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## iPSC-Based Drug Screening for Huntington's Disease

Ningzhe Zhang, Barbara J. Bailus, Karen L. Ring, and Lisa M. Ellerby

Buck Institute for Research on Aging, 8001 Redwood Blvd, Novato, CA 94945

### Abstract

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, caused by an expansion of the CAG repeat in exon 1 of the huntingtin gene. The disease generally manifests in middle age with both physical and mental symptoms. There are no effective treatments or cures and death usually occurs 10–20 years after initial symptoms. Since the original identification of the Huntington disease associated gene, in 1993, a variety of models have been created and used to advance our understanding of HD. The most recent advances have utilized stem cell models derived from HD-patient induced pluripotent stem cells (iPSCs) offering a variety of screening and model options that were not previously available. The discovery and advancement of technology to make human iPSCs has allowed for a more thorough characterization of human HD on a cellular and developmental level. The interaction between the genome editing and the stem cell fields promises to further expand the variety of HD cellular models available for researchers. In this review, we will discuss the history of Huntington's disease models, common screening assays, currently available models and future directions for modeling HD using iPSCs-derived from HD patients.

### Keywords

Huntington's disease; Stem cells; Induced pluripotent stem cells; Drug screening; Phenotypes

### Introduction

Huntington's disease (HD) is a devastating, dominantly inherited neurodegenerative disease caused by a polyglutamine expansion in the N-terminus of the huntingtin protein (HTT) (1, 2). Individuals with 36 CAG repeats or fewer are not affected, while those with 40 or more CAG repeats will eventually develop HD at some point in their lives. The HD mutation is fully penetrant with the time of onset correlating to repeat number. A higher number of CAG repeats results in an earlier age of onset and a more severe form of the disease. In HD patients, neurons in both the striatum and cortex are affected (Figure 1). Progressive atrophy of both the striatum and cortex results in movement incoordination (also known as chorea), a decline in cognitive function, and psychiatric disorders. Typically, most HD patients are

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Corresponding author: [lellerby@buckinstitute.org](mailto:lellerby@buckinstitute.org).

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diagnosed as middle age adults (40–45 years) and the disease slowly progresses over 10–20 years after initial symptoms (1). However, about 10% of HD cases occur in juveniles who typically have a more severe form of the disease.

Unfortunately, there is no effective disease modifying therapy available for HD. One reason for these deficiencies may be the lack of accurate HD models that display all the phenotypes and pathology seen in human patients with HD. Post-mortem brain tissues from HD patients are useful to identify neuropathology of HD. However, post-mortem tissues generally provide late stage pathological changes for HD, which makes understanding early disease changes challenging. Phenotypes observed in post-mortem tissues may represent secondary phenotypes while primary causes might not be uncovered (3, 4). The use of post-mortem tissues as HD model is further impeded by limited availability. Patient derived cell types available are usually limited to blood and spinal fluid. Animal models that mimic some but not all HD phenotypes are used to help elucidate the mechanisms for HD pathogenesis. The HD mouse models most commonly used include the short HTT fragment transgenic models, full length HTT BAC/YAC transgenic models, and HTT knock-in models (5). However, no single HD mouse models is able to recapitulate all aspects of human HD disease progression most likely due to the fundamental genetic differences between rodents and humans (6). Additionally, and perhaps revealing why these models are inadequate, the HD rodent models require much longer CAG expansions to elicit phenotypes, and even then, disease phenotypes are still mild compared to human HD patients (7). Particularly striking is the fact that in human patients, there is a 90% loss of medium spiny neurons (MSNs) while in most rodent models the loss of MSNs is 10% (8).

The breakthrough in human embryonic stem cells (ESCs) technology (9) in the late 1990s when scientists were able to isolate and culture ESCs, provided new possibilities for modeling the various human neurological diseases in a dish, including HD (10–14). However, the use of human ESCs is hindered by ethical concerns regarding the destruction of human blastocysts in the process of ESC derivation and the fact that the disease relevant genotypes may not be represented. In 2006, the discovery of induced pluripotent stem cells (iPSCs) provided a more attractive and efficient way to model human diseases utilizing patient-derived stem cells without the previous ethical concerns. This technology can reprogram adult human somatic cells into ESC like iPSCs by ectopic expression of four transcription factors (15) without the use of embryonic tissue. The advancement of iPSC technology has truly revolutionized the disease-modeling field, making disease relevant human cell types and genotypes available for mechanistic studies and drug screening. Methods to improve and optimize the generation of iPSCs are ongoing (16–22).

The HD field has benefitted substantially from the recent growth and breakthroughs in the human stem cell field (23–31). The technologies in the stem cell field have allowed for the generation of more HD models, customization of specific HD models and the ability to study HD in many relevant cell types without the need of biopsies. This review will detail the current HD stem cell based models, considerations for screening technologies using these models, and the future directions for stem cells in HD modeling.

## iPSC based human HD models

Many screens including high throughput screening (HTS) and high content screening (HCS) have already been conducted in HD invertebrate models, primary cells, or immortalized cell lines with chemical or genetic modifiers (32). However, lower organisms like yeast, worms, and fruit flies have limitations and may not accurately reflect the pathogenesis of HD in humans. Primary cells are physiologically relevant to HD, but their sources such as human biopsy samples or animal dissections are usually associated with poor availability, low quality, and inconsistency. Immortalized and easily available rodent and human cell lines such as HeLa, PC12 and HEK293 cells can be genetically modified to carry the HD mutation, but their relevance in the context of HD has not yet translated into a treatment for HD. Another consideration in terms of a cell lines suitability to model HD is the process of immortalization, which may introduce unpredictable variables (such as genetic mutations) to these cell lines (33). Likely due to nonphysiological expression of HTT and the prior mentioned drawbacks of immortalized cell line and invertebrate models, hits identified by previous screens has not yet translated into a treatment for HD (34). With these considerations, it is necessary for the field to have better models with which to understand the pathology of HD and screen for potential treatments.

Human pluripotent stem cells (PSCs), including ESCs and iPSCs, exhibit an unlimited potential for self-renewal and can differentiate into all cell types in the body including neurons. Therefore, they are an excellent source of cells to screen for HD therapies. One example is a screen in wild type human ESCs derived neural stem cells (NSCs) for chemical inhibitors of the transcriptional repressor REST (repressor element-1 silencing transcription factor) using a luciferase-based assay. One potent compound was identified, which could increase expression of brain-derived neurotrophic factor (BDNF) (35). This may be highly relevant to HD as BDNF is a key neurotrophic factor that is depleted during disease progression and restoring BDNF levels ameliorates disease phenotypes. Compared to ESCs, iPSCs have many advantages. As mentioned the generation of iPSCs does not require the destruction of human blastocysts, thus avoiding the major ethical issues associated with using human ESCs. More importantly, iPSCs have the same genetic background as the donor's somatic cells. These autologous cell lines may be the foundation for the development of personalized diagnosis, screening and therapy.

Shortly after the discovery of human iPSCs, Park et al. generated a group of human iPSC lines from patients with various diseases including HD (31). Following this development, we demonstrated that HD-iPSCs when differentiated into NSCs had a number of features relevant to HD pathology, including susceptibility to cell death upon growth factor withdrawal as measured by caspase activity (30). In the years following, many iPSC-based human HD cell models have been generated and utilized (23–30, 36). These models together with human ESC based HD models are summarized in Table 1. Among these HD cell models, the ones generated by the HD iPSC Consortium are the best characterized. The HD Consortium generated fourteen iPSC lines derived from fibroblasts originating from seven individuals, healthy or affected by HD, representing cell models ranging from asymptomatic controls to HD models with varying CAG repeat numbers and disease severity. NSCs were differentiated from these iPSC donor lines with CAG repeat numbers 21, 33, 60, 109, and

180. These NSC lines were analyzed by microarray and clustered-based analysis of gene expression. Control NSCs (21 and 33 CAG repeats) were shown to be distinct from HD NSCs (60, 109 and 180 CAG repeats). It was found that HD NSCs and HD-derived neurons exhibited decreased cell adhesion and adenosine triphosphate (ATP) production, increased caspase-3 activation, increased cell death after prolonged culture or BDNF withdrawal, and increased vulnerability to stress/toxicity. Significantly, these disease-associated phenotypes correlated to CAG repeat number: more severe phenotypes were found in HD cells with longer CAG repeats (24). These iPSC lines are currently deposited at Coriell and represent a rich resource for the HD community including their use in potential drug screens.

At this time, most HD iPSC lines are studied without any genetic modification and require a direct comparison to age and sex-matched healthy control iPSC lines. Studies comparing disease and control iPSC lines from separate individuals have shown that genetic background differences between these individuals, even when controlled for by age, sex, and ethnicity, can skew results when trying to identify specific disease mechanisms. To circumvent this issue, isogenic iPSC lines, where disease-causing mutations are genetically corrected to produce a wild type allele, have been generated to produce genetically identical disease and control iPSC lines. Theoretically, any phenotypes identified in these isogenic iPSCs can be solely attributed to the disease-causing mutation. These cell lines are particularly suitable for screening of drugs or identifying mechanisms that target phenotypes caused by the disease mutation. With regards to HD, we generated two human HD isogenic iPSC lines using a homologous recombination based genetic correction method in which a 72 CAG repeat was replaced with a normal 21 CAG repeat in the gene *HTT* (25). It was demonstrated that at the NSC stage, genetic correction of the HD mutation could reverse disease-associated phenotypes such as elevated cell death and caspase-3/7 activity as well as lower BDNF levels and energy metabolism (25). As discussed further below, new genome editing tools such as CRISPR/Cas9 have made it easier to produce genetically modified cell models, which will allow for the efficient generation of iPSC allelic series of varying CAG repeat lengths and new models of HD (37).

An additional alternative to human HD iPSCs is HD iPSC lines derived from monkeys. These iPSCs were derived from skin cells of a transgenic monkey HD model with human *HTT* exon 1 with 84 CAG repeats (25). The iPSCs were able to differentiate into neurons and HD phenotypes were observed including aggregate formation of mutant *HTT* (mHTT) protein and increased cell death (38). These primate HD iPSC models provide another platform for drug screening with possibility of further exploring promising candidates in a non-human primate HD models. This is a potentially important drug-screening step as many candidate drugs are screened in non-human primates before entering Phase I human clinical trials.

## HD phenotypes for screening

A pivotal consideration when utilizing HD stem cell models for drug screening is the selection of appropriate measurable phenotypes to use as endpoints. This may relate to the biology of the *HTT* protein or to well characterized phenotypes found in HD disease progression. Phenotypic endpoints may have benefits for drug discovery when compared to

using known targets. Recent analysis of approved drugs suggests a decline in new drugs brought to market, which may be due to a switch in the pharmaceutical companies from using phenotypic assays to target-based assays for drug development (39, 40). After selecting endpoints, assays can be either designed or utilized for the most efficient screening methods. Previous studies have provided researchers with a robust selection of disease associated phenotypes as end points in primary cells and immortalized cell lines. The commonly used phenotypic endpoints for human stem cell derived HD models are listed in Table 1. Several of these commonly screened phenotypes first identified in HD post mortem or other models have also been validated in HD iPSC cell models.

HTT aggregation is arguably the most commonly used phenotype for HD screening. Similar to other neurodegenerative diseases, HD is characterized by abnormal protein (mHTT) folding, aggregation, and clearance (Figure 1). The aggregates formed by the N-terminal fragments of mHTT are found in cortical neurons and striatal medium spiny neurons in HD patients and various HD models (41). The clearance of mHTT aggregates correlates with disease reversal in HD mouse models (42). In some screens, aggregation of mHTT is monitored by fluorescence image based high content screening (43–45). Alternatively, sodium dodecyl sulfate (SDS) insoluble mHTT aggregates can be detected by a filter retardation assay coupled by dot immunoblotting (46–48). Research supports the concept that the mHTT aggregates are actually a coping mechanism for the cells and are less toxic than the smaller and more soluble HTT fragments (49, 50). When screening for potential therapeutic compounds, it would be advisable to screen for aggregation formation, mHTT, HTT post-translational modifications and toxic fragment levels. Since mHTT protein is the cause of HD pathology, screening for compounds capable of decreasing mHTT levels has been reported (32). Time-resolved fluorescence resonance energy transfer (FRET) assays that detect HTT levels were developed to screen small molecule libraries in the HN10 neuronal cell line in a high throughput format (51, 52). A western blot based assay aimed at reducing an N-terminal HTT fragment was utilized to screen a RNA interference (RNAi) library that targeted all human protease genes and it identified matrix metalloproteinases (MMPs) as modifiers of HTT proteolysis (53). These assays offer the researcher a variety of choices from which to select the best assay for their screening purposes.

Cell death and neuronal toxicity are other hallmark phenotypes of neurodegenerative diseases including HD. In HD, neuronal cell loss is observed in the cortex and striatum of patient brains. The decrease in striatal and cortical volume correlates with cell death and can be detected in patients many years before behavioral symptoms manifest (54). Early markers of cell death and upregulation of the apoptosis pathway including activation of caspases are typically associated with different kinds of HD cell models (41). Due to their obvious significance to HD pathology, cell death and neuronal toxicity are popular endpoints for screening. Using an HD PC12 cell model with an endpoint based on a fluorescent cell viability assay, five structurally distinct compounds were found to prevent apoptosis induced by mHTT. Their common target protein, disulfide isomerase, was further identified to be pro-apoptotic and is involved in the pathogenesis of a number of neurodegenerative diseases (44). An assay for caspase-3/7 activity, which are key execution caspases in apoptosis, was employed to screen a kinase inhibitor library in immortalized striatal cells from the Hdh<sup>111Q/111Q</sup> knock-in HD mouse model. The screen identified that DGK inhibitors

decreased caspase-3/7 activity (55). The same caspase-3/7 activity assay was used on a larger scale to screen siRNA pools against over 7000 druggable human genes (56). Another chemical screen performed by the same group applied both a caspase-3/7 activity assay and an ATP assay, a measurement indicating mitochondrial function, in Hdh<sup>111Q/111Q</sup> striatal cells. This screen identified a number of structurally related compounds that may act through the serotonin receptor signaling pathway (57). All of these cell death and neuronal toxicity assays have been essential in understanding HD pathology. These assays can be utilized in further studies on HD iPSCs and corresponding disease derived cell types.

Autophagy is one of the major methods for cells to clear misfolded protein. In HD, the autophagy system is impaired by mHTT, which causes dysregulation in the clearance of misfolded and aggregated proteins including mHTT itself (58, 59). Inhibition of mammalian target of rapamycin (mTOR) can activate autophagy. The mTOR inhibitor rapamycin and its analog CCI-779 have been shown to reduce mHTT accumulation and toxicity in HD fly and mouse models (60). However rapamycin has many side effects due to its broad activity (61). It would be ideal to have a highly specific autophagy inducer for treating HD. Several screens in HD cell models have identified pharmacological (62, 63) or genetic (64) modifiers of autophagy. One group has also identified mTOR independent targets for autophagy induction in a screen of FDA-approved drugs (65).

## Phenotypes in human derived HD-iPSCs cells types

There are many phenotypes in human PSC derived HD models that can be measured by specific assays and these are listed in Table 1. The most commonly screened phenotypes in both human ESC and iPSCs were mentioned previously and are neuronal toxicity and HTT aggregation. Additionally, there are more phenotypes compatible with high throughput screening in iPSC based HD models. One of these phenotypes is BDNF expression, which is suppressed in HD NSCs compared to isogenic corrected NSCs with a normal CAG repeat length (30, 66). This effect is probably due to transcriptional dysregulation caused by mHTT (25) and has been confirmed in other human HD derived neurons from iPSCs (36). In HD NSCs, when growth factors in normal medium (full medium) are taken away, cells show phenotypes relevant to the disease. As shown by immunocytochemical staining in Figure 2, phenotypes such as increase in cleaved HTT, activated caspases and decrease in BDNF and polymer F-actin as measured by phalloidin conjugated fluorophore are suitable endpoints for screening in HD-NSCs (Figure 2). ATP levels were lower in NSCs bearing a longer CAG expansion (38), which indicates impaired mitochondrial function, as mitochondria are the major power source inside cells that produce ATP. Furthermore, neurite outgrowth was impaired in neurons differentiated from HD patient derived iPSCs (26), a phenomenon resembling defects in neuronal maturation and axon outgrowth in HD. These examples represent endpoints suitable for HD screens in a high throughput format, which is especially valuable when screening thousands of potential therapeutic candidates. Other phenotypes that have been identified in MSNs derived from human HD-iPSCs are increased expression of  $\gamma$ H2AX and elevated oxidative stress (67). In this study the A<sub>2A</sub>R-selective agonists protected MSNs through the cAMP/PKA-dependent pathway (67). Also observed in human HD NSCs is a deficit in manganese-dependent activation of p53 (68).

Recently, an unsuccessful screen funded by the California Institute for Regenerative Medicine (CIRM) and conducted by Numerate, Inc. demonstrated the importance of using more caution in screening plan design. This screen tried to identify novel small molecule modifiers of toxicity in neurons derived from HD patient iPSCs. In their counter screen, all previous hit compounds behaved the same as in their original screen, and thus these hits were identified as false positives (69). To reduce commonly seen false positive hits from primary screens, it is critical to plan the screening strategy carefully. It is advisable to have multiple end points for the primary screening. Alternatively, HD cell models from multiple sources can be screened simultaneously, to help account for cell line specific hits. Implementing these strategies should significantly lower the chance of generating false positive results. A counter screen should be included in the secondary screen to validate hits from the primary screen. Certainly expanding the number of end points and/or cell types will increase screening costs, but the cost will be even more if false positive candidates are carried over to later stages of the drug discovery process, where one starts to utilize model organisms and produce large quantities of the compounds.

Technological development of high content screening has made multiple end points in primary screen more feasible. For example, multiple disease biomarkers can be imaged simultaneously, and live imaging can be coupled with other conventional high throughput format screens. All these advances will make designing a successful screening plan more readily available and effective for potential iPSC based HD screens.

## From iPSCs to differentiated cells

One important detail that should be considered when studying iPSC based HD models is the differentiation stage of the stem cells. In Table 1, the majority of HD disease phenotypes are not detected in pluripotent stem cells, but in either differentiated neurons and glia or lineage defined multipotent progenitor cells like NSCs. As one example, there is no significant difference in caspase-3/7 activity after growth factor deprivation between HD and corrected cells in iPSCs. The change in caspase-3/7 activation is only observed at the multipotent NSC stage (Ellerby lab unpublished data). This is in agreement with the fact that HD symptoms occur in the mature brain, affecting primarily the committed neural lineages. It should be noted that more subtle changes are detected in HD-iPSCs when compared to controls and this is noted in a number of signaling pathways such as TGF- $\beta$ ,  $\beta$ -cadherin, oxidative stress proteins and the p53 (66, 70). This information suggests that when using iPSC-based models for HD screening, it is essential to choose the appropriate differentiation stage starting from iPSCs and the method of differentiation.

Striatal neurons, specifically MSNs, are the main cell type in the brain affected by HD and MSN-like cells are likely the best candidates for the development of effective and predictive HD drug screens. MSNs of the striatum originate in the lateral ganglionic eminence (LGE) in the ventral part of telencephalon (71, 72). Sonic hedgehog (SHH) is one of the most important morphogens that determines dorsal-ventral patterning in developing neural tube through a concentration gradient with a high level of SHH on the ventral side and a low level of SHH on the dorsal side (73, 74). LGE formation is also under the control of the right concentration of SHH (75). In contrast to SHH, WNT signaling is critical in dorsal

telencephalon development (76). Ectopic activation of WNT signaling in the ventral telencephalon can repress expression of ventral markers and induce expression of dorsal markers (77). Measurement of these proteins can be used to validate the correct development stage of iPSC-derived MSNs before using them to screen for therapeutic compounds.

Guided by knowledge of striatal development in the brain, multiple protocols are published that can derive striatal neurons from human ESCs or iPSCs. These ESC/iPSC-derived striatal neurons are positive for the MSN marker dopamine and cAMP-regulated neuronal phosphoprotein 32KD (DARPP-32) (Table 2) (64, 78–85). However, the efficiency of generating DARPP-32-positive striatal neurons in these protocols is still being optimized for conducting cell-based screening. The default *in vitro* differentiation of human ESCs to telencephalic progenitors is predominantly a dorsal fate because of endogenous WNT signaling (79). Therefore, almost all protocols aiming to generate striatal neurons from ESCs or iPSCs require supplementation of recombinant proteins or chemicals to suppress WNT signaling and activate SHH signaling at the appropriate concentration. Besides extrinsic signaling pathways, differentiation of striatal neurons is also regulated by intrinsic cues such as transcription factors. DLX1, DLX2, ASCL1, GSX2, FOXP1, and CTIP2 are all transcription factors that play key roles in the development of LGE progenitors and striatal neurons in the brain (86–93). Additionally, a recent study showed that Activin A is a critical factor for generating MSNs from human ESCs and iPSCs (83). One study involving direct conversion (or transdifferentiation) of human fibroblasts to striatal neurons involved expression of the brain enriched microRNA miR-9/9\*-124 together with transcription factors DLX1, DLX2, CTIP2 and MYT1L (94). This strategy could also be used to optimize protocols for generating striatal neurons from iPSCs in combination with modulation of SHH and WNT signaling. Optimization of these pathways will allow for drug screening to take place in the most disease relevant cell type, increasing the likelihood of finding effective therapeutics.

Cortical pyramidal projection neurons are also affected in HD. Specifically, the cortical layers III, V, and VI atrophy during HD disease progression and as much as 30% of cortical neurons in those layers die (95, 96). The cortex and striatum are linked by the corticostriatal pathway. The striatum receives excitatory glutamatergic inputs from the cortex via pyramidal projection neurons that innervate and excite MSNs. Dysfunction in striatal and cortical circuits or in striatal or cortical neurons can advance HD disease progression and neuropathology (97). Due to these findings, many studies of HD pathogenesis now incorporate toxicity screenings in cortical neurons as well as striatal neurons. Multiple protocols are published that can generate cortical neurons with high efficiency and purity from human ESCs and iPSCs (93, 98–100). By mimicking human cortical development, these protocols can generate cortical stem and progenitor cells that further differentiate into multiple classes of cortical projection neurons that express markers such as the glutamatergic marker vGlut1, deep-layer cortical neuron markers Tbr1 and CTIP2, and upper-layer neuron markers Cux1, Satb2, and Brn2. A commonly used method developed by Shi et al. was able to generate primary neuroepithelial cells and secondary cortical stem/progenitor cells using a method of dual SMAD inhibition to push cells towards the anterior neuroectodermal lineage (98). The primary cortical progenitor cells generated by this method express the transcription factors Foxg1, Pax6, Otx1/2, and Tbr2. The incorporation



of cortical neurons along with striatal MSNs in HD drug screens will provide a more thorough model of pathology of HD, which may lead to more effective therapeutics.

In the process of making neurons, it is critical to monitor the maturity of these neurons. Terminally differentiated, mature neurons are usually mitotically inactive, have elaborated processes, form synapses with neighbouring neurons and are capable of firing action potentials. Mature neurons differ from immature neurons not only in function and marker expression, but also in their capacity to handle stress and apoptotic signals. Mature neurons are more resistant to cytotoxic stimuli and more likely to survive in stressful conditions (101). This is particularly important for HD models as immature and mature neurons may behave significantly different in screening assays. However, neuron aging is a distinct phenomenon, representing a decline rather than maturation of functions. In aging neurons, there is an increase in reactive oxygen species (ROS), DNA damage, misfolded proteins, mitochondrial instability and dysregulation of ion homeostasis. All of these factors contribute to neuron dysfunction and death (102). HD is a neurodegenerative disease associated with aging. Typically age of onset for HD is in middle age despite the presence of mHTT protein from birth. Elevated levels of ROS and senescence associated inflammatory cytokines are markers of aging and might also be targeted for accelerating aging of freshly differentiated neurons (103). Due to the association of age with onset of HD, it might be useful to age neurons *in vitro* cell models to more accurately recapitulate HD pathology.

While striatal and cortical neurons are the main cell types affected in HD, evidence of phenotypes in glia, specifically astrocytes, have also been reported. In human post-mortem HD brains, astrocytes express mHTT and gliosis is often found in late stage HD brains and in HD mouse models (104–106). Interestingly, HD mouse models that expressed mHTT in striatal astrocytes experienced a decrease in expression of glutamate transporters and a reduction in glutamate uptake. This resulted in increased striatal neuron dysfunction due to the inability of astrocytes to protect neurons from glutamate excitotoxicity (106, 107). *In vitro*, astrocytes derived from human HD iPSCs exhibit phenotypes such as a higher number of cytoplasmic vacuoles compared to wild type astrocytes (29). Multiple protocols that efficiently generate glial fibrillary acidic protein (GFAP) positive astrocytes from human ESCs and iPSCs have been published (Table 2) (108–110). Thus endpoints such as the presence of cytoplasmic vacuoles or reduced glutamate transporter expression could be used to screen HD astrocytes derived from patient iPSCs.

Investigating other glial cell types such as microglia and oligodendrocytes could prove worthwhile with regards to developing HD screens. Microglia are activated in presymptomatic HD patients (111) and microglial activation is correlated with HD disease severity (112). In HD mice, mHTT impairs microglia migration in response to injury (113). The potential role that oligodendrocytes play in HD is more obscure. One study suggests that in preclinical stages of HD, myelin breakdown and increased iron levels occur and cause toxicity that could contribute to HD pathogenesis. During HD progression, neuronal loss is complemented by glial expansion of astrocytes and oligodendrocytes, which could further cause toxicity in HD patients, running endpoint assays in these cells will help elucidate if a therapeutic will be effective on all the cell types affected by HD (114). Protocols to generate

oligodendrocytes are available (115, 116) and microglia from human ESCs or iPSCs are currently being developed.

Thus when considering how to model HD *in vitro* using ESCs or iPSCs, there are multiple different cell types to consider. As is the case with other neurodegenerative disorders, co-culture models where multiple cell types are cultured together could provide more sophisticated models of HD and more phenotypic endpoints for drug screening. Amyotrophic lateral sclerosis (ALS) is a prime example of the power of co-culture experiments for disease modeling. By culturing astrocytes from SOD1 mutant ALS mice with healthy motor neurons (the cell type affected in ALS patients), scientists discovered that superoxide dismutase 1 (SOD1) mutant astrocytes secrete toxic factors that cause the death of healthy motor neurons (117). Conducting a screen using this type of co-culture model could identify drug candidates that prevent astrocyte-mediated motor neuron death in ALS. In the case of HD, culturing glial cells with either striatal neurons, or cortical neurons, or both could be beneficial in a similar manner to ALS.

Another alternative to co-culture is the generation of cerebral organoids, which are 3D brain structures derived from human ESCs or iPSCs that exhibit similar developmental stages to endogenous neural development and generate multiple brain tissue types and regions (118, 119). Brain organoids can survive long periods in culture, thus providing “aged” neurodegenerative models with more mature, functional neurons. This system has already been utilized to model microcephaly, and organoids generated from microcephaly patient iPSCs exhibited premature neuronal differentiation (120). In a 3D organoid system using HD iPSCs, screens could be developed with neuron function or survival as endpoints among the others listed previously. Additionally, other cell types affected such as glial cells could be easily monitored at the same time. Due to the difficulty of obtaining human living brain tissue samples, cerebral organoids currently offers one of the most comprehensive model conditions to represent in-vivo HD pathogenesis. Further development of this technology and optimization for high throughput screening would be very beneficial not only for HD but for many other neurodegenerative diseases.

## Genome Editing Tools for HD Models and Screening

When creating an ideal HD cell model for screening purposes, limiting the genetic background variance would be ideal while simultaneously having multiple CAG repeat lengths represented (37). Until very recently, creating a set of varying CAG repeat HD iPSC isogenic lines was a time consuming process and occurred with a low efficiency rate. However, the rapidly expanding genome engineering field has made the creation of multiple variations of a genetic disease model practical. The latest generation of genome engineering tools is referred to as “clustered regularly interspaced short palindromic repeats” (CRISPR) (121). Unlike the previous genome engineering tools, zinc finger nucleases (ZFNs) and TALENs (transcription activator-like effector nucleases), CRISPR is easier to utilize by the scientific community. The system targets any site in the genome with high efficiency, can be made to order, and has minimal off target effects (122). Cost and time have been drastically reduced, while accuracy and efficiency have been increased when utilizing this technology. CRISPR technology is a gene editing nuclease system, which uses a bacterial nuclease to cut

at a specific site in the DNA. This specific site is dictated by a guide RNA (gRNA), which specifies 18–21bp in the DNA, making it possible to target a single unique site in the genome (123). Recent studies have shown that the off-target effects of CRISPR are minimal with as few as one off target site(s) that were cleaved (124, 125). Having a low off target cut rate is critically important when introducing a mutation for disease model creation, to ensure that only one mutation is introduced into the genome. Both ZFNs and TALENs are complicated to design, assemble and they exhibit significant off target binding and cleavage, making them less than ideal tools for disease model creation. The advances in genome sequencing would allow for each potential model to be fully sequenced ensuring that only one donor insertion event has occurred. CRISPR has been used successfully to make several disease relevant iPSC models and mammalian models including the generation of a mouse model with multiple specific mutations in a single generation (126–129). The high efficiency and accuracy of CRISPR makes it the ideal system to utilize for making a library of varying length CAG repeat models for HD (37) or engineering HD reporter cell lines for drug screening needs.

Increased availability of varying CAG repeat length models for HD and multiple isogenic cell lines will be valuable for understanding HD disease mechanisms and will improve the chances of discovering effective treatments for HD (37) (Figure 3). It is well documented that the CAG repeat length impacts the time of onset, progression and severity of HD, and thus it is possible that the repeat length may also affect drug response. Additionally, drug response may be different in individuals with modifier mutations (130). Many modifier genes have been identified for HD, meaning their expression or structure can affect when and how the disease manifests (131, 132). Models that reflect subsets of modifier genes may prove useful in drug screening as this subpopulation could respond differently to certain drugs. New genome editing tools such as CRISPR have made knock-in/knock-out of modifier genes in HD iPSC models more efficient and easier than before. CRISPR has also provided a powerful tool to screen genomes for HD genetic modifiers in an unbiased way. In fact CRISPR has been utilized in a genome wide knock-out screen to identify genes essential for viability to human PSCs (133). The same method can be readily adapted to screen for disease modifier genes in human iPSCs.

Another application for genome editing tools in HD drug screening is generating reporter cell lines. The ease of genetic engineering would allow for the tagging of several potential therapeutic target proteins with fluorophores or other selection markers in HD. The flexibility of gRNA directed genomic modification with CRISPR makes it simple to insert selection markers either in endogenous loci or a safe harbour locus such as the AAVS1 site. Tagging certain proteins with a fluorophore, or even multiple proteins with different fluorophores in the same cell could allow for multiple fluorophore monitoring during screening of compounds (134). These are only a few of the potential benefits of the recent advances in the genome engineering field, which can significantly impact screening strategies and our basic understanding of HD.

## Conclusion

The contributions that stem cells and specifically iPSCs have made to the HD field is substantial and has impacted our current understanding of HD and the therapeutic directions being pursued. As a tool available to researchers for under a decade, human iPSCs have been utilized in drug screens in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) (135) and familial dysautonomia (FD) (136). Given the advantages of iPSCs, including more relevant human phenotypic endpoints and the variety of differentiated cell types, iPSCs and their derivatives are a promising new direction for HD drug screening. The potential of iPSCs is just beginning to be thoroughly explored, both in phenotypic screens and in very early genetic manipulations to create even more representative disease models. iPSCs and their cell derivatives will undoubtedly help form the foundation on which new therapeutic investigations will occur and new insights to HD will be understood.

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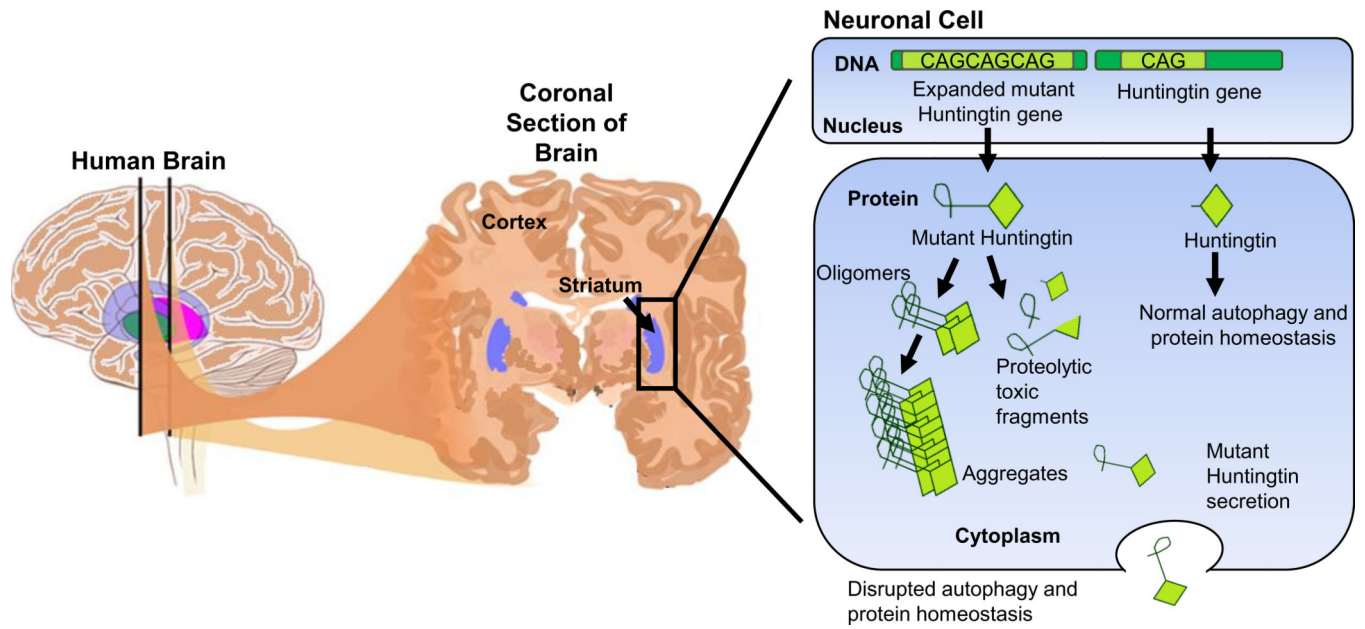
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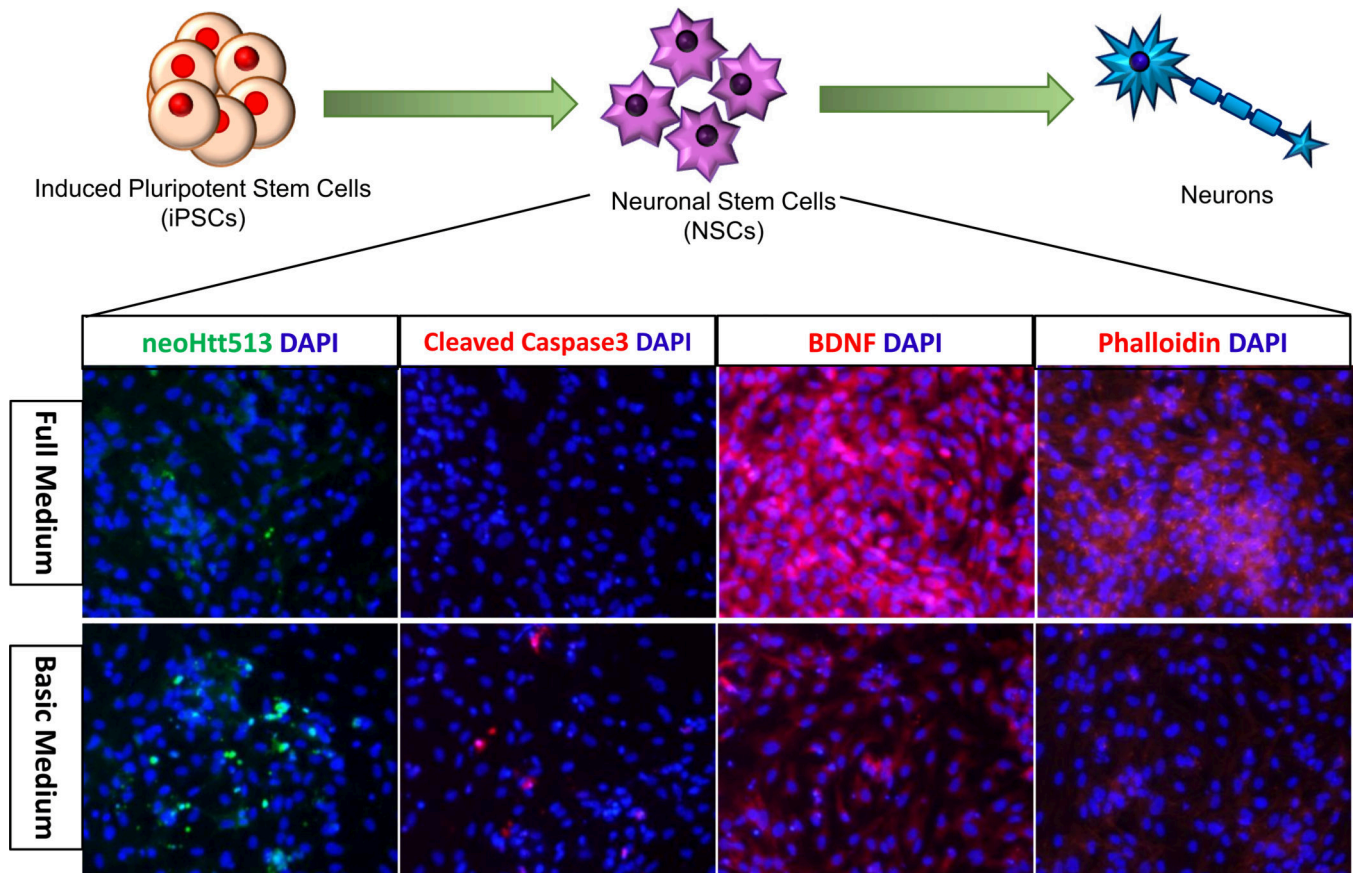
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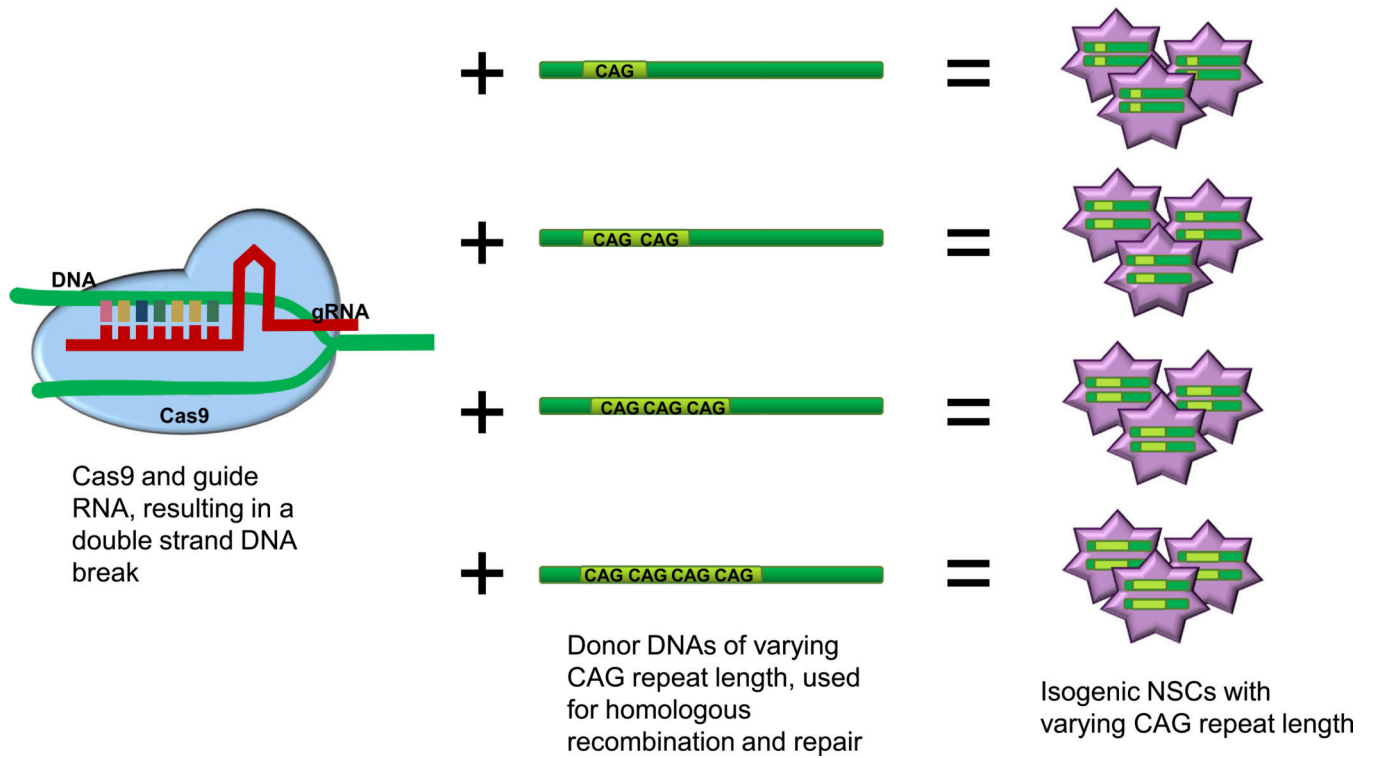


**Figure 1. A schematic representation of the brain highlighting the striatum and the pathology of HD in a neuronal cell**

In affected neuronal cells, the mutant *HTT* gene with an expanded CAG trinucleotide tract is transcribed and translated to mutant HTT (mHTT) protein. mHTT protein does not fold correctly and forms aggregates and fragments which cannot be cleared properly from the cell and as a result is thought to cause certain disease phenotypes.



**Figure 2. Diagram of iPSC differentiation and potential endpoints at the NSC stage**  
 Human HD iPSCs were differentiated into NSCs, then neurons and glia. At the NSC stage, we observed disease-associated phenotypes. When stressed (change from full medium to basic medium without growth factors) NSCs derived from HD iPSCs demonstrated upregulation of the N-terminal 513aa Htt fragment (recognized by neoHtt513 antibody) and cleaved caspase-3 and downregulation of BDNF and phalloidin labelled F-actin.



**Figure 3. A representation of a potential genome editing method to create multiple isogenic HD iPSC lines**

CRISPR technology can target and modify specific genes by inducing DNA double strand breaks which triggers the cells DNA-repair machinery to correct the break via homologous recombination. With CRISPR and *HTT* specific gRNAs, introduction of a donor template DNA with varying lengths of CAG repeats allows generation of isogenic iPSC lines, which differ only in CAG repeat length in exon 1 the *HTT* gene.

**Table 1**

Human stem cell models of Huntington's disease and their phenotypes reported in the literature.

Pluripotent Cell Type	CAG Repeat	Genetic Modification	Differentiation Stage and Phenotypes	Reference
ESCs from HD embryos	37 and 51	no	Differentiated neurosphere cells showed expanded CAG repeat length instability	11
ESCs from HD embryos	40, 45, 46 and 48	no	No phenotype described	12
ESCs from HD embryos	37 and 51	no	CAG <sub>51</sub> forebrain neurons had elevated glutamate-evoked responses such as intracellular calcium levels	13
H9 ESCs	23, 73 and 145	<i>HTT</i> exon1 under CAG promoter delivered by ePiggyBac system	CAG <sub>73</sub> and CAG <sub>145</sub> neurons showed EM48 positive aggregates and increased cell death upon growth factor deprivation compared to CAG <sub>23</sub> neurons	14
ESCs from HD embryos	15/21, 18/21, 22/22, 12/40, 17/46, 17/48, 23/45, 19/41, 19/46, 21/42	no	Proteomic analysis had mitochondria dysfunction in HD affected ESCs shown by reduction components of electron transport chain complex I, III and IV. Neurons from HD affected ESCs showed vulnerability to nonspecific kinase inhibitor STS. There were transcription dysregulation such as histone H1 family members and actin cytoskeletal signaling proteins in HD neurons	10
iPSCs from HD patient	72	no	No phenotype described	31
iPSCs from HD patient	72	no	Increased caspase activity upon growth factor deprivation in NSCs	30
iPSCs from HD patient	50 and 109	no	Increased cytoplasmic vacuolation seen in differentiated astrocytes	29
iPSCs from HD patient	15/17, 15/18, 17/45, 39/43, 42/44	no	No difference in growth rate, differentiation, caspase activation in iPSCs. Higher lysosomal activity in HD-iPSCs and derived neurons shown by LysoTracker dye. Increased autophagosome formation in HD-iPSCs	28
iPSCs from HD patient	72	no	HD iPSCs form EM48+ aggregates upon treatment of proteasome inhibitor MG132. NPCs from HD iPSCs and developed EM48+ HD pathology characteristic at later stage of transplantation into rat quinolinic acid-induced HD model	27
iPSCs from HD patient	72	no	Dysregulated proteins involved in oxidative stress response, apoptosis in HD-iPSCs. Decreased neuronal differentiation and neurite outgrowth and increased apoptosis (TUNEL) in HD-iPSCs and neurons. Reduced cytoskeleton associated proteins in HD-neurons	26
iPSCs from HD patient	72	Expanded CAG was genetically corrected byHR	At NSC stage, HD cells showed increased apoptosis (TUNEL), caspase activity, decreased BDNF expression, maximal oxygen consumption rate, altered TGF- $\beta$ and cadherin signaling. Corrected NSCs reversed these DhenotvDes	66
iPSCs from HD patient	17/21, 21/28, 18/33, 18/60, 19/109, 18/180	no	Microarray analysis showed distinct gene expression pattern in differentiated NSCs. HD NSCs showed decrease in energy metabolism (lower intracellular ATP level and ATP/ADP ratio) and cell adhesion (smaller clump size). HD neurons had higher risk of cell death after prolonged culture (stained cleaved caspase-3), BDNF withdrawal (condensed nuclei and caspase activation) or stressed by H <sub>2</sub> O <sub>2</sub> or 3-MA (condensed nuclei). Physiological or pathological glutamate treatment would increase percentage of HD neurons with calcium dyshomeostasis	24

Pluripotent Cell Type	CAG Repeat	Genetic Modification	Differentiation Stage and Phenotypes	Reference
iPSCs from HD patient	72	no	mHTT formed EM48 positive aggregates in differentiated neurons which could be reduced by microRNA miR-196a	23
iPSCs from HD patient (non-integrating method)	18/21, 18/28,18/33, 18/60, 19/109, 18/180	no	More Nestin+ neural cells after differentiation of HD iPSCs. Withdrawal of BDNF caused more apoptosis (TUNEL) in HD neurons and reduced numbers of Nestin positive cells.	36

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**Table 2**  
Differentiation methods to produce medium spiny neurons, cortical neurons, astrocytes, and oligodendrocytes from ESC and iPSCs.

Starting Cell Type	Differentiated Cell Type	Differentiation Method	Markers	Efficiency	Reference
ESC	MSN	d0-12: co-culture hESC with MS cells in DMEM-F12/KSR; d12-21: culture in DMEM-F12/N2; d21: isolate neural rosettes and plate on poly-ornithine/laminin surface; d21-46: culture in DMEM-F12/N2 with BDNF, SHH, DKK1; d46-62: differentiate into neuron in DMEM-F12/N2 with BDNF, db-cAMP, VPA	DARPP32 GABA calretinin calbindin	53% DARPP32+ in MAP2+ cells	78
ESC	MSN	d0-6: EB in suspension; d6: attach on plastic surface; d6-10: culture in DMEM-F12/N2; d10-30: induce ventral telencephalic progenitor in DMEM-F12/N2 with SHH, DKK1; d30-42: differentiate into neuron in Neurobasal/N2/B27/BDNF/GDNF/IGF1	DARPP32 GAD65/67 GABA	not described	79
ESC	MSN	d0-12: primitive neuroepithelia induced in DMEM-F12/N2; d12-26: SHH or purmorphamine added in DMEM-F12/N2; d26-33: Neurobasal/VPA; d33-: Neurobasal/BDNF/GDNF/IGF/cAMP	DARPP32 GAD65/67 GABA Meis2	89.7% DARPP32+ in GABA neurons	85
ESC and iPSC	MSN	d0-3: PSCs expanded in MEF conditioned medium with Y27632; d3-15: KSR medium with dorsomorphin and SB431542; d8-29: SHH and DKK1 added; d29-d80: terminal differentiation in N2/B27 medium with BDNF	DARPP32 GAD65/67 GABA calbindin CTIP2	20% DARPP32+	80
Immortalized NPC line	MSN	Differentiation in DMEM-F12/N2/B27/Insulin; d0-d11: BDNF (30ng/ml)/SHH/DKK1; d0-d1: additional Y-27632 added; d11-60: BDNF (50ng/ml)	DARPP32 GABA Calbindin	21% DARPP32+, 96% GABA+, 84% Calbindin+ in MAP2+ neurons	82
ESC and iPSC	MSN	d0-d5: 80% confluent PSCs induced in DMEM-F12/Neurobasal (2:1) supplemented with N2/B27/SB431542/LDN-193189; d5-d9: SB431542 removed; d9-28: LDN-193189 replaced by activin A; d28-36: BDNF/GDNF added	DARPP32 CTIP2 Calbindin	20-50% DARPP32+	83
ESC and iPSC	Cortical Neuron	Differentiation in 3N medium (DMEM-F12/Neurobasal, 1:1, N2/B27/insulin/vitamin A). d0-d11: 95% confluent monolayer PSCs on Matrigel differentiated in 3N with retinoids, SB431542 and dorsomorphin or noggin; d11-13: replated to poly-ornithine/laminin in 3N with bFGF; d13-100: maintained in 3N	Pax6 O1×1/2 Enx1 Foxg1 Tbr2	>95% Pax6+, O1×1/2+by d15	98

Starting Cell Type	Differentiated Cell Type	Differentiation Method	Markers	Efficiency	Reference
ESC and iPSC	Cortical Neuron	d0-16: monolayer PSCs on Matrigel differentiated in DMEM-F12/N2/B27/B27/BSA/Glutamax/noggin; d16-24: same medium with noggin removed; d24: replat cells to poly-L-lysine/laminin in same medium with noggin removed and Y27632 added; every 5-7d, half medium changed with Neurobasal/B27/glutamine	Pax6 Otx1/2 Emx1/2 Foxg1 Tbr1/2 vGlut1/2	Mostly Pax6+, Otx1/2+, Emx1/2+, Foxg1, Tbr1/2, 60% vGlut1 + d44	99
NSC	Astrocyte	NSCs on poly-L-ornithine/laminin or Geltrex were induced for astrocyte differentiation with StemPro/activin A/Heregulin $\beta$ /IGF1	GFAP S100 $\beta$	62% GFAP+	108
ESC and iPSC	Astrocyte	d0-6: EB in suspension; d6: EB plated on Matrigel in Neurobasal/N2; d6-9: bFGF/EGF added; d9-12: bFGF/EGF/CNTF added; d12-15: bFGF/CNTF added; d15-: CNTF alone;	GFAP S100 $\beta$ GLT-1	78% GFAP+ at 5weeks	109
iPSC	Astrocyte	iPSCs were induced with dorsomorphin/SB431542/NAC for 5-7d before enzymatic dissociation to form neurospheres; retinoic acid added to neurospheres for 7-12d; neurospheres mechanically chopped and replated in NSCR (AdDMEM-F12/N2/B27/NEAA/Glutamax) with EGF/LIF for 2-4w; propagated in NSCR with EGF/bFGF; dispase dissociation and CNTF induced differentiation	GFAP sioop Vimentin NF1A	>90% GFAP+, S100 $\beta$ +	110
ESC and iPSC	Oligodendrocyte progenitor cells (OPCs), Oligodendrocytes (OL)	d0-d8: neural induction using dual SMAD inhibition and 100 nM all-trans RA in adherent conditions; d8: added SAG to medium; d12: lift cells for sphere formation in medium with RA and SAG; d30: plate spheres on poly-L-ornithine/laminin dishes in PDGF medium for OPC formation; d75+: glial medium for oligodendrocyte maturation	OPC: NKX2.2 SOX 10 OLIG2 OL: O4 MBP	40-70% O4+	115
ESC and iPSC	Oligodendrocyte progenitor cells (OPCs), Oligodendrocytes (OL)	d0-d5: EB formation in ESC medium; d5-d19: neuroepithelial generation in NIM, FGF2; d19-d29: pre-OPC generation in neural induction medium (NIM), RA, B27, purmorphamine; d29-d40: pre-OPC expansion in NIM; bFGF, B27, purmorphamine; d40-d160: OPC generation in glial induction medium (GIM), T3, NT-3, IGF, PDGF-AA, purmorphamine; d160-d180: terminal differentiation in reduced GIM	OPC: OLIG2 NKX2.2 SOX 10 PDGFRa OL04 MBP	OPC: >45% ESC derived >70% iPSC derived	116