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Induced pluripotent stem cells to model gene-environment interactions in Huntington's disease

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Abstract
Huntington's disease (HD) is a neurodegenerative disorder with hyperkinetic symptoms due to loss of medium spiny neurons (MSNs) in the caudate and putamen of the striatum. HD is caused by a polyglutamine tract expansion with longer expansions leading to earlier age at onset (AO). However, a large AO variability still exists between patients with the same size expansion. Environmental modifiers have been shown to play a large role in AO variability. Few gene-environment interactions have been studied for HD partly due to the difficulty in performing such studies in animal models. Induced pluripotent stem cells (iPSCs) can be generated from a patient's dermal cells and can be subsequently differentiated into neuronal cells. Directed differentiation of human embryonic stem cells has already been shown to generate medium spiny neurons (MSNs), the cells selectively degenerated in HD, in culture. Having a supply of HD patient-specific medium spiny neurons will allow for gene-environment interaction screening in cells containing nearly an identical expression profile and genetic background to *in vivo* patient cells. Clinical applications can then be developed, which could potentially delay AO and decrease disease severity.

Keywords: *Huntington's disease, gene-environment interactions, induced-pluripotent stem cells, directed differentiation, medium spiny neurons*

Huntington's Disease

Huntington's disease (HD) is a dominantly inherited genetic disorder that affects approximately 6 persons per 100,000[1]. A "dance-like" hyper-kinetic symptom known as chorea, one chief characteristic in the diagnosis of HD, was first accurately described by George Huntington in 1872[2]. The motor and behavioral phenotypes of this disease are caused by neurodegeneration in many brain areas but most notably the loss of medium spiny neurons found in the caudate and putamen of the striatum[3]. More recent MRI studies have found varying degrees of degeneration in nearly all brain regions in early disease[4].

In 1993, the genetic cause of the disease was narrowed to the *IT-15* gene, which encodes for a protein now known as huntingtin[5]. HD patients have an expansion in the glutamine repeat region (polyQ) in the huntingtin gene (*HTT*). Huntingtin is expressed in all tissues and is necessary for embryonic and neural development[6-8]. Huntingtin has many diverse functions, and for this reason, the mutant form leads to many diverse cellular pathologies including calcium signaling abnormalities, mitochondrial dysfunction, neurotrophic factor reduction, excitotoxicity, transcriptional dysregulation, protein aggregate formation, and altered autophagy[3,9-13]. Currently, no treatments have been proven effective for HD patients, and the specific mechanism underlying the selective degeneration is undetermined. HD occurs in individuals with repeat lengths greater than 35 glutamine codons (CAG). Individuals with 36-39 repeats show incomplete penetrance and those with >70 repeats typically display HD symptoms in childhood[14]. However, one study reported a patient with a post-mortem diagnosis of HD had a repeat length of 29 on the longer allele[15]. This observation hints toward more complexity in pathogenesis than merely repeat length.

Modifiers of HD Age at Onset

Although repeat length of the polyglutamine tract has a strong inverse relationship to the age at onset (AO)[16], additional genetic and environmental factors play a critical role. Association studies using large HD patient cohorts have attributed 50-72% of the AO variability to the length of the polyQ repeat[8,16,17]. Environmental modifiers account for 60% of the remaining AO variability[17]. This gene-environment modifier contribution to AO is thought to increase with shorter repeat length causing a larger AO variability. In fact, patients with a repeat length of 40 CAG can have an AO between 32-69 years of age[17]. Even monozygotic twins with HD have shown differences in both rate of disease progression and symptomatic manifestation despite identical genetic background[18-21]. These data taken together provide evidence that environmental modifiers play a large role in HD disease progression.

Few environmental modifiers have been found for HD, though environmental enrichment in transgenic rodent models is one robust HD modifier. Rodents with mutant huntingtin provided with running wheels and novel objects showed decreased disease severity compared to rodents not exposed to an enriched environment[22]. Reduced production and trafficking of brain-derived neurotrophic factor (BDNF) is a major phenotype of HD, and enrichment has been linked to increases in BDNF expression[13,23,24]. Dietary restriction has also been shown to increase BDNF levels in wildtype mice, and essential fatty acid supplementation prevents some motor deficits in an HD mouse model[25,26]. Environmental factors can therefore have a therapeutic role in HD.

In addition to these protective effects of environment, many environmental compounds can synergistically increase the progression of neurodegeneration. In some neurodegenerative diseases, such as Parkinson's disease (PD), environmental toxins are thought to be a primary causative agent[27]. Metals are known to increase the progression of many neurodegenerative disorders. Small concentrations of divalent metals (Fe^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+}, etc.) can greatly increase oxidative stress, a major disease mechanism of many neurodegenerative diseases[28]. Some of these metals such as iron(II) and copper(II) are known to convert superoxide and hydrogen peroxide molecules into more reactive hydroxyl radicals that can damage proteins, lipids, and DNA[28]. Mitochondrial dysfunction can also be caused by oxidative stress and is another HD disease mechanism[12].

Many neurodegenerative diseases involve altered metal ion homeostasis. Copper (II) and iron (II) are found to have increased accumulation in brains of Alzheimer's, PD, and HD patients and mouse models[28-31]. Copper can also inhibit lactate dehydrogenase leading to reduced metabolic function and accumulation of lactate, which are two phenotypes of HD[30]. Wilson's disease, which involves the accumulation of copper, often leads to

HD-like striatal degeneration, and manganism, an overexposure to manganese, has Parkinsonian-like symptoms[30,32]. One study previously reported a decrease in manganese (II) levels in mouse striatal cells due to mutant huntingtin expression[33]. Reduced Mn^{2+} concentration was also shown to selectively occur in the striatum of an HD mouse model[33]. Many enzymes for metabolism (e.g. pyruvate decarboxylase, glutamine synthatase, and arginase) and antioxidation (e.g. SOD1 and SOD2) need metal cofactors. Therefore, both excess and insufficient concentrations of certain metal ions can also lead to reduced metabolic function and increase oxidative stress. The overlapping characteristics between metal toxicity and neurodegenerative disease as well as altered metal ion homeostasis in many neurodegenerative diseases provides a firm foundation for studying gene-environment interactions between mutant htt and metal ions.

Previous HD Models

Animal models have provided many important insights into HD disease mechanisms; however, a complementary human-based model would overcome several obstacles in HD research[34]. Model systems have included 3-nitroproprionic acid lesioned rodents transgenic mice, rats, fish, flies, and monkeys as well as immortalized murine striatal cell culture[35-37]. The divergent evolutionary time between rodents and humans has allowed for large genetic differences, and these interspecies genetic differences could alter disease mechanisms and gene-environment interactions. Furthermore, murine HD models with similar size polyQ repeats can have completely different phenotypes[3]. Interestingly, the endogenous murine huntingtin only has a polyQ expansion of 7 CAG repeats while the human gene averages around 20, and the functional significance behind this difference could also alter disease mechanisms and environmental disease modification based on species[3].

Using human cells and tissues would circumvent these genetic difference issues. Post-mortem tissues have provided an important supply of human HD culture material; however, since HD patients lose the majority of their striatal volume during disease progression, these tissues are even more limited. Peripheral tissues such as dermal fibroblasts can be propagated in culture for a limited time period, but differences in expression pattern between CNS and dermal cells reduce their usefulness for modeling neurodegeneration.

Induced Pluripotent Stem Cells

Until recently, post-mortem and peripheral tissues were the only human models available for neurodegenerative studies, but with the advent of induced pluripotent stem cell (iPSC) technology, this paradigm is changing. In 2007, Shinya Yamanaka's lab first demonstrated the direct reprogramming the epigenetic status of human somatic cells into iPSCs[39,40]. In this landmark study, four transciption factors (OCT4, KLF4, SOX2, and c-MYC) were introduced using retroviral integration into cultured human dermal fibroblasts. Within 30 days, iPSC colonies appeared in culture[41]. After establishing these colonies into stable cells lines, the endogenous pluripotency genes were found to be epigenetically active and the retroviral constructs were silenced. Further studies have validated that the expression pattern, telomerase activity, mitochondrial regulation, and pluripotency of hiPSCs is similar to that of human embryonic stem cells (hESCs)[40,42,43].

Like hESCs, iPSCs can be differentiated into any cell type in the body. Patient-specific iPSCs have also been generated (e.g. HD, ALS, PD, Down syndrome, spinal muscular atrophy) providing researchers with patient-specific cell lines for *in vitro* disease modeling (Figure 1)[44-47]. Recent studies have also provided increased reprogramming efficiencies by small molecule incubation and integration-free iPSC induction techniques using cell permeable proteins and non-integrating DNA vectors[48-50]. A recent study has also shown induction of iPSCs from culturing T cells found in a single milliliter of blood[51]. These technical advancements will soon provide a simple, integration-free patient-specific generation method for HD iPSC lines that can be differentiated into relevant cell types.

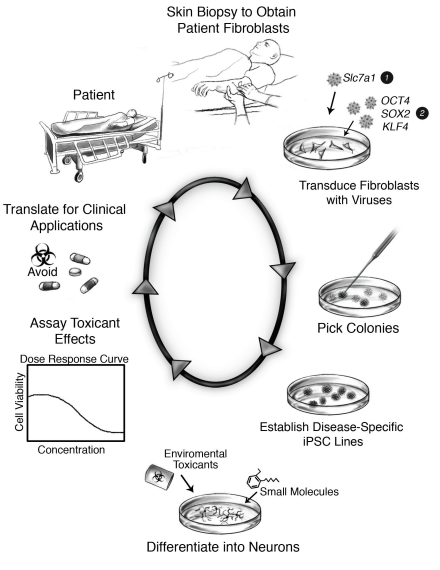

Fig 1. A schematic representation of iPSC neurodegenerative disease modeling

Neuronal Differentiation

With the correct incubation of signaling molecules, HD iPSC lines can then be differentiated into striatal neurons. Neurodevelopment requires many spatial-temporal signaling events that are complicated by the multiple roles of signaling molecules. Unfortunately, the majority of neurodevelopmental research has been based on murine embryo brain development, and the timing and function of human homologs cannot be assumed from these data. Using human pluripotent stem cells *in vitro* allows for elucidation of both normal and disordered human neurodevelopment. Several laboratories have already used human recombinant signaling proteins and small molecules to exogenously direct differentiation of iPSC and ESC *in vitro* cell cultures to enriched populations of motor neurons, midbrain dopaminergic neurons, and forebrain GABAergic and glutamatergic neurons[45,46,52-56]. Neurons differentiated from HD and control iPSC could be used for *in vitro* studies of disease mechanisms, gene-environment interactions, drug-screening, and regenerative medicine.

The first stage of human stem cell differentiation is toward one of the three germ layers: neuroectoderm, mesoderm, and endoderm. Neuroectoderm has been found to be the default tissue fate of epiblast culture, and cultured hESCs have been shown to be in an epiblast stage[57,58]. Signaling by the TGF-β super family, including bone morphogenic proteins (BMPs), inhibits the formation of neuroectoderm[59]. *In vivo*, proteins like noggin bind to BMPs to block their signaling allowing default neural tissue to form. Initial neural induction studies used either the formation of embryoid bodies or coculture with stromal cells. Unfortunately, embryoid bodies induce small numbers of neural cells, and stromal cells take several weeks to cause efficient neural induction followed by laborious mechanical picking of neural rosettes[60]. These difficulties were overcome when Chambers *et al.* induced ~80% PAX6-expressing cells, a neuroectodermal markers, in less than a week in monolayer hESC culture[53,61]. This was achieved using noggin and a TGFβ small

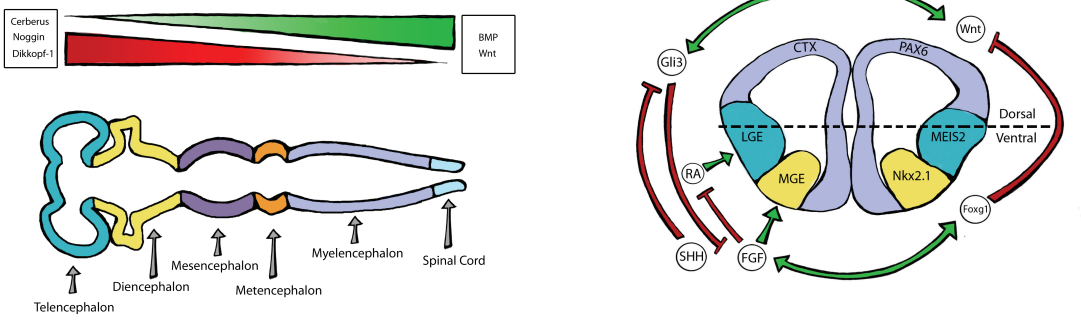

Fig 2. ***(A)*** **Several important signaling molecules in anterior-posterior axis formation. Wnt and BMP promote posterior formation, and Cerberus, noggin, and dikkopf-1 inhibit the function of these posteriorizing molcules leading to anterior formation.** ***(B)*** **Major telencephalic signaling molecules. The three major regions are labeled on the left cortex (CTX), lateral ganglionic eminence (LGE), and medial ganglionic eminence (MGE). To the right are specific markers of these regions. Sonic hedgehog (SHH), fibroblast growth factors (FGF), retinoic acid (RA), Wnt, Foxg1, and Gli3 have both active and repressive roles in setting up the dorso-ventral and medial-lateral axes.**

molecule inhibitor[53]. Noggin can also be replaced with dorsomorphin, a small molecule that blocks the downstream Alk-Smad pathway of BMP signaling[53,55]. Unfortunately, iPSC lines have been found to be highly variable in their neural induction efficiencies[62]. However, this variability may be attenuated with the production of iPSC lines screened by more strict validation methods. In any case, a large percentage of neural progenitors can be produced from iPSC lines.

Medium spiny neurons (MSNs), which are particularly vulnerable in HD, develop in the lateral ganglionic eminence of the ventral telencephalon. Gradients of signaling molecules give developing progenitors positional identity. LGE progenitors need to be patterned by signaling molecules that lead to an anterior, ventral, and finally lateral position during the neural patterning process. The anterior-posterior axis is set up by anterior expression of Wnt inhibitors, Cerberus (also a BMP inhibitor) and dikkopf-1 (DKK1), and BMP inhibitors inducing anterior fate (Figure 2A)[63]. Sonic hedgehog (SHH), is secreted from the floorplate of the neural tube, setting up the dorso-ventral axis by inhibiting the dorsally expressed Gli3 repression of fibroblast growth factor (FGF) signaling (Figure 2B)[64]. FGF is the major ventralizing signal of the CNS. FGF receptor knockouts lose most of the ventral region of the telenchepalon64. However, incubation of chick lateral ganglionic eminence (LGE) explants, which normally develops into striatum, with FGF8 causes expression of medial ganglionic eminence (MGE) markers[65]. Several laboratories have incubated hESCs with exogenous DKK-1 and SHH to produce cells expressing ventral telencephalic markers[52,54].

To specify LGE identity over MGE requires the signaling molecule retinoic acid (RA)[65]. RA expression in chick embryos causes expansion of the LGE at the expense of both MGE and dorsal regions[66]. Additional studies have found RA receptor antagonists block the formation of LGE[65]. Few studies have used RA for the specification of LGE-like progenitors despite its apparent necessity. The ventral telencephalon has been shown to endogenously produce RA, which could lead to LGE patterning *in vitro*[67,68]. However, for a more robust directed differentiation protocol, RA will almost certainly be necessary.

After patterning, neural progenitors must be induced to terminally differentiate into mature neurons. BDNF is a key molecule in striatal differentiation. It induces the expression of DARPP-32, and its loss decreases striatal projection neuron markers and MSN dendritic arborization complexity[69-71]. Additionally, exogenous expression of BDNF and noggin in the adult striatal ventricular zone has been found to reactivate nascent progenitors to become new MSN's[72]. The only report of DARPP-32 positive MSN generation from human ES cells used a combination of BDNF with valproic acid and dibutryl-cyclic-AMP, two compounds known to increase striatal neurogenesis[52,69,73]. This paper from the Perrier lab showed that MSN-like differentiation is possible; however, the protocol was highly inefficient and took nearly two months to complete. More refined protocols will be needed to generate sufficient quantities of MSNs and provide insights into HD striatal developmental since abnormalities have been reported in HD mice[74].

Modeling Disease with Patient-Specific iPSC Derived Neurons

Neurons generated from HD iPSCs must demonstrate recapitulation of an HD phenotype. Several disease-specific iPSC lines have been generated, but only a few pioneering studies have investigated phenotypes in iPSC models of neurological disease. Using iPSCs from a spinal musclar atrophy (SMA) patient and control, Ebert *et al.* reported deficits in SMN protein in an the iPS cell state[46]. This recapitulated deficits seen in the patient's dermal fibroblasts. This study also reported reduced motor neuron differentiation in the SMA mutant iPSC line. This difference is potentially due to the variability of potency between the two lines since only a single clonal line of each genotype was used[62]. Alternatively, viral construct integration could have inactivated genes altering the properties of the cell line. These issues can be mitigated in the short term by using multiple clonal lines, which will have different integration sites, and in the long term by utilizing integration-free iPS induction methods.

The Studer lab used more robust methodology in modeling familial dysautonomia (FD). Two clonal iPSC lines each from an affected and a control individual were differentiated into several tissue types, and the lowest levels of the affected transcript were found in neural crest progenitors. This correlates with the phenotype of peripheral autonomic and sensory degeneration seen in FD patients[47]. Similar genotype-phenotype interactions have not yet been demonstrated in a CNS neurodegenerative disease iPSC model.

Establishing a genotype-phenotype correlation will give validity to an HD iPSC model. Testing for 3-NP selective vulnerability in the differentiated HD iPSC lines would recapitulate phenotypes seen in current HD models[33,35]. Similar toxicant treatments have been performed on hES-dervied dopaminergic neurons; however, hES lines are not amenable to producing disease-specific lines[75]. Comparisons between disease-specific and controls cells are now possible with iPSC technology.

Conclusion

Induced pluripotent stem cells will provide an important model for the study of Huntington's disease. These cells can be generated from patients with well-documented genotypes and symptoms in much less time than generating a transgenic mouse. They can be propagated indefinitely and differentiated into medium spiny neurons. *In vitro* differentiation allows for better understanding of human neurodevelopment and how disease and toxicants affect developmental time points. Maintenance of the human genetic background allows for an adequate model to study gene-environment interactions, and these studies could lead to treatments that delay disease onset and progression for HD patients.

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