRISPR-Cas9-based gene editing of iPSCs, to correct an IDD-related mutation in patient-derived cells, or introduce the mutation

into control cells. While CRISPR-based gene editing can introduce unwanted off-target changes in the genome, analysis has shown low rates of unwanted changes in gene-edited hPSCs (Smith et al., 2014, 2015). To reduce the likelihood of these confounding effects, alternative editing systems, including Cas9 variants with enhanced specificity or systems foregoing double-strand DNA breaks can be used (Table S3) (Chaudhari et al., 2020). It is also feasible to sequence the genome of engineered lines at reasonable cost to detect such changes (Banan, 2020; Broeders et al., 2020). However, gene-editing technology cannot currently correct disorders involving multiple genes or large mutations (e.g., CNVs). An alternative strategy involves recruiting mosaic individuals for iPSC generation. Somatic cells in mosaic individuals all derive from a single zygote and thus are genetically identical, except for the presence or absence of the mutation. iPSCs derived from mosaic individuals are particularly valuable if mosaicism arose early in development, because cells with and without the mutation have gone through similar developmental processes *in vivo*. Isogenic controls provide a powerful tool to validate disease-related phenotypes in iPSC-based experimental systems.

Cell line variability

While iPSCs resemble human embryonic stem cells (hESCs), their distinct source must be considered. Variability may arise in either the somatic cell of origin or reprogramming method. The contribution of different



Table 1. Recommendations for characterizing iPSCs, NPCs, and neurons include molecular, cellular, and functional methods with the goal of improving reproducibility in iPSC culture and differentiation.

	iPSC characterization	NPC characterization	Neuronal characterization
Molecular and cellular	G-banded karyotyping	–	–
	KaryoStat assays	–	–
	STR profiling	–	–
	hPSC genetic analysis kit	–	–
	CNV qPCR assays and arrays		
	SNP microarray	–	–
	RNA sequencing	RNA sequencing	RNA sequencing
	RT-PCR: OCT4, Nanog, SSEA3/4	RT-PCR: Sox2, Nestin, Pax6, vimentin	RT-PCR: TUBB3, NeuN, MAP2, neuronal subtype specific genes
	mycoplasma/sterility	sterility	sterility
	immunostaining	immunostaining	immunostaining
Technical	cell source	–	–
	reprogramming method	–	–
	number of clones (patients, controls)	–	–
	cell line gender	–	–
	patient demographic info	–	–
	morphology (presence of spontaneously differentiating cells)	–	–
	passaging method	–	–
	media composition	media composition	media composition
	extracellular matrix	extracellular matrix	extracellular matrix
	seeding density for differentiation	seeding density	seeding density
	passage number	passage number	–
	–	technical repeats	technical repeats
	–	–	differentiation method
	Functional	embryoid body formation followed by trilineage qPCR	–
teratoma formation (optional)		–	morphometric analysis
–		–	multi-electrode array
–		–	electrophysiology (number of neurons)
–		–	calcium transients (number of regions of interest)

derivation methods to genetic or epigenetic variability of iPSC lines has not been systematically assessed. However, researchers generally agree that reprogramming methods that avoid genome integration are preferred. Clonal iPSC

lines generated by reprogramming can exhibit variable morphology, proliferation rates, stem cell marker expression, and differentiation potential (Hu et al., 2010). To study IDD-related developmental deficits, iPSCs must



readily differentiate into neural cell types. Newly generated iPSC lines can be screened for robust neural differentiation potential, as measured by frequencies of iPSC-derived NPCs expressing PAX6 and/or SOX2, excluding poorly differentiating clones (e.g., <80% PAX6+/SOX2+ NPCs) from further work. Alternatively, expression of specific microRNAs distinguishes iPSC lines with differential neurogenic differentiation propensity (Du et al., 2013). Multiple clonal lines should be characterized to identify the best clones for experimental use.

Two technical variables that influence iPSC performance are the maintenance media and use of feeder-free culture versus culture on mouse embryonic fibroblast (MEF) feeder layers. Initial protocols involved culture on MEF feeders in media containing 20% serum. However, as serum composition is not controlled and exhibits lot-to-lot variability, alternatives include serum-free media of defined composition (e.g., knockout serum replacement plus basic fibroblast growth factor [bFGF]). Currently, feeder-free culture in defined media, such as mTeSR (STEMCELL Technologies) and on Matrigel (Hannoun et al., 2010; Inzunza et al., 2005; Ludwig and Thomson, 2007; Thomson et al., 1998) lessen both variability and labor. A survey of the IDDRC Human Stem Cell Working Group established that all centers currently culture iPSCs under feeder-free conditions, with 12 of the 13 groups using Matrigel (or equivalent) substrate. Three groups also use MEF feeder layers routinely. The predominant use of Matrigel-like substrates versus human recombinant substrates (e.g., vitronectin or rhLaminin-521), likely reflects their widespread availability from commercial sources and relative affordability. The effect of different feeder conditions is poorly understood, as suggested by recent findings of differences in organoid production between iPSCs cultured under different feeder conditions (Watanabe et al., 2019). Heat-stabilized bFGF can reduce fluctuations in cell signaling and spontaneous differentiation (Daniszewski et al., 2018; Lotz et al., 2013). Studies have assessed specific aspects of iPSC culture, including effects of different maintenance media on gene expression (Daniszewski et al., 2018) and of different somatic cells of origin on motor neuron differentiation (Bardelli et al., 2020). Systematic, comprehensive review and meta-analysis will be needed to understand how iPSC derivation methods and/or culture conditions affect neural differentiation. Given the large number of independent variables, extensive cross-site collaborative efforts may be necessary to assess their relative contributions.

Development of human cortical neurons

Balanced inhibitory and excitatory neuronal activity in the cerebral cortex is needed for normal neurological function and often disrupted in IDD, due to altered development and/or function of either or both neuronal subtypes. Therefore, many IDD cell-based experimental approaches focus

on mimicking aspects of these developmental programs. During cortical development, progenitors generate different neuronal layers at distinct developmental stages. Excitatory glutamatergic projection neurons are derived from the cortex in the dorsal forebrain, while inhibitory GABAergic cortical interneurons originate from the subcortical ventral forebrain. Excitatory neurons arise from progenitor cells in the cortical ventricular zone and migrate radially to their cortical layer destination, with early-generated neurons residing in deep layers, while late-generated neurons reside in upper layers. During cortical progenitor differentiation from iPSCs, this temporal sequence of neuron specification is conserved (Anderson and Vanderhaeghen, 2014; Gaspard et al., 2008), such that differentiating upper layer cortical neurons can take several months. Each temporally defined progenitor population can be differentiated into layer-specific neurons by inhibiting cell division to synchronize differentiation (Borghese et al., 2010; Ogura et al., 2013). This characteristic can be exploited in study design to focus on the most relevant neuronal subtypes affected in patients with a specific IDD. Initial iPSC studies used differentiation protocols that led to mixtures of primarily deep-layer GABAergic and glutamatergic neurons. However, as many IDDs affect upper layer neurons and interneurons, these studies may have overlooked disorder-related phenotypes or implicated phenotypes not relevant to IDD etiology. While protocols for generating spinal motor neurons, dopaminergic neurons, and cortical excitatory and inhibitory neurons are well developed, differentiation of other IDD relevant neurons, such as hippocampal, serotonergic, and hypothalamic neurons, is not yet well established. Programs for generating glial cells, including astrocytes and oligodendrocytes, have also been developed, but are used less extensively in IDD studies. Furthermore, while iPSC-derived neurons that express many inhibitory cortical interneuron subtype markers can be readily generated, these may not represent mature or inclusive subtypes, by comparison with those present in fetal brain. Finally, while three-dimensional (3D) cortical organoids are now in use, these represent substantially more complex and heterogeneous mixtures of different neural cell types, and so were not incorporated into our cross-manuscript comparison (Lancaster et al., 2013; Sidhaye and Knoblich, 2021). In summary, it is important for studies to build on patient clinical phenotypes and draw comparisons between fetal- and iPSC-derived neuronal cell types.

Differentiation of cortical neurons from iPSCs

Protocols for generating iPSC-derived neurons *in vitro* to study IDDs involve manipulating growth factor signaling to mimic developmental cues or, more recently, inducing neurons directly from iPSCs by overexpressing neurogenic transcription factors (TFs) for cell fate conversion. Each



protocol has multiple intervention points that vary between laboratories, researchers, or experiments, resulting in differential efficiencies with which neurons are generated, and in their characteristics, maturation, and functionality (Figure 3; Tables S4, S5, and S6). Variation must be documented and controlled to enable reproducible results and post hoc meta-analyses, especially to compare data across different IDD studies. We summarize technical variables for protocol standardization and benchmarking of iPSC-derived neurons.

In vitro iPSC differentiation protocols endeavor to mimic spatial signals present during brain specification and patterning *in vivo* (Campbell, 2003; Gaspard et al., 2008; Watanabe et al., 2005). iPSCs transition into NPCs before maturing into neurons. When handled and monitored properly, NPCs can be expanded and/or cryopreserved, which is advantageous for assays requiring large cell numbers, and minimizes differentiation batch effects between assays. The most widely used strategy to derive neural ectoderm from iPSCs involves inhibiting SMAD TFs acting downstream of the Activin/Nodal and bone morphogenetic protein (BMP) transforming growth factor β (TGF- β) signaling pathways (so-called dual SMAD inhibition [Chambers et al., 2009]). Further modulating morphogen signaling, including SHH and WNT, specifies NPCs with different dorsoventral and anterior-posterior regional identities, resembling progenitors found in different locations of the developing brain. NPCs can then be differentiated into cortical neurons that are predominantly glutamatergic without morphogens, or differentiated into GABAergic cortical neurons or specific interneuron subtypes with particular morphogens (Figure 3; Tables S4, S5, and S6).

Most iPSC differentiation protocols promote NPC cell cycle exit to generate neurons (Borghese et al., 2010; Ogura et al., 2013). If a relatively homogeneous neuronal culture is required for downstream analyses, some protocols also incorporate additional steps to eliminate proliferating progenitors and/or isolate mature neurons from the culture, including adding AraC (cytarabine) to kill dividing cells or purifying neurons expressing cell surface markers. Maturing neurons remains a major challenge, as these cells typically resemble human fetal neurons rather than those of later developmental stages (Handel et al., 2016). Co-culturing iPSC-derived neurons on astrocyte feeders (mouse/rat or human) can promote functional maturation (Johnson et al., 2007). Maturing iPSC-derived neurons may require more complex culture conditions involving more than one neuronal subtype and glial population. Some researchers address this challenge by using 3D organoid cultures, but these neurons are still not fully mature, mimicking neurons in second trimester fetal brain (Amiri et al., 2018; Kaya et al., 2019; Velasco et al., 2019).

Key technical variables

Many differentiation protocols are used to generate cortical excitatory neurons and cortical interneurons. Key technical variables (detailed in Tables S4 and S5) include signaling cues/morphogens and basal media, time course, 3D versus monolayer culture, molecular markers used to benchmark specification and differentiation, efficiency with which NPCs and neurons express these markers, and assays used to assess functional neuronal properties. Technical variability influences the specificity, efficiency, and timing of patterning, and/or affects characteristics of the resulting NPCs and neurons. For example, for cortical excitatory NPC specification, some protocols modulate only BMP signaling (Griesi-Oliveira et al., 2015), while most modulate both BMP and Activin/Nodal TGF- β signaling (Table S4). Likewise, current protocols for specifying cortical interneuron NPCs use variable timing and regimens for SHH modulation (Liu et al., 2013; Maroof et al., 2013; Meganathan et al., 2017; Nicholas et al., 2013) (Table S5). Even protocols that ostensibly modulate the same signaling pathways over the same time frame vary, with some using recombinant proteins while others use small-molecule agonists or antagonists (Tables S4 and S5). Finally, vendor and lot-to-lot variability, as well as variable toxicity, may effect experimental outcomes (Zhang et al., 2012). These variables can be addressed by fully disclosing materials, including vendor and catalog number, and providing detailed differentiation protocols with a clearly annotated timeline for experimental procedures.

Another fundamental variable involves whether cells undergo patterning as a plated monolayer (ML), a floating 3D embryoid body (EB), or an adherent, plated EB (aEB), during either NPC specification and/or neuronal differentiation (Tables S4 and S5). While EB methods arguably more closely mimic cell-cell interactions that occur in the developing neural tube, EBs can be heterogeneous. Moreover, as the signaling mechanisms in each paradigm are not fully appreciated, there may be subtle differences in performance, such that it is critical to benchmark the neuronal outcome.

Induced neurons: Bypassing development

Overexpressing neurogenic TFs can directly convert iPSCs into induced neurons or "iNs" (Pang et al., 2011; Xu et al., 2015). Commonly used TFs to produce cortical excitatory iNs include Neurogenin-2 (NGN2), NGN1, and NEUROD1 (Tables S6). Similarly, GABAergic neurons or astrocytes can also be effectively induced using other TFs (Araujo et al., 2018; Canals et al., 2018; Yang et al., 2017). iNs are relatively homogeneous (e.g., >90% neurons), and reach later developmental stages more quickly than development-based differentiation protocols. Thus, iNs are useful for applications like high-content imaging or high-throughput screening, which require large numbers of neurons with

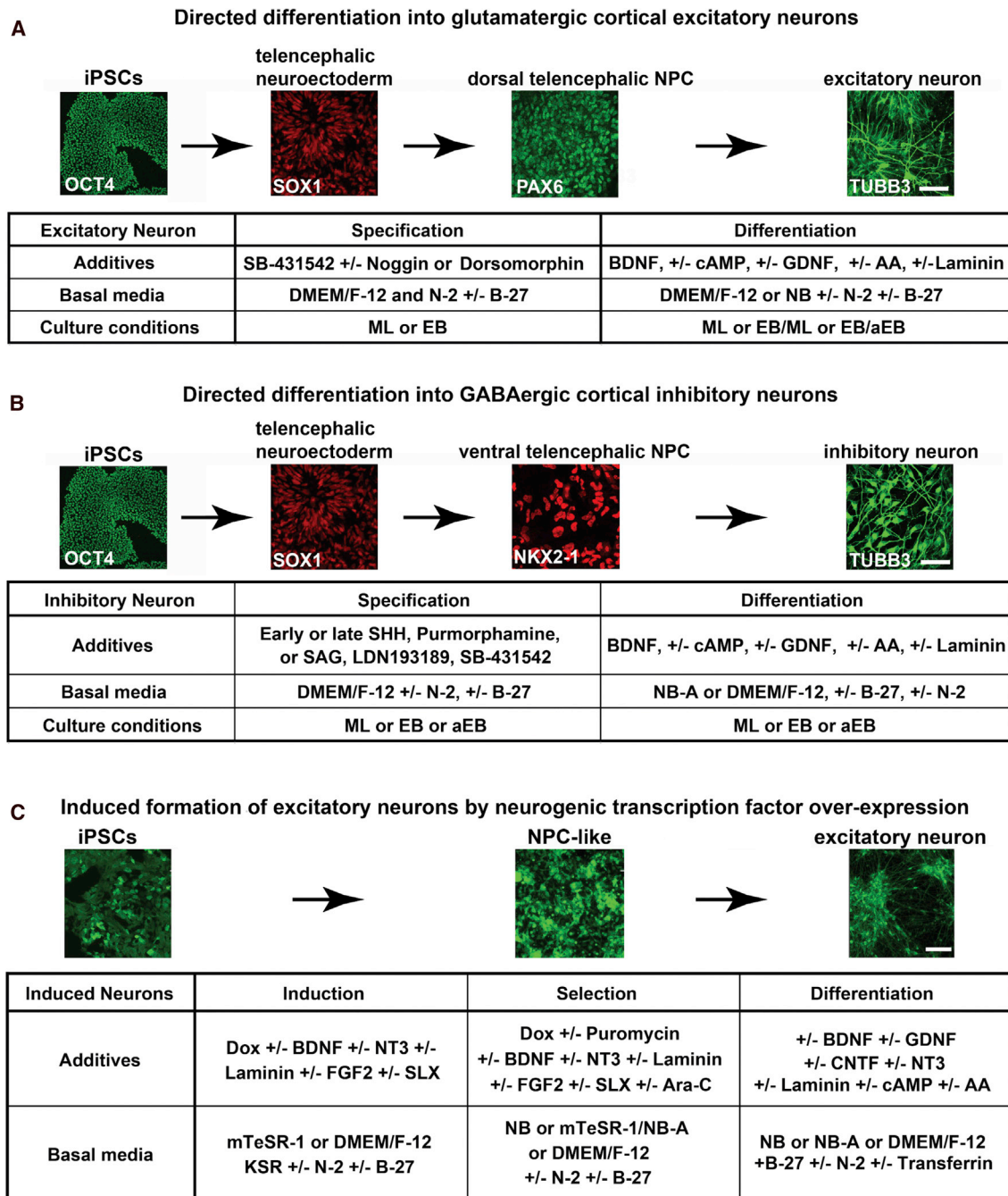


Figure 3. Technical variables that differ by laboratory and protocol during generation of neurons from iPSCs

(A and B) Variables that differ during specification and differentiation of iPSCs into (A) glutamatergic cortical excitatory neurons and (B) GABAergic cortical inhibitory neurons are shown. These include signaling cues/additives, basal media, culture conditions, and whether differentiation is conducted in monolayer (ML), or adherent or non-adherent embryoid body (aEB/EB) culture.

(C) Variables that differ during generation of cortical excitatory neurons from iPSCs by NGN2 overexpression. This figure summarizes data from studies assessed in Tables S4, S5, and S6. Abbreviations and definitions of terms are in Table S8. Scale bars, 50 μ m in (A and B) and 130 μ m in (C).



low batch-to-batch variability. However, since most iN protocols are designed to generate a single homogeneous neuronal subtype, iNs may require co-culturing to facilitate maturation and functionality. Furthermore, as published protocols used by various laboratories differ in multiple ways, the resulting iNs may also differ (Table S6).

Comprehensive analysis of iNs, including single-cell sequencing, is needed to define their identity and developmental stage, and to determine whether these cells resemble neurons *in vivo*. When studying IDD with patient-derived iNs, exogenously expressing a master transcriptional regulator of neurogenesis, such as NGN2 may override and mask IDD-related defects in neuronal development, particularly those occurring upstream of NGN2 activities (Wilkinson et al., 2013). Moreover, since most IDDs involve altered neurodevelopment, some phenotypes may only be detected by differentiating iPSCs through an NPC intermediate. As iN protocols involve a significantly shortened progenitor stage, these protocols are not suitable for characterizing NPC-specific anomalies and can confound use of iNs to define IDD-linked cellular phenotypes (Schafer et al., 2019).

TF overexpression remodels the epigenetic landscape. Coupled with antibiotic selection, iNs exhibit lower batch-to-batch and line-to-line variability than neurons derived by modulating signaling. However, similar technical variables (e.g., iPSC quality, media conditions, extracellular matrix environment, and cell organization) likewise impact differentiation propensity and cell identity. For example, using both dual SMAD inhibition-mediated differentiation media and NGN2 overexpression in concert increases generation of mature neurons (Nehme et al., 2018). Single-cell sequencing also revealed molecular heterogeneity and challenges the notion that iNs are homogeneous. Since iNs can still sense extrinsic signals, it is perhaps not surprising that cell fate can be influenced by both culture conditions and TF reprogramming. Furthermore, different expression vector integration sites can cause variable expression of neurogenic TFs, increasing iN heterogeneity. Use of genome editing to introduce an inducible TF expression construct into genomic safe harbor loci (e.g., AAVS1) can overcome this shortcoming.

Several unique variables apply to these transcription factor-based protocols (Table S6). Similar to growth factor-mediated differentiation, cell-seeding density profoundly affects iN maturation and health. Viral titer and transduction efficiency, antibiotic selection dosage and time frame (if used), and doxycycline dosage and time frame to induce TF overexpression must all be optimized to minimize iPSC toxicity and generate robust iN populations. While activities of key TFs are expected to be largely conserved between species, direct comparisons have not been made, complicating comparisons between studies using human

versus mouse NGN2 overexpression. Moreover, tetracycline-inducible transcriptional activation can exhibit leaky basal transcription in an uninduced state, potentially compromising iPSC pluripotency (Costello et al., 2019; Tian et al., 2009). Therefore, it is important to select lines with low basal TF expression when establishing iPSC lines transduced with iN factors and to monitor spontaneous differentiation when culturing uninduced iPSCs.

Recommendations and benchmarks to address variability

Given many potential sources of variability, it is essential to benchmark iPSC quality, efficiency of progenitor specification, regional progenitor character, neuronal differentiation efficiency, final cell identity and culture composition, and neuronal maturity and functionality (Muratore et al., 2014; Tao and Zhang, 2016; Topol et al., 2015). Recommendations for best practices in data collection, QC, and benchmarking are summarized in Table 1, and a template for data documentation is provided in Table S7.

Genetic abnormalities can arise during reprogramming, passaging, or genome editing of iPSCs. Therefore, cell line authentication and quality control for pluripotency and genome stability are essential. Verification of cell line identity with the patient-derived sample by short tandem repeat (STR) analysis is required, as is validation of stem cell marker expression. Trilineage differentiation potential confirms iPSC pluripotency. Importantly, accumulation of common chromosomal abnormalities should be closely monitored as aneuploidy (most frequently of chromosomes 12, 17, 20, and X) is common (Assou et al., 2020; Taapken et al., 2011). At a minimum, G-banded chromosome analysis should be carried out immediately after iPSC derivation, after each cell expansion for banking, and after every 5–10 passages during culture (Martins-Taylor et al., 2011; McIntire et al., 2020). In addition, a recent study has shown similar numbers of *TP53* mutations in cells grown with and without feeder cells (Merkle et al., 2017), but further studies are required. Clump passaging and avoidance of enzymatic dissociation when culturing iPSCs is advisable, as dissociation to single cells may select for the best adapted cells, contributing to genomic variation (Bai et al., 2015; Garitaonandia et al., 2015). It is also advisable to track each line's passage number and avoid working with lines at >40 passages when aneuploidy and other mutations are more likely to arise (Martins-Taylor et al., 2011; McIntire et al., 2020; Taapken et al., 2011). For this reason, it is crucial to expand and cryopreserve large numbers of vials of working stocks at low passage number for each iPSC line. Furthermore, CNVs are present as low-frequency somatic genomic variants in parental fibroblasts and manifested in the corresponding iPSC lines due to the colonies' clonal origin (Abyzov et al., 2012). Experimental design should account for differences between clonal lines derived from the same patient by



replicating findings across at least two independent clonal lines, with more than four patient/control pairs when available (biological replicates), and more than three independent differentiation batches (technical replicates) per experiment (Germain and Testa, 2017). For phenotyping NPCs and neurons derived from multiple iPSC lines, it is important to differentiate each, if not all, patient/control pairs simultaneously to minimize experimental variability. It is also ideal to derive iPSCs from the same somatic cell type via the same reprogramming method, and to maintain these in the same manner, to reduce potential variability. Furthermore, for phenotyping in small well format (96- or 384-well), robotic liquid handling and electronic pipettes with speed and volume control can decrease well-to-well variability.

For neural differentiation, pilot testing of reagents should be conducted and, whenever possible, the same validated lots of reagents throughout a set of experiments should be used. Once NPCs are generated, in addition to gene expression, they should be assessed for the desired NPC identity (e.g., >80% of cells expressing PAX6 or NKX2-1 for excitatory and inhibitory NPCs, respectively) by fluorescence-activated cell sorting (FACS) or immunocytochemistry (Tables S4 and S5). If NPCs or transduced iPSCs for generating iNs are cultured, passage number and culturing duration should be limited to avoid cell state drift. Once neurons have been differentiated, the efficiency of differentiation, neuron maturity, and functional properties should be assessed, starting with qPCR or RNA sequencing (bulk or single cell). Next, it is standard practice to report both the overall percentage of neurons (e.g., expressing TUJ1 and MAP2) and percentage expressing markers of the neuronal subtype of interest (e.g., VGlut or VGAT for excitatory and inhibitory neurons, respectively; Tables S4 and S5). If purification approaches, such as FACS or microbead-bound antibodies, are used to isolate cells during differentiation, a detailed timeline for differentiation, cell purification, and experimental assays should be reported. Finally, functional assessment by patch-clamp or multi-electrode array should be reported to characterize electrophysiological properties of the neurons. In sum, quantitative and qualitative assessment of the cell population is required throughout differentiation. Genetic and epigenetic background can modify not only the neuronal differentiation efficiency, but also the timing of neuronal maturity and acquisition of subtype identity and functional properties, even within excitatory or inhibitory neuron populations. Therefore, comprehensive analyses utilizing multiple neuronal markers, in combination with appropriate iPSC controls, are necessary to distinguish an IDD mutation-associated phenotype.

Quantitative data should be supported by adequate statistics. As a best practice, statistical tests should be per-

formed with at least four independent biological replicates (iPSCs from different individuals) (Germain and Testa, 2017). ISSCR guidelines encourage some journals (e.g., Stem Cell Reports) to require data from two to three patients and two to three controls, with two to three clones per donor analyzed. These numbers may be smaller when studying rare diseases or when using isogenic pairs of iPSC lines. More patients may be needed when studying polygenic disorders, unless a rigorous patient/control selection strategy is used.

Conclusion

Patient-derived iPSCs provide a unique and powerful model to study IDD etiology, both incorporating complex genetic contributors to IDDs in individual patients and recapitulating aspects of human brain development otherwise inaccessible to experimentation. The issues we raise are broadly applicable to other brain disorders. With knowledge accumulated from prior studies and advances in technology, we now have a wide repertoire of tools. Diverse techniques can sometimes unveil serendipitous discoveries that are informative, but the data are then not easily accessible and/or cross-comparable. Therefore, it is important to implement standard quality control measures and to methodically document experimental design and outcomes in publications. Such standards enable meta-analysis across groups and/or across different IDDs to identify commonly affected pathways for developing interventions. Adherence to these standards requires increased resources, and so we recommend that journal editors provide additional time for experimentation and that funding agencies provide additional funding to fortify the reproducibility of future studies. Now, more than ever, the stem cell research community needs to develop shared standards, approaches, and benchmarks to ensure rigor and reproducibility in stem cell-based IDD research, so that these experimental systems can be used to define reproducible alterations of neurodevelopment and neural cell function linked to IDDs and to assess potential avenues for diagnosis and treatment.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.stemcr.2021.03.025>.

AUTHOR CONTRIBUTIONS

A.B., K.L.K., and M.S. conceptualized and supervised this project. P.F.C. created the PubMed search term and compiled a publication list. N.C.A., P.F.C., and W.A.S. verified and reviewed selected papers. N.C.A. and P.F.C. designed the reviewing methodology and scored the papers. N.C.A., P.F.C., K.L.K., K.M., A.J.P., and W.A.S. created tables and figures. A.B., N.C.A., P.F.C., K.L.K., M.S., and K.M. wrote the manuscript with input from all authors. Members



of the Cross-IDDRC Human Stem Cell Working Group reviewed the final version of this manuscript.

CONFLICTS OF INTEREST

M.S. reports grant support from Novartis, Roche, Pfizer, Ipsen, LAM Therapeutics, and Quadrant Biosciences unrelated to this project. M.S. served on Scientific Advisory Boards for Sage, Roche, Celgene, Aeovian, Regenxbio, and Takeda. N.C.A., P.F.C., W.A.S., K.L.K., A.J.P., K.M., and A.B. report no competing interests.

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