Title
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Publication Date
2013-09-01

DOI
10.1016/j.mcn.2013.02.005

Peer reviewed

Molecular and Cellular Neuroscience 56 (2013) 50–64

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Modeling Huntington's disease with induced pluripotent stem cells

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ARTICLE INFO

Article history:
Received 19 September 2012
Revised 14 February 2013
Accepted 18 February 2013
Available online 28 February 2013

Keywords:
Huntington's disease
Induced pluripotent stem cells
IPSC
MSN
Stem cell models
Neurodegenerative disease

ABSTRACT

Huntington's disease (HD) causes severe motor dysfunction, behavioral abnormalities, cognitive impairment and death. Investigations into its molecular pathology have primarily relied on murine tissues; however, the recent discovery of induced pluripotent stem cells (iPSCs) has opened new possibilities to model neurodegenerative disease using cells derived directly from patients, and therefore may provide a human-cell-based platform for unique insights into the pathogenesis of HD. Here, we will examine the practical implementation of iPSCs to study HD, such as approaches to differentiate embryonic stem cells (ESCs) or iPSCs into medium spiny neurons, the cell type most susceptible in HD. We will explore the HD-related phenotypes identified in iPSCs and ESCs and review how brain development and neurogenesis may actually be altered early, before the onset of HD symptoms, which could inform the search for drugs that delay disease onset. Finally, we will speculate on the exciting possibility that ESCs or iPSCs might be used as therapeutics to restore or replace dying neurons in HD brains.

Contents

Introduction

Huntington's disease (HD) is a devastating and incurable neurological disorder caused by a CAG expansion in the gene encoding the protein huntingtin (Htt). HD is inherited in an autosomal-dominant

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http://dx.doi.org/10.1016/j.mcn.2013.02.005

fashion and leads to sleep disturbances (Arnulf et al., 2008), motor dysfunction, cognitive impairment and psychiatric abnormalities (Vonsattel and DiFiglia, 1998) and ultimately premature death (DiFiglia et al., 1995). The age of onset (typically 30–50 years) and severity correlate with the number of CAG-encoded glutamine repeats in mutant Htt (mHtt) (Andrew et al., 1993; Snell et al., 1993). Normal, unaffected individuals have 35 or fewer CAG repeats, and affected individuals have more than 36 (Langbehn et al., 2004). Individuals with longer expansions have an earlier onset of disease (Langbehn et al., 2004). Cellular pathologies associated with HD result in the death of striatal medium spiny neurons (MSNs) in the basal ganglia (Augood et al., 1997), the executive center for orchestrating motor memory, learning and function in the brain (Packard and Knowlton, 2002). At later stages, mHtt also affects other cell types, such as cortical neurons and pyramidal projection neurons (Han et al., 2010). Although the mutation that causes HD is known, no therapies that delay or slow the disease progression currently exist (Mestre et al., 2009).

Htt participates in numerous biological processes, such as regulating apoptosis (Hickey and Chesselet, 2003), regulating transcription and enhancing microtubule-based transport or scaffolding of cytoskeletal molecules at synapses (Cattaneo et al., 2005). Hallmarks of mHtt-affected cells include abnormal gene expression (Cha, 2007; Thomas, 2006), aberrant protein folding, degradation and clearance (Finkbeiner, 2011), and the formation of large protein aggregates called inclusion bodies (DiFiglia et al., 1997).

It has been hypothesized that the polyglutamine expansion in mHtt alters its conformation, leading to aggregation and abnormal protein-protein interactions (Miller et al., 2010) and may also alter Htt's normal function (Kratter and Finkbeiner, 2010). In turn, misfolded mHtt may disrupt cellular homeostasis by overwhelming the cell's proteostasis machinery by interfering with protein-clearance pathways (Finkbeiner, 2011). The accumulation of misfolded forms of mHtt may also stress protein homeostasis pathways so that unrelated proteins cannot fold or function properly, thus compromising cell health. As a consequence, the cell sequesters mHtt through the formation of IBs that help mitigate the stress on the proteostasis network by aggregating mHtt into forms that are more inert than misfolded monomers (Arrasate et al., 2004). In addition, mHtt is known to interact promiscuously with other molecules, and thereby may impede their expression or change their function (Li and Li, 2004). For example, mHtt interferes with CREB activation of its gene targets (Sugars et al., 2004). Thus, mHtt disrupts multiple fundamental cellular functions, making it challenging to conceive of simple therapeutic strategies.

As with other neurodegenerative diseases, we have learned a great deal about HD from transgenic animals and rodent primary neuronal cultures. However, as diseased human cells are not easily obtainable, much of the work to understand the molecular insults of mHtt has been done in murine tissues. Despite successful modeling of several aspects of HD *in vitro* and *in vivo*, therapies that appeared beneficial in these models have been disappointing in human clinical trials.

Until recently, studies in human tissue have been restricted to immortalized or transformed cells, fixed post-mortem tissues and blood samples. While valuable in some respects, immortalized cells have fundamentally different biological properties than brain cells and fixed tissues are subject to the degradation of DNA and RNA. Blood samples have been valuable for detecting gene-expression changes that can be used as biomarkers of disease progression (Borovecki et al., 2005; Long et al., 2012; Lovrecic et al., 2009) but these changes only recapitulate some molecular characteristics of HD. Further, the availability of such tissues is limited. The dearth of robust human tissue-based systems derived directly from HD patients has slowed the validation of promising therapeutics.

The discovery of induced pluripotent stem cell (iPSC) technology provides exciting new opportunities to develop *in vitro* HD models that more accurately reflect the human disease. Cells obtained directly from HD patients can be propagated indefinitely and differentiated into the susceptible neuronal subtypes. Although HD is a late-onset disease, recent evidence suggests that these comparatively young HD iPSCs accurately recapitulate several aspects of the human disease (An et al., 2012; The HD iPSC, 2012). Here, we will examine the practical implementation of iPSCs to study HD, including methods to differentiate embryonic stem cells (ESCs) or iPSCs into MSNs, the cell type most susceptible to the pathogenic effects of mHtt. We will explore the HD-related phenotypes identified in iPSCs and ESCs, and review how brain development and neurogenesis may be altered early, before the onset of HD symptoms. Finally, we will explore the exciting possibility that ESCs or iPSCs might be used as therapeutics to restore or replace dying neurons in HD brains. The development of human HD models using iPSCs could lead to critical advances in our understanding of HD progression at its earliest stages and, in turn, could lead to the development of relevant small-molecule or cell-based therapeutics that delay disease onset and progression.

Modeling human disease in rodents

While transgenic animals and rodent primary neuronal cultures have been valuable systems for studying HD, therapies developed using these models have been disappointing in human clinical trials. Clearly, rodents are not humans, having diverged ~96 million years ago (Nei et al., 2001), and the cumulative effect of small and infrequent base-pair changes over time might affect how a putative small-molecule therapeutic interacts with that cell's proteome. Thus, an effective drug in rodent cells may not work as well or as specifically in humans. Furthermore, many single amino-acid polymorphisms alter molecular stability and global protein–protein interactions (Ferrer-Costa et al., 2002). For example, two single-nucleotide polymorphisms in *APOE4* increase the risk of Alzheimer's disease and significantly alter APOE structure and protein function (Mahley et al., 2006). Further, these polymorphisms yield different functional consequences for protein toxicity in the human versus the rodent (Vance and Hayashi, 2010).

In addition to functional differences between human and rodent proteins, the complexity of the human brain far exceeds that of the rodent at the cellular and systems levels. Thus, there are many examples in which murine models do not recapitulate the physiology or anatomy of the human brain (Dolmetsch and Geschwind, 2011). For example, anatomically, the human brain is simply bigger and far more complex in shape than that of a mouse. There are significant differences in the development and patterning of the cerebral cortex and subventricular zones (Clowry et al., 2010; Dolmetsch and Geschwind, 2011) (Table 1). The human brain contains more glial subtypes and a higher ratio of glia to neurons than rodents (Oberheim et al., 2006), while some human neuronal subtypes appear to be completely lacking in rodents altogether (Table 1).

In terms of gene-expression profiles in specific cell types, humans and mice can have global differences in the expression of genes involved in pluripotency, cell-cycle regulation, and apoptosis (Ginis et al., 2004) as well as in innate and adaptive immunity (Mestas and Hughes, 2004) and response to inflammatory stress (Zermeno et al., 2009). While some reports show a high conservation in expression of orthologous genes (Zheng-Bradley et al., 2010), there are many examples of divergence of gene function, alternate splicing events and differences in copy number variants between mice and humans (Gharib and Robinson-Rechavi, 2011) (Table 1). In addition, binding sites for highly conserved transcription factors can vary significantly between rodents and humans (Odom et al., 2007). Thus, it is not surprising that so many drugs fail once they reach clinical trials given the heavy reliance on model systems that are so different from human tissues.

Does the HD mutation affect brain development?

Historically, HD has been thought of as a late-onset disorder, with the initiation of the disease marked by the first appearance of

Table 1
Fundamental differences between rodents and humans.

Event	Examples	Reference
Patterning and development of the subventricular zone (SVZ)	The SVZ in humans is larger, develops faster and has structures not observed in rodents.	Clowry et al. (2010)
Patterning and development of the cortex	Cortical structures in the human brain, such as the subplate, develop more connections and become more elaborate compared to other mammals.	Clowry et al. (2010) and Dolmetsch and Geschwind (2011)
Neuronal types	Von Economo neurons have only been found in humans, whales and primates but are absent in mice and rats.	Allman et al. (2011) and Nimchinsky et al. (1999)
Diversity of cell types	Astrocytes in the human brain are much more abundant, larger and more complex than in mice.	Oberheim et al. (2006)
Gene expression	Regulation of gene-expression regulation varies widely between mice and humans. There is also a divergence of gene function, alternate splicing events and differences in copy number variants between rodents and humans.	Gharib and Robinson-Rechavi (2011), Ginis et al. (2004) and Odom et al. (2007)
Neurogenesis	In the human brain, inhibitory interneurons are generated in cortical progenitor zones; in mice they are formed outside of the cortex and migrate in.	Clowry et al. (2010)
Immunity	There are fundamental differences in the innate and adaptive immune systems between mice and humans, including differences in the concentration and balance of lymphocytes, neutrophils, B and T cells, cytokines and defensins.	Mestas and Hughes (2004)
Inflammatory response	Gene expression changes in response to stresses, such as burns, injuries, endotoxins and infection, vary drastically between murine models and humans.	Seok et al. (2013)

symptoms. However, detectable physiological changes likely occur much earlier than previously thought. For example, behavioral disturbances such as depression are often evident and can predate the onset of motor dysfunction (Duff et al., 2007; Julien et al., 2007), suggesting that there are molecular changes that take place in HD brains long before clinical onset of disease symptoms. The longitudinal study PREDICT HD (Nopoulos et al., 2011) investigated intracranial volume (ICV) in HD carriers, which is a measure of early childhood brain development (Giedd, 2004; Rushton and Ankney, 1995). Male HD carriers who were not yet symptomatic had smaller ICVs than non-expanded controls (Nopoulos et al., 2011). Further, children with 39 or more CAG repeats had smaller head circumference, weight and body mass index than non-affected children (Lee et al., 2012). Thus, mHtt appears to affect brain development, but the mechanisms by which this occurs are unclear.

In mice, null deletion of Htt results in gastrulation defects and embryonic lethality (Duyao et al., 1995; Zeitlin et al., 1995). shRNA-mediated knockdown of Htt expression in the central nervous system (CNS) of late-stage embryos (i.e., embryonic (E) day 12.5) leads to a near-complete loss of neuronal migration from the ventricular zone (VZ) to the cortical plate (CP), while reducing cellular proliferation and increasing cell death in the developing cortex (Tong et al., 2011). To identify Htt's specific role in maturing tissues, $Hdh^{-/-}$ (*Htt* knockout) ESCs were injected into the blastocysts of normal mice. Although many cells survived until adulthood, they were mostly found in the cerebellum and hindbrain and rarely in brain regions most affected in HD, such as the cortex and basal ganglia (Reiner et al., 2001), suggesting that Htt is important in the maturation and development of these cell types. Interestingly, Htt is also a key player in regulating mitosis and neuronal fate (Godin et al., 2010). During mitosis, Htt localizes to the spindle poles during mitosis and aids in the proper orientation of cell division and localization of microtubule-based motors within cells. Loss of Htt expression results in the loss of cortical progenitors in the developing neocortex of the mouse brain (Godin et al., 2010), which directly ties Htt to neurogenesis (see below). Further, in ES Hdh knock-out cells subjected to a neuronal differentiation paradigm, loss of Htt expression results in cultures that contain fewer mature neurons but, interestingly, more glial-like cells, than wildtype ES cultures (Conforti et al., 2013).

How might mHtt alter brain development? Misfolded mHtt could induce developmental abnormalities in the striatum, predisposing those neurons to later degeneration. In one study of *Hdh-Q111* mice, which contain 111 polyglutamine repeats, distinct differences during embryonic maturation were observed in the expression and localization of several markers in the striatum, including the phosphoprotein DARPP-32, mGluR1 and the neuronal markers NeuN, β-tubulin and Islet-1 (Molero et al., 2009). Furthermore, striatal progenitors from *Hdh-Q111* mice were less likely to mature into specific cell types and more likely to maintain their proliferative potential at later stages of development (Molero et al., 2009). A selective delay in neuronal maturation limited to the striatum could have profound implications for the establishment of brain circuitry and function, which could affect brain processes from a very early stage. Recently, *in vivo* diffusion kurtosis imaging was used to see how a truncated mHTT fragment (51 CAG repeats) affected development in rat pups over time. This methodology can elucidate the structural components of brain tissue in living samples by tracking the distribution of water diffusion (Basser and Pierpaoli, 1996). The study found significant differences in gray and white matter distribution, suggesting that mHTT causes defects in myelination as a result of developmental abnormalities (Blockx et al., 2012). Thus, mHtt expression might compromise oligodendrocyte function during development and alter neuronal maturation in the developing brain, each of which could profoundly affect brain architecture and function before the point in time classically associated with disease onset.

Normal striatal development

Much of what we have learned about the process of differentiation and maturation of the brain comes from studying chick or murine tissues during neural development. This begins with induction of the neural plate during gastrulation that, in turn, gives rise to the neural tube. The neural tube is lined with instructive signals that delineate the anterior and posterior axes of the animal and instruct the subdivision of the three main structures of the brain, namely the 1) forebrain (prosencephalon), which consists of the telencephalon and the diencephalon, 2) midbrain (mesencephalon) and 3) hindbrain (rhombencephalon) (Rubenstein et al., 1998).

The striatum develops from within the telencephalon. The telencephalon is first made up of two distinct domains, the pallium (dorsal telencephalon) and subpallium (ventral telencephalon), which give rise to cortical structures and the basal ganglia respectively (Jain et al., 2001). The subpallium comprises the medial ganglionic eminence (MGE), which is established first, followed by subdivision into the lateral ganglionic eminence (LGE), and the caudal ganglionic eminence (CGE) (Sousa and Fishell, 2010). Neural cells born in the subventricular zone (SVZ) and ventricular zone (VZ) of the ganglionic eminences subsequently migrate into the developing striatum, cortex and other forebrain regions, where they integrate into neural circuits (Marin and Rubenstein, 2001). MSNs are derived from the LGE (Olsson et al., 1998). In mice, striatal neurogenesis begins around E12 and peaks around E14–E15 (Fentress et al., 1981). Once fully developed, the adult mouse striatum will contain almost 1.4 millon neurons (Fentress et al., 1981) of which ~95% are MSNs (Gerfen, 1992a).

What are the factors that govern MSN development in vitro?

An array of morphogens and transcription factors act within the telencephalon to drive LGE patterning and MSN differentiation. For a more detailed summary on normal striatal development, see Evans et al. (2012). To direct MSN differentiation from iPSCs and ESCs, some of the key factors are reviewed below (An et al., 2012; Aubry et al., 2008; The HD iPSC, 2012).

Brain-derived neurotrophic factor (BDNF)

BDNF is a secreted protein that has long been known for its role in promoting survival, axon guidance, synapse formation and neuronal plasticity (Huang and Reichardt, 2001). BDNF binds Trk receptors that, in turn, activate downstream signaling events in the CNS. But until recently, BDNF's role in the developing striatum was unclear. Exposure to BDNF activates BDNF-dependent signaling pathways in embryonic striatal cultures, suggesting that it may be important for striatal differentiation (Ciccolini and Svendsen, 2001). In transgenic mice carrying a CNS-specific conditional knockout of the BDNF gene, striatal neurons were much smaller and had fewer branching dendritic segments and reduced spine density (Rauskolb et al., 2010), suggesting a prominent role for BDNF in striatal neuron maturation. During embryonic development, selective removal of the BDNF receptor TrkB from the LGE led to a massive loss of striatopallidal MSNs and a large increase in apoptosis in the SVZ and VZ, suggesting a requirement for BDNF in cell survival pathways as well (Baydyuk et al., 2011). In adult mice, ectopic expression of BDNF in the VZ significantly increased neurogenesis in the striatum and, in particular, DARPP-32-positive MSNs (Benraiss et al., 2001). Thus, BDNF is key to striatal neuron differentiation.

Fibroblast growth factors (FGFs)

FGFs are a family of ligands that act through their cognate receptors (FGFRs) to promote cell proliferation and differentiation (Mason, 2007). They participate in many stages of patterning, starting with neuronal induction, gastrulation and finally telencephalic specification, where they influence patterning of both the dorsal and ventral regions of the telencephalon, and later, the basal ganglia (Hoch et al., 2009; Mason, 2007). During telencephalon formation, FGF signaling induces proliferation, promotes survival, and activates ventral gene expression (Hoch et al., 2009; Shinya et al., 2001). There are five FGFs thought to guide telencephalic formation, some of which probably act redundantly, as loss of a single FGF does not terminate development of the telencephalon. However, simultaneous knockout of *Fgfr1, Fgfr2,* and *Fgfr3* in mice results in abnormal gene expression and severe defects in telencephalic development (Paek et al., 2009).

FGF-2 has typically been used to generate neural progenitors from ESCs that give rise to neurons *in vitro* (Sanalkumar et al., 2010). However, the use of FGFs to generate neural progenitors is complicated, as there appears to be different instructive roles for different FGFs (Sterneckert et al., 2010). Some studies show that they inhibit differentiation of the neural lineage, whereas others show that they are required for neural patterning, and their use in ESC culture remains controversial (Sterneckert et al., 2010). We use FGF-2 along with EGF to generate and maintain a neural progenitor cell (NPC) population of iPSCs (The HD iPSC, 2012).

Sonic hedgehog homolog (SHH)

SHH, one of the best-studied CNS morphogens, acts early in development to establish a dorsal–ventral axis in the embryo, and at later stages participates in cell proliferation, axon guidance, neurogenesis and neuronal specification (Sousa and Fishell, 2010). During early patterning, SHH is expressed in the ventral telencephalon and activates Gli transcription factors that are critical for establishing the LGE and MGE (Sousa and Fishell, 2010). Genetic studies revealed that the loss of SHH expression leads to a loss of ventral cell types as well as expression of the LGE markers Dlx2 and Gsh2 (Rallu et al., 2002), whereas ectopic SHH expression in the telencephalon induces the expression of LGE and striatal markers, such as GAD67 and Dlx2 (Kohtz et al., 1998).

This patterning effect persists in culture when differentiating stem cells into forebrain neurons. In ES cultures, inhibition of SHH signaling promotes the differentiation of dorsal telencephalic structures that give rise to cortical neurons (Eiraku and Sasai, 2012; Eiraku et al., 2008; Gaspard et al., 2008), whereas exposure to SHH stimulates the expression of subpallial LGE markers (Danjo et al., 2011; Ma et al., 2012). Addition of the small-molecule agonist purmorphamine to human neural stem cell cultures also significantly increased the percentage of DARPP-32-positive neurons after differentiation (El-Akabawy et al., 2011; Ma et al., 2012). Further, exposure of ESC-derived primitive neuroepithelial cells to SHH triggered ventralization and, in a subset of cells, promoted differentiation into DARPP-32-expressing GABAergic neurons (Li et al., 2009). Nevertheless, the timing of exposure and concentration of SHH seems to be critical for the patterning of cells towards the LGE fate. In studies aimed at defining the optimal SHH exposure conditions that promote LGE differentiation *in vitro*, exposure of mouse ESCs to exogenous SHH during striatal differentiation is critical, as is the eventual removal of exogenous SHH to induce terminal maturation into GABAergic DARPP-32-positive cells (Danjo et al., 2011).

Wnt inhibitors

Wnts are a group of secreted palmitoylated signaling proteins that bind to Frizzled receptors which, in turn, signal beta-catenin translocation to the nucleus. Once there, beta-catenin activates the transcription of many target genes involved in cell fate, differentiation, proliferation and cell migration (Logan and Nusse, 2004). In early telencephalic development, Wnts induce the expression of PAX-6 and other genes that stimulate dorsal development and patterning (Gunhaga et al., 2003; Li et al., 2009) that will give rise to cortical neurons.

Wnts are required for neurogenesis and proliferation of the MGE (Gulacsi and Anderson, 2008). During subdivision of the MGE and LGE, inhibiting Wnt signaling in dorsal structures is key to promoting LGE patterning. Specifically, inhibition of Wnt signaling in the pallium results in the ectopic expression of ventrally associated genes such as Gsh2, Mash1, and Dlx2 (Backman et al., 2005), whereas inducing Wnt activity in ventral LGE-derived explants can stimulate the formation of dorsal telencephalic structures *in vitro* (Gunhaga et al., 2003). Finally, inhibiting Wnt activity during the differentiation of ESC cultures seems key to promoting the MSN fate, as exposure to the Wnt inhibitor DKK-1 along with SHH can induce dorsal progenitor cells to adopt a ventral fate and eventually give rise to GABAergic neurons (Aubry et al., 2008; Li et al., 2009; Watanabe et al., 2005).

Other compounds that induce striatal development include the anti-convulsant and mood-stabilizing drug valproic acid, which when ectopically expressed, increases the neurogenesis of several cell types in the brain (Zhang et al., 2010a). Valproic acid was shown to increase the production of GABAergic neurons from cultured primordial stem cells more than fivefold (Laeng et al., 2004). The morphogen retinoic acid (RA) stimulates the expression of Nolz1, a key transcription factor that promotes striatal differentiation in LGE-patterned cells (Urban et al., 2010). Further, RA activates the expression of GAD67, an enzyme involved in GABA neurotransmitter synthesis and GABAergic neuron differentiation *in vitro* (Chatzi et al., 2011). Many ESC differentiation methods include RA to increase GABAergic neuronal differentiation (Ma et al., 2012; Shin et al., 2011).

MSN differentiation in culture

To successfully model HD in culture, the ability to differentiate iPSCs or ESCs into inhibitory GABAergic MSNs—the most susceptible cell type in HD pathogenesis—along with other affected cell types, including cortical neurons, astrocytes and possibly oligodendrocytes, will be key. Protocols for differentiating these cell types have been described (Czepiel et al., 2011; Krencik et al., 2011; Shi et al., 2012) and are being further refined as iPSC technology becomes more widely used. Protocols to differentiate ESCs into GABAergic neurons generally follow a multi-step process of 1) induction of the neural lineage, 2) regional patterning and differentiation of neuronal progenitor cells and 3) specialization of mature neurons (Aubry et al., 2008; Carri et al., 2013). Differentiated GABAergic neurons may be generated by several methods, including the embryoid body (EB), monolayer, five-stage (Shin et al., 2011), and cell-clustering stepwise differentiation methods (The HD iPSC, 2012).

The EB method fosters the formation of aggregates or clusters of cells that recapitulate the 3D structure and intracellular signaling processes that occur during native development to produce all three germ layers (Itskovitz-Eldor et al., 2000). After EB generation, morphogens such as RA are added to induce neural patterning (Bain et al., 1995), after which cells are plated to induce GABAergic specification (Strubing et al., 1995). Similar to the EB method, the monolayer protocol utilizes patterning morphogens, such as FGF, in defined concentrations and for specific periods of time to induce GABAergic specification (Westmoreland et al., 2001). This methodology is thought to generate neural cells more efficiently than other methods as the media and conditions are more defined and likely more closely recapitulate neural development (Abranches et al., 2009). Further, these cells tend to be less aggregated and more uniform, making them easy to characterize. The five-stage method involves EB formation that is followed by dissociation and monolayer formation. This protocol also utilizes instructive cues, such as FGF, to direct neuronal patterning, followed by FGF deprivation to induce GABAergic specification (Okabe et al., 1996). Other GABAergic differentiation protocols incorporate feeder layers for neuronal induction (Aubry et al., 2008); however, these methods can introduce unknown factors that limit differentiation potential and restrict the resulting neural cell types to a midbrain or hindbrain fate (Pankratz and Zhang, 2007). All of these methods have been used with different iterations of varying morphogens and media.

Although all of these methods produce GABAergic neurons, the first protocol designed to specifically direct the differentiation of ESCs towards GABAergic MSNs was established using ESCs co-cultured with murine MS5 stromal cells - (Aubry et al., 2008). The protocol is divided into several stages, beginning with the generation of neural rosettes, then the patterning towards telencephalic progenitors, and a final stage of induction of specialized, mature striatal cells using morphogens known to drive striatal neuron patterning, such as SHH, BDNF, valproic acid and Dkk-1. Cultures differentiated in this way are composed of ~20% mature MAP-2 positive neurons that stain for GABA, calbindin and calretinin, with only about ~half of these are DARPP-32-positive after ~ 2 months. Given that the vast majority of striatal neurons are GABAergic MSNs (Gerfen, 1992b), the proportion of DARPP-32-positive cells produced with this differentiation method is quite low. Further, high levels of Nestin- and PAX-6-positive cells continue to propagate, even after 2 months in culture, suggesting that many cells in these cultures are still dividing and differentiating. Indeed, when injected into the rat striatum, younger, less differentiated populations of cells never became DARPP-32-positive and instead formed teratomas, whereas cells injected at later stages of maturation produced no teratomas and differentiated into DARPP-32-positive cells *in vivo* (Aubry et al., 2008). Still, massive overgrowth and proliferation eventually occurred even with the more mature cells, demonstrating that they do not receive, or are unreceptive to, the appropriate signals that induce functional integration in the brain. In another study, a slightly different version of this protocol (that subjected the cells to the EB stage and did not co-culture with MS5 stromal cells for neural induction), was used to differentiate HD iPSCs and a similar-sized subset (~10%) became DARPP-32-positive (Zhang et al., 2010b).

The HD iPS consortium used the protocol developed by Aubry et al. (2008), but also did not use a stromal cell line for the neural induction of ES cells; rather, iPSCs were first differentiated into a population of intermediate renewable neural stem cells (NSCs) that were then exposed to striatal patterning morphogens (The HD iPSC, 2012) (Fig. 1). Differentiating MSNs from NSCs makes the production of striatal neurons much more feasible, as it is less time consuming to maintain NSCs in culture than ESCs/iPSCs. Using this method, the HD iPS consortium reported the generation of cultures containing ~5% DARPP-32-positive neurons after ~2.5 months.

Other methods have also been reported for generating MSNs from ESCs. A monolayer method was used to differentiate mouse ESCs into GABAergic neurons, of which a subset (~30%) became DARPP-32-positive after just three weeks (Shin et al., 2011). Another protocol using hESCs reports a significantly increased differentiation efficiency, yielding cultures composed of ~ 75% DARPP-32-positive cells that were capable of functionally integrating and restoring motor function in the QA-lesion-induced HD model (Ma et al., 2012). While still time consuming, this differentiation protocol is not as lengthy (~1.5 months) as the Aubry et al. (2008) method.

Most recently, a very elegant study relied on the expression of markers found in the developing human brain to gauge the maturation of striatal MSNs from hESCs differentiated using a monolayer method (Carri et al., 2013). Although the protocol is quite long (~2.5 months) and the efficiency of generation of DARPP-32-positive cells is similar to the Aubry et al. (2008) protocol (~ 10%), the characterization of the cells generated is by far the most thorough to date. The group performed extensive co-staining experiments to define striatal identity, comprehensive electrophysiology that revealed a subset of cells containing MSN-like properties, and determined that these cells were able to integrate and form synaptic connections upon transplantation into the rat brain (Carri et al., 2013).

Although these different protocols underscore the potential of this technique for generating MSNs, they are fairly time consuming and resource intensive, both of which make the generation of these neurons highly susceptible to failure. These obstacles need to be addressed in order to develop these cells as an effective platform for genetic or drug screening.

What markers do MSNs express once they mature?

To monitor the differentiation success of ESCs or iPSCs into striatal neurons, it is important to define the gene-expression patterns indicative of mature MSNs. For details on the transcription factors that influence LGE patterning and genes that are expressed during MSN development see Carri et al. (2013), Evans et al. (2012), and Jain et al. (2001).

The most commonly used marker of mature MSNs of either type is DARPP-32, which is expressed in ~95% of all MSNs (Ouimet et al., 1984, 1998). Other general MSN markers include Rhes, GPR88, GAD-65/67, CALBINDIN and CTIP-2 (Table 2). Expression of many of these genes is

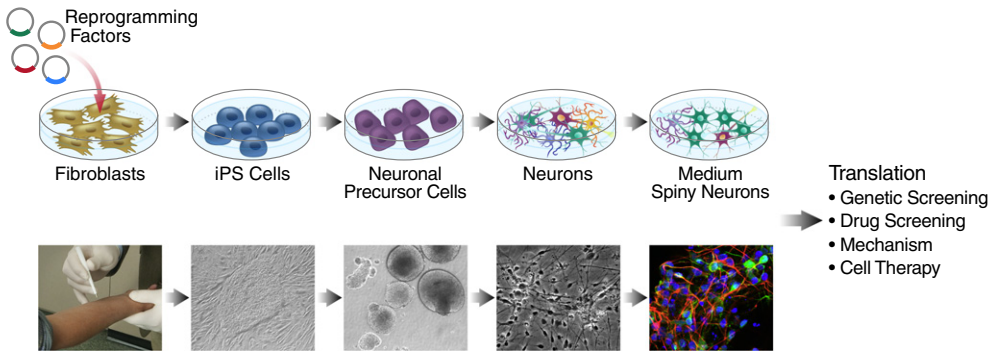

Fig. 1. Modeling HD in culture using IPS cells: from patients to cultured medium spiny-Like neurons. Top panel, cartoon of differentiation process. Skin fibroblasts are reprogrammed by expressing "reprogramming factors" that convert the differentiated cells into iPS that are capable of becoming any cell type. To make striatal-like cultures, iPS cells are exposed to growth factors that induce patterning of neural lineage. Addition of growth factors and neurotropins induce development of neurons and eventual maturation of cultures that contain MSNs. Bottom panels, skin cells are safely removed from a patient (far left) and transformed into self renewing iPS cells (micrograph second panel from left). NSCs (middle) are converted into neurons (micrograph second panel from right) and matured into DARPP-32-positive cells (right). Confocal image of fixed and stained iPSs differentiated for 60 days in culture (The Hd Ipsc, 2012). Blue = DAPI, green = DARPP-32, and red = MAP-2.

Table 2
Genes enriched in mature MSNs.

Gene	Protein	Expression	Expression change in HD	Reference
Adora2a	Adenosine A2a receptor	Enriched in striatopallidal MSNs but is also expressed in the olfactory tubule, cortex and hippocampus and peripheral tissues	mHtt reduced expression of the core promoter of the A2a receptor in primary striatal neurons *in vitro*	Blum et al. (2003), Chiang et al. (2005), Heiman et al. (2008), Lobo et al. (2006), and Wei et al. (2011)
CALB	Calbindin-$_{D28k}$, calcium binding protein	Enriched in spiny neurons of the striatal matrix but also widely expressed in the brain and nervous system and peripheral tissues	Expression increases in both HD mouse models and HD brains	Ferrante et al. (1991), Huang et al. (1995), Pickel and Heras (1996), Schwaller et al. (2002), and Sun et al. (2005)
CTIP-2	COUP TF1-interacting factor 2	Expressed in early postmitotic migratory MSNs during development; expression increases and is maintained in the adult. Also expressed in cortex, hippocampus and olfactory bulb	Expression decreases in mouse models of HD	Arlotta et al. (2008) and Desplats et al. (2008)
DARPP-32	Dopamine and adenosine 3, 5-cyclic monophosphate (cAMP)-regulated phosphoprotein, 32 kDa	Highest in mature, post-mitotic MSNs, but also expressed in other cell types in the cerebellum, cortex. In addition, some expression is found in glial cells	Expression decreases in HD mouse models	Bibb et al. (2000), Luthi-Carter et al. (2000), Ouimet et al. (1984, 1998)
DRD1	Dopamine receptor 1	Highly enriched in striatonigral MSNs but expressed in other cell types in the cortex and basal ganglia	Expression decreases in HD brains	Augood et al. (1997), Gerfen et al. (1990), Heiman et al. (2008), Hodges et al. (2006), Lobo et al. (2006) and Meador-Woodruff et al. (1996)
DRD2	Dopamine receptor 2	Highly enriched in striatopallidal MSNs but also expressed in other parts of the basal ganglia	Expression decreases in HD brains	Augood et al. (1997), Gerfen et al. (1990), Heiman et al. (2008), Lobo et al. (2006) and Meador-Woodruff et al. (1996)
Enkephalin (Penk)	Neuropeptide	Highly enriched in striatopallidal MSNs	Expression decreases in mouse models of HD	Heiman et al. (2008), Hodges et al. (2006), Lobo et al. (2006) and Menalled et al. (2000)
GAD65/67	Glutamic acid decarboxylase	Enriched in striatal neurons of the basal ganglia but expresses in all GABAergic neurons	Expression does not change in the striatum of mouse models of HD but is altered in other parts of the brain	Gourfinkel-An et al. (2003), Menalled et al. (2000), Mercugliano et al. (1992) and Pickel et al. (1980)
GPR6	G protein-coupled receptor 6	Enriched in striatopallidal MSNs	Expression decreases in HD brains	Hodges et al. (2006) and Lobo et al. (2007)
GPCR88	G protein-coupled receptor 88	Enriched in the caudate and putamen but also expressed in the olfactory tubercule, nucleus accumbens and inferior olive nucleus	Expression decreases in HD brains	Hodges et al. (2006), Mizushima et al. (2000) and Van Waes et al. (2011)
Rhes	Rhes homolog enriched in the striatum	Enriched in adult striatum but also expressed in many other parts of the brain including the cortex, hippocampus and cerebellum	Unknown	Vargiu et al. (2001)

downregulated in mouse models of HD as well as in human HD brains (Table 2), but alterations in the expression of these genes in MSNs differentiated from HD iPSCs have not yet been detected (An et al., 2012; The HD iPSC, 2012; Zhang et al., 2010b). Antibodies against most of these proteins are available; however, some are better than others. We generally rely on immunohistochemical staining of DARPP-32 and CTIP-2 to assess the differentiation of striatal-like neurons in iPSC cultures (The HD iPSC, 2012). Because many of these proteins, such as DARPP-32, calbindin, and CTIP2, are not solely expressed in MSNs but are also found in other neuronal cell types (Arlotta et al., 2008; Liu and Graybiel, 1992; Ouimet et al., 1984; Rajput et al., 2009), co-staining of multiple striatal markers is ideal. A recent study examined both the simultaneous expression of CTIP-2 with DARPP-32 to better confirm MSN identity (Carri et al., 2013; Rajput et al., 2009). Further, to ascertain that LGE patterning is occurring during differentiation, the examination of proteins expressed in striatal precursors, such as FOXP1 (Carri et al., 2013; Tamura et al., 2004), FOXP2 (Carri et al., 2013; Takahashi et al., 2003), and GSX2 (El-Akabawy et al., 2011; Waclaw et al., 2009) would be optimal.

Mature MSNs are functionally classified as either striatopallidal or striatonigral MSNs, which make up the indirect or direct pathways of the striatum, respectively (Gerfen et al., 1990). There is evidence of a differential susceptibility of these two MSN populations to the effects of mHtt, whereby striatopallidal neurons are lost first while striatonigral neurons are spared until later in the disease course (Albin et al., 1992). Many genes are selectively enriched in one of these two neuronal populations (Lobo et al., 2006; Heiman et al., 2008) (Table 2). It will be intriguing to probe this potential differential susceptibility using neuronal cultures derived from HD versus WT iPSCs.

Does the HD mutation affect neurogenesis?

Changes in the architecture of synapses and circuitry during neurogenesis could predispose the HD-affected brain to cell dyshomeostasis and eventual death, so a thorough understanding of whether mHtt affects these processes is critical for the development of HD iPS cell models. There is increasing evidence from both murine and human studies that neurogenesis is altered in both maturing and adult HD brains. However, there seems to be a discrepancy between the sorts of changes that occur in humans versus mice.

Among the first rodent models of HD were the R6/1 and R6/2 transgenic mouse lines. R6/1 mice express a variant of human mHtt that contains ~115 repeats within exon 1, and show HD-like symptoms around 15–21 weeks of age (Mangiarini et al., 1996). R6/2 mice express the same mHtt fragment but with 28 more CAG repeats, which results in a more severe phenotype (Mangiarini et al., 1996). Neurosphere assays, which show how many neural stem cells (NSCs) are present in tissues, revealed that the number of hippocampal precursor stem cells was significantly greater in R6/1 mice compared to WT mice, while no change in the number of SVZ stem cells was observed between WT and R6/1 mice (Walker et al., 2011). However, curiously *in vivo* bromodeoxyuridine (BRDU) labeling- revealed that neurogenesis in the hippocampus was significantly decreased compared to WT animals, suggesting that although cellular proliferation is altered, a latent stem cell population in the hippocampus is maintained as a response to mHtt (Walker et al., 2011). The same reduction of cell proliferation in the hippocampus of R6/1 mice was observed by another independent study (Lazic et al., 2004).

Another group studying cell proliferation in the hippocampus of R6/2 mice found that as early as 3.5 weeks of age, R6/2 mice had fewer proliferating cells in the dentate gyrus than WT animals. Most of the proliferating cells stained for NeuN, confirming that they were neuronal precursors (Gil et al., 2005). The reduction of proliferating cells resulted in significantly fewer neuroblasts in the dentate gyrus at 10 weeks. However, similar to the study by Walker and colleagues, there were no differences in the number of proliferating cells in the SVZ in R6/2 or WT mice (Gil et al., 2005). Likewise, direct comparison of proliferating BRDU-positive and differentiating DCX-positive cells in the R6/2 showed similar numbers of newly born cells in the SVZ (Phillips et al., 2005).

In mice, mHtt may also affect neurogenesis in parts of the brain other than the SVZ and hippocampus. For example, impaired neurogenesis was observed in the piriform cortex, which is required for odor perception and olfactory memory (Lazic et al., 2007), in both R6/2 (Phillips et al., 2006) and R6/1 mice (Lazic et al., 2007). This finding is consistent with the observation that HD patients have defects in olfactory discrimination (Lazic et al., 2007; Nordin et al., 1995).

In contrast, a study looking at neurogenesis in the forebrain subependymal layers in the SVZ of R6/2 mice contradict the preceding studies but are consistent with observations in human tissues. They showed a significant *increase* in the number of proliferating NSCs in the neurosphere assay compared to WT animals, which directly corresponded to the onset of symptoms. No neurosphere differences were observed at 4 weeks of age, when symptoms are subtle, but by 8 weeks—at about the time when motor symptoms start to become apparent—these changes became significant (Batista et al., 2006). Interestingly, NSCs that normally migrate to the olfactory bulb were redirected to the striatum (Batista et al., 2006), suggesting that the striatum can recruit proliferating cells, possibly in reaction to the cellular insults caused by mHtt. In agreement with this, after injection of quinolinic acid (QA) – a potent neurotoxic substance that creates lesions in the brain to model HD – neuroblast proliferation increased and the migration of these cells appeared to be redirect to the striatum from the SVZ (Tattersfield et al., 2004).

Indeed, studies in human tissues suggest proliferative rather than inhibitory changes in neurogenesis in response to mHtt expression. Before the HTT gene was mapped, Ferrante et al. (1991) examined the subcellular changes in postmortem HD brains at different disease stages. Single-section Golgi staining of the striatum of HD patients revealed two surprising morphological changes compared to controls: (1) a substantial increase in spine density in a subset of dendrites from "Grade 2"—stage HD MSNs, and (2) an increase in the size of growth-cone-like processes with filipodia on the tips of distal dendrites, suggesting proliferation rather than degeneration in neurons from HD brains (Ferrante et al., 1991). Although, in the more severe grades of HD, the majority of structural changes observed in MSNs were those associated with degeneration, such as spine loss, abnormal recurving of distal dendritic segments and aberrant branching of dendrites (Ferrante et al., 1991).

The first study to show that neurogenesis takes place in the brain as a direct consequence of cell death was in postmortem brain tissue from HD patients. Curtis et al. (2003) observed a significant increase in cell proliferation in the subependymal layer (SEL) or (SVZ) adjacent to the caudate in HD brains compared to unaffected controls (Curtis et al., 2003), and this increase was positively correlated with the number of CAG repeats. Further, these proliferating cells gave rise to both astrocytes and newborn neurons. The extent of neurogenesis was highly significant, such that HD brains exhibited a 2.6-fold increase in the number of newborn neurons in the SVZ compared to controls (Curtis et al., 2005). Interestingly, a study examined the number of proliferating cells in the hippocampal subgranular zone (SGZ) of HD human postmortem brains, and found no differences in cellular proliferation compared to controls. (Low et al., 2011). This contrasts with what has been observed in murine studies of hippocampal neurogenesis in the presence of mHtt.

In summary, the majority of murine-based HD models report alterations of neurogenesis in the hippocampus while SVZ neurogenesis is spared. Whereas in contrast, in the brains of humans with HD, changes in neurogenesis are found in the SVZ but not in the hippocampus. The reason for these discrepancies between murine and human neurogenesis in the context of HD is unclear. One possibility is that because symptoms of disease appear only after decades of exposure to endogenous levels of mHtt in humans, whereas transgenic HD models experience a much shorter time course of disease progression and typically have higher levels of transgenic mHtt, different signaling pathways and coping mechanisms are triggered. A finding consistent with this possibility was observed in

the knock-in Hdh-Q111 model, in which mHtt expression is under the control of the endogenous promoter. In these mice, neurogenesis within the striatal SVZ was shown to be lower in young animals (E11.5), but at later stages (E15.5) increased compared to WT (Molero et al., 2009), suggesting that rodent mHtt-expressing models may better recapitulate the human disease state when mHtt is expressed at near-physiological levels.

However, another study suggests that this explanation still falls short. Using a mouse model in which full-length Htt carrying 128 CAG repeats is expressed under the control of the human promoter (YAC-128 model), leading to reduced mHtt expression compared to R6 mice, YAC-128 mice exhibited a marked reduction in neural precursor proliferation and differentiation in the hippocampus, but no changes in the SVZ were observed (Simpson et al., 2011).

The differences observed may alternatively be due to CAG repeat length, as most rodent models have upwards of ~100 CAG repeats, whereas in humans CAG, repeats generally range from 40 to 50. It would be of interest to look at neurogenesis in juvenile cases of HD where extreme CAG repeat lengths are observed. Finally, it is likely that the lack of access to early-stage (i.e., young to mid-adult) post-mortem human tissue makes resolving these discrepancies difficult, given that increases in human neurogenesis may take place only very late in the disease course, and therefore, a comparable time frame may be challenging to witness in mice. Regardless, these discrepancies illustrate the challenges with using rodents to model human disease, which in our view may be circumvented by the development of *in vitro* models that rely on human stem cells from HD patients.

Stem cells and HD

ESCs are self-renewing pluripotent cells derived from embryos that are capable of giving rise to all cell types from the three germ layers. They have been used extensively to model development, differentiation and some disease states *in vitro* (Ben-David et al., 2012). The first stem cells made from HD patients were part of an effort to make ESC lines from individuals with different genetic abnormalities (Verlinsky et al., 2005). Since then, other HD lines have been made from donated embryos containing 40–51 repeats (Bradley et al., 2011; Mateizel et al., 2006; Niclis et al., 2009). One line was generated from blastocysts that were fertilized *in vitro* and propagated on mouse feeder layers (Mateizel et al., 2006), and since then several of these lines have been successfully differentiated into neurons (Bradley et al., 2011; Niclis et al., 2009), confirming the potential of stem cells to generate neurons carrying mHtt. Importantly, some of the HD ESC lines retain stable CAG repeats over multiple passages and following neuronal differentiation (Seriola et al., 2011). Although many human lines have been generated to date, no HD-related phenotypes have been reported with these human ESC lines.

However, murine ESC-based models can reveal HD-related phenotypes. ESCs generated and propagated *in vitro* from mice with either 150 or 77 CAG repeats (*Hdh CAG150* and *Hdh CAG77,* respectively), showed similar neural proliferative properties to human SVZ cells in HD brains (Curtis et al., 2003, 2005). Specifically, at the ESC stage, the HD cell lines showed no differences in cell-cycle progression compared to cells from a WT *Hdh CAG7* line. However, after neuronal differentiation, more cells became positive for Sox-3 and β-tubulin in the *Hdh CAG150* and *CAG77* lines than in WT cells, suggesting that the HD lines transitioned faster to the neural progenitor and neuron stages (Lorincz and Zawistowski, 2009). NSCs isolated from the SVZ of mice at postnatal week 20 exhibited similar differentiation properties, indicating that these phenotypes were not specific to ESCs. After subjecting the ES cells to only ~1 week of a neuronal differentiation paradigm, increased cell death was observed in the *CAG150* line suggesting that the polyglutamine expansion becomes toxic as soon as the cells take on a neuronal fate. Finally, transcriptional profiling revealed many gene-expression changes in pathways regulating axonal growth and neuronal differentiation, suggesting that the increase in neurogenesis observed in mutant cells may be a result of mHtt-induced transcriptional dysregulation. As changes in gene expression are a hallmark of HD in human brains (Cha, 2007), these findings demonstrate that ESCs can recapitulate key aspects of the disease.

In another study, a mES cell line carrying 150 CAG repeats in HTT (*HdhQ150*) was used to confirm the activity of a compound identified in a screen for molecules capable of enhancing mHtt degradation. This compound induced the expression of heat-shock protein 70 (HSP70) and reduced mHtt expression in *HdhQ150* ESCs differentiated into neurons (Baldo et al., 2012).

Recently, Jacobsen et al. created a series of isogenic mouse ES-cell lines carrying 7-111 CAG repeats, as well as a line in which Htt was completely knocked out (Jacobsen et al., 2011). With these lines, they uncovered gene-expression changes attributable specifically to CAG-repeat size and not loss of Htt function. In doing so, they were able to parse out how the loss of Htt expression versus varying CAG-repeat lengths alter biological pathways in distinct or overlapping ways (Jacobsen et al., 2011). This allelic series was subsequently used to investigate the effects of mHtt during neuronal differentiation (Conforti et al., 2013). Changes in cell death, measured by caspase activation and transcription, were observed in mHtt cells compared to WT, although no relationship unique to CAG length was detected. However, both high CAG-repeat length and loss of Htt expression resulted in similarly decreased proportion of mature neurons, where a loss of mHtt resulted exclusively in increased numbers of glial cells (Conforti et al., 2013). This allelic series will be useful for exploring other CAG-dependent phenotypes, and efforts are underway to establish similar isogenic lines in human iPSCs (unpublished data).

Although still a relatively new undertaking, these results suggest that ESCs can be used to explore the effects of mHtt in cells that are renewable and fully expandable, which could be extremely valuable for drug-screening efforts. Given that ESCs from a single genetic background can be differentiated into multiple cell types, we can tease out the mechanisms by which mHtt exerts its cell-type-selective effects in a controlled genetic background. Further, ESC-based models will allow for the study of mHtt's effects on cells during early differentiation and development, which may reveal a novel insight into HD pathogenesis.

iPSCs and HD

The revolutionary discovery by Yamanaka and colleagues (Takahashi et al., 2007) that human fibroblasts could be reprogrammed into self-renewing pluripotent cells displaying many of the properties of ESCs has transformed our ability to study disease in human cells. The reprogramming of fibroblasts into iPSCs depends on the expression of 4–6 factors that silence genes involved in cell differentiation while activating genes that promote pluripotency (Takahashi et al., 2007; Yu et al., 2007). Like human ESCs (hESCs), iPSCs can self-renew for long periods of time and differentiate into nearly all cell lineages (Takahashi et al., 2007; Yu et al., 2007).

Importantly, iPSC technology enables models to be created of both genetically inherited and sporadic diseases *in vitro* (Ebert et al., 2009; Park et al., 2008a). Since iPSCs can be derived directly from patients with a given disease, they carry all of the associated genetic components that make certain cell types susceptible to a disease state, thereby representing the most genetically precise model of a disease (Fig. 1).

Despite the promise of this technology for modeling complex neurodegenerative diseases such as HD, several considerations must be addressed. The first is: can we observe disease-relevant phenotypic differences in patient-derived iPSCs compared with healthy controls? There are many limitations of cell culture that might interfere with this possibility. Cells growing in a dish lack the complexity of neural cells that have developed *in vivo.* The complex 3D environment of the brain and the plethora of intrinsic and extrinsic cues may be important to fully understand how the insults of mHtt alter cellular function. Second, there are many other cell types within the brain other than neurons that may contribute to the neurodegenerative disease process. The stoichiometry and

concentration of these different cell types may be important for detecting HD-related pathology and these features may be hard to recapitulate in culture. Finally, a third important consideration is the role of aging in neurodegeneration. Since the symptoms of HD typically emerge in late adulthood, years rather than weeks of mHtt expression may be necessary to fully recapitulate disease. It is possible that the effects of mHtt may immediately disrupt cellular function, but an organism's ability to deal with the insults of mHtt may be offset more readily in young versus aged cells; in this way, symptoms can be staved off until late age. Thus, how easily will we be able to capture the phenotypes associated with the initiation of late-onset disease symptoms in a short-term culture? So far, iPSCs that have been generated from patients with various neurodegenerative disorders indeed display robust phenotypes that replicate many features of their respective diseases (An et al., 2012; Bilican et al., 2012; Brennand et al., 2011; Ebert et al., 2009; Egawa et al., 2012; Lee et al., 2009; Marchetto et al., 2010; Park et al., 2008b; The HD iPSC, 2012). We will review the progress made to model HD using this exciting new technology.

Fibroblasts from HD patients have shown HD-related phenotypes, such as alterations in proteasome activity (Seo et al., 2004) and altered gene-expression profiles (Valenza et al., 2005), that conceivably could hinder the successful generation of iPSCs. However, proof of principle that iPSCs can successfully be made from individuals with severe neurodegenerative disorders has been demonstrated, including pluripotent HD lines that exhibit no apparent difference from control iPSCs (Park et al., 2008a). It was not until these HD iPSCs were subjected to a neuronal differentiation paradigm that differences between WT and HD iPSCs emerged (An et al., 2012; Chae et al., 2012; Jeon et al., 2012). Proteomic analysis revealed hundreds of distinct changes in protein expression in the HD iPSCs subjected to a short neuronal induction protocol (Chae et al., 2012). Further, neuronal differentiation efficiency and neurite length were reduced in HD iPSCs (Chae et al., 2012).

An independent group developed iPSC-based models from the HD lines originally developed by Daley and colleagues (Park et al., 2008b). These lines were subjected to a modified differentiation protocol to generate MSNs (Aubry et al., 2008) that express DARPP-32 (Zhang et al., 2010b). As mHtt specifically affects striatal neurons, differentiated HD cultures might be expected to contain fewer DARPP-32-positive neurons if these cells are more susceptible than other cell types in culture. However, no difference in the percentage of DARPP-32-positive neurons was reported between HD and WT cells. We use a slightly different version of the protocol by Aubry et al. (2008) to generate striatal neurons (The HD iPSC, 2012), and observed that the cells cluster into tightly bound 3D structures rather than a monolayer of neurons. We find that these structures make it difficult to accurately estimate the percentage of DARPP-32-positive cells within defined areas of each well (The HD iPSC, 2012).

However, Ellerby and colleagues (An et al., 2012; Zhang et al., 2010b) found other HD-related phenotypes in the lines made by Daley and colleagues (Park et al., 2008a). Removing growth factors at an early NSC stage resulted in more caspase 3/7 activity in the HD compared to WT Daley iPSCs, suggesting that cells with mHtt are more susceptible to stress even at a very early stage of differentiation (Zhang et al., 2010b). In a subsequent study, additional HD-related phenotypes were reported and the CAG expansion of the Daley HD-iPSC line was corrected to WT lengths through homologous recombination. Restoration of the CAG repeat to WT length was able to restore distinct transcriptional changes in the E-cadherin and TGF-beta signaling pathways to WT levels, prevented the cell death, increased BDNF transcription, rescued mitochondrial dysfunction and normalized the elevated caspase 3/7 activity observed in the HD-iPSC lines (An et al., 2012), suggesting that these alterations were directly induced by CAG expansion and were not caused by inter-cell-line variability.

Other HD iPSCs have been generated and examined, including lines generated from two individuals homozygous for mHtt (repeat sizes between 39 and 44 CAGs) and a heterozygote with 44 repeats (Camnasio et al., 2012). Surprisingly, this group reported few differences between these HD and WT lines, as neuronal differentiation, growth rate and caspase activation were indistinguishable. However, lysosomal activity was higher in HD iPSCs, suggesting that cellular coping mechanisms, including the protein-clearance machinery, may be abnormal in HD.

Scientists from the HD and iPSC communities have united to form a consortium under the auspices of the National Institutes of Neurological Disorders and Stroke (NINDS). The so-called HD iPS consortium has generated a large panel of HD iPSC lines and HD fibroblasts that are being reprogrammed into iPSCs, and made these lines available to the public through the Coriell Institute for Medical Research, which maintains a cell repository (http://ccr.coriell.org/) (The HD iPSC, 2012). This collaborative effort discovered numerous differences between HD and WT iPSCs, and most importantly, uncovered HD-related phenotypes in several HD iPSC lines. Whole-transcript expression profiling and analysis of genes involved in signaling, neuronal development and axon guidance confirmed previously described aberrant changes in HD tissues compared to WT, but new and interesting changes were also observed that were CAG expansion-dependent. Further, decreased ATP/ADP levels were detected in the HD-derived NSCs, revealing that changes in energy metabolism may be an early component of HD. The group further differentiated the NSCs to produce neurons, and found alterations in their ability to fire both spontaneous and evoked action potentials and calcium dyshomeostasis in the HD iPS cells. In cultures differentiated for longer periods of time to yield DARPP-32-positive neurons, HD-derived lines exhibited properties seen in cells harboring disease-associated CAG expansions, including increased cell death and increased susceptibility to cell stressors such as glutamate and trophic factor removal, and calcium dyshomeostasis (The HD iPSC, 2012). These findings reveal that HD iPSCs can exhibit many of the biological properties found in the human HD brain.

These previous studies report phenotypes in cells differentiated towards a neuronal lineage. However, other cell types are affected by mHtt expression, such as astrocytes (Bradford et al., 2010). iPSCs made from a juvenile onset (109 repeats) and an adult-onset (50 CAG repeats) HD patient and differentiated towards an astrocytic lineage displayed elevated cytoplasmic vacuolation that increased over time (Juopperi et al., 2012). Differentiating HD iPSCs into different cellular lineages may reveal novel insights into the cell-specific effects of mHtt.

One of the most widely documented phenotypes in human HD brains and HD mouse models is the formation of mHtt aggregates (Arrasate and Finkbeiner, 2012; DiFiglia et al., 1997). However, in human HD iPSCs, the spontaneous formation of aggregates has not been observed. Recently, the addition of a stressor, the proteasome inhibitor MG132, was shown to induce aggregation in iPSCs containing 72 repeats, as revealed by immunostaining using the mHtt-specific antibody EM48 (Gutekunst et al., 1999) (Jeon et al., 2012). It may be that the effects of endogenous levels of mHtt—or the exposure time to mHtt during typical iPSC culture—are not sufficient to disrupt cell proteostasis and promote aggregation in the absence of stress. Interestingly, the same study observed EM48-positive aggregates after transplantation of HD iPSCs into mouse brains after ~9 months in vivo (Jeon et al., 2012), suggesting that cellular age may be a key factor in identifying aggregates in human HD iPSCs. Curiously, aggregates in iPSCs generated in HD models from other species have been observed in culture. For example, iPSC lines have been generated from skin cells obtained from transgenic rhesus monkeys expressing Htt exon 1 (Chan et al., 2010) and from R6/2 mouse tissues (Castiglioni et al., 2011). Interestingly, in both models, mHtt-specific aggregates were not detectable in pluripotent cells, but were seen only after the cells differentiated into neurons.

Finally, other phenotypes in iPSCs from mouse models have been observed. In the R6/2 iPSC lines, transcriptional dysregulation—a hallmark of HD—was observed for genes involved in the cholesterol and lysosomal pathways (Castiglioni et al., 2011). Curiously, the expression of several genes linked to HD pathogenesis, such as BDNF, was unaffected in these cells (Castiglioni et al., 2011; Zuccato et al., 2001, 2005).

Applications for elucidating mechanism and drug discovery

iPSC technology allows cells obtained directly from patients with HD to be propagated indefinitely and differentiate into the susceptible neuronal subtypes (An et al., 2012; The HD iPSC, 2012), offering the promise of discovering unique, human-specific effects of polyglutamine-expanded Htt that cannot be revealed by rodent models. A human model may eventually reveal how mHtt affects the molecular changes that lead to alterations in behavioral, cognitive, and motor impairments characteristic of patients with HD. iPSCs carrying mHtt may also form the basis for improved high-throughput drug screening, bioinformatics, and global gene-expression analyses. They have been proposed as potentially powerful diagnostic tools for patient stratification in clinical trials and personalized medicine approaches. For example, a potential therapy might be tested first in iPSCs from a patient to determine whether they respond to the compound and should therefore be included in a clinical trial. If the therapy becomes an approved drug, the same approach could be used to determine who should receive it, thereby avoiding potentially harmful side effects in patients who don't stand to benefit.

The next challenge will be to employ HD iPSCs to screen for drugs that attenuate mHtt-induced pathology. Exposure to IGF-1 and gentamicin has been shown to restore normal glutamatergic synapse numbers in iPSCs derived from Rett's syndrome patients (Marchetto et al., 2010), and high-dose BDNF can increase cell-survival rates in HD iPSCs (The HD iPSC, 2012). Altered RNA metabolism observed in ALS iPSCs can be attenuated by exposure to the transcriptional inhibitor anacardiac acid in differentiated motor neurons generated from ALS patients (Egawa et al., 2012). Exposure of iPSCs from patients with familial dysautonomia to kinetin led to reduced expression of the aberrantly spliced form of the IκB kinase complex-associated protein (Lee et al., 2009). These studies suggest that iPSCs can be used to find small molecules that are active in primary human cells and can reverse phenotypes relevant to a given disease process. The critical unanswered question is whether drugs discovered this way will be more successful in clinical trials than the preclinical model systems that have been used previously. Importantly, the next steps en route to developing iPSCs as a screening platform will necessarily involve establishing the best methodologies to differentiate large numbers of cells with minimal variability, and to devise sensitive, high-throughput approaches to detect relevant phenotypic changes. iPSCs may be well-suited to identify reversible phenotypes that appear early in the course of a disease and at a time when pharmacological intervention might be feasible and effective, long before their devastating late-stage consequences take hold.

The next steps: early phenotypes in HD iPSCs

For future efforts involving iPSC-based systems to model HD, it may be useful to focus on changes in HD iPSCs that occur in patients well before cell death or the onset of symptoms. For example, alterations in gene transcription are present early on before symptoms of HD begin (Cha, 2007), and some are thought to predate symptoms. Postmortem *in situ* hybridization studies of presymptomatic or early-HD human tissue helped to establish the Vonsattel grading system, in which grade 0 denotes no detectable abnormality, grade 1 suggests only microscopic changes such as gliosis of the caudate and putamen, while grade 3 indicates more severe neuropathology (Vonsattel and DiFiglia, 1998). At early stages, there was a significant reduction in the expression levels of enkephalin and substance-P mRNAs. Similarly, D1 and D2 receptor expression was significantly decreased in early-stage HD tissue without degeneration or apparent cell loss (Augood et al., 1997). In a comprehensive gene-expression study, principal-component analysis of microarray data from blood samples of both early and late presymptomatic individuals revealed distinct gene- and HD-specific clustering patterns, suggesting that significant transcriptional changes were present early on. Further, the group identified a cohort of 12 biomarker genes that could distinguish the stages of disease progression from very early to advanced HD (Borovecki et al., 2005).

In R6/2 mice, postnatal MSNs had abnormally large NMDA currents and current densities just 2 weeks after birth, suggesting the presence of aberrant synaptic function early in development (Starling et al., 2005) that could lead to excitotoxicity, mitochondrial damage and cell death (Bano et al., 2011). There is also evidence for bioenergetic changes caused by mHtt before HD symptoms start. For example, asymptomatic R6/2 mice as young as 4 weeks old have been shown to display increased levels of phosphocreatine and creatine that precede a decrease in ATP levels in the striatum and cortex (Mochel et al., 2012). Interestingly, decreased glucose uptake was observed in the cortex and striatum of presymptomatic HD patients, suggesting that altered metabolic changes take place in the brain long before symptoms begin (Ciarmiello et al., 2006). This study also reported a substantial loss in the volume of white matter in the brains of presymptomatic HD carriers compared to controls (Ciarmiello et al., 2006), supporting the idea that cell loss and alterations in brain structure are occurring well before disease onset. Other reports revealed changes in cellular trafficking as well as significantly altered staining patterns for dynamin and pascin1 in the cortex of presymptomatic HD brains, suggesting that axonal transport is impeded (DiProspero et al., 2004). This already long list of molecular changes is growing. Uncovering such phenotypes in iPSCs, and adapting these cells for use in assays to test therapeutics, may be key to finding drugs that can offset HD at its earliest stages.

In addition, it may be that improved methods to generate *bona fide* MSNs will be needed to identify phenotypes associated with the early-disease stage of HD. Thus far, investigations of HD-related phenotypes in human HD iPS cells has used methodologies that produce DARPP-32-positive cells or cell types that exhibit action potentials with features characteristic of immature cells (The HD iPSC, 2012), and it is not clear how similar these iPSC-derived cells are to native MSNs in the human brain. Investigations of HD phenotypes should be performed in cultures where comparison studies of human fetal or newborn MSN phenotypes and their electrophysiological properties were used to gauge the extent of development and maturation of iPSC-derived MSNs, as recently reported in ES cells (Carri et al., 2013).

Stem cells as therapeutics: transplantation

iPSCs and ESCs are not just invaluable tools for modeling HD *in vitro*, but also hold promise as cell-replacement therapies for treating HD. Some of the first human cell-implantation studies used fetal neural tissues that were grafted into the striatum of HD patients (Bachoud-Levi et al., 2000; Sramka et al., 1992). These efforts showed modest results: about half of the patients showed some clinical improvement for ~2 years post-surgery, but deterioration resumed thereafter (Bachoud-Levi et al., 2006). Although the grafts seemed to integrate, they did not protect the surrounding tissues from degeneration (Strubing et al., 1995). Fetal striatal neurons have proved to be the most efficacious cell type for use in transplantation studies (Evans et al., 2012); however, the most intractable problem associated with fetal stem cells is their source: in addition to the controversy that surrounds the use of brain tissues from aborted fetuses, the availability of high-quality material is limited (Evans et al., 2012; Dunnett and Rosser, 2007). Thus, a more reliable source of pluripotent cells will be required to replace dying or damaged striatal tissues in humans.

Studies in rodent HD models have yielded modest but promising results using NPCs or ESCs (Dunnett and Rosser, 2007; Kim et al., 2008) that, while less mature than fetal striatal tissues, are more easily obtainable. Adult NPCs isolated from the SVZ and injected into QA-lesioned rats not only can survive but also can migrate and successfully differentiate in the striatum, and most importantly, can significantly improve motor impairment (Vazey et al., 2006). A similar study was performed using hESCs differentiated into NPCs and injected into the striatum of

QA-lesioned animals. Like NPCs, these hESCs differentiated into mature cell types that rescued behavioral deficits (Song et al., 2007). However, some of the most exciting studies have employed stem cells genetically engineered to deliver trophic factors to support the survival of existing cells and to induce new cell growth. An impressive study engineered mouse NPCs (mNPCs) to express glial-derived neurotrophic factor (GDNF). When these GDNF-expressing mNPCs were injected into the striatum of presymptomatic mice containing 82 polyglutamine repeats (N171-82Q), the GDNF-producing cells significantly reduced weight gain, latency to fall in the rotarod test and neurodegeneration within the striatum (Ebert et al., 2010).

Injecting ESCs directly into patients could be potentially harmful since these cells have the ability to self-renew, proliferate, migrate and become tumorigenic (Kulbatski, 2010). It is well-established that ESCs can also accumulate chromosomal abnormalities in culture over time, raising the risk of eventual transformation (Ross et al., 2011). Further, a common measure of pluripotency of an ESC or iPSC line is the formation of teratomas after injection into immunocompromised mice (Ross et al., 2011; Takahashi et al., 2007). This potential for unregulated growth must be addressed prior to their application as cell-based therapies in humans.

One way to circumvent this is to differentiate ESCs and iPSCs into a mature non-dividing state prior to transplantation. Noggin-treated hESCs injected into the QA-lesioned rat striatum were found to migrate, mature into MAP-2-positive cells and survive for up to 8 weeks post-transplantation, but did not form tumors; in contrast, noggin-untreated hESCs were hyperplasic and migrated outside of the transplantation site (Vazey et al., 2010). However, a different study revealed that ESCs, which were differentiated into mature DARPP-32-positive cells, eventually formed highly proliferative teratomas, but only 3–5 months after injection (Aubry et al., 2008). Thus, following the graft site throughout the life of injected animals is crucial to determine the long-term consequences of stem cell-based therapies. A recent study used a different striatal protocol to generate DARPP-32-positive GABAergic neurons that were then transplanted into the striatum of QA-lesioned mice (Ma et al., 2012). The grafts rescued motor deficits in the animals; however, it was unclear how long the grafts remained intact after transplantation or if they eventually formed tumors. Although ESCs hold great promise as clinical HD treatments, better differentiation protocols are needed that will select for the cell type least likely to exhibit hyperplastic overgrowth and tumorigenesis, while relieving the symptoms of disease.

Another promising cell type is the human multipotent stromal cell/mesenchymal stem cell (MSC) derived from bone marrow, thought to promote repair by releasing trophic cytokines and chemokines. Injection of hMSCs into a mouse model containing 82 polyglutamine repeats (N171-82Q) increased neurogenesis in the striatum and prevented striatal loss (Snyder et al., 2010), in part by inducing expression of the neurotrophins FGF-2 and VEGF in the striatum. Interestingly, the majority of injected hMSCs did not survive for more than 5 days within the striatum, whereas the neuroprotective changes were observed up to 30 days post-injection; thus, these cells probably exerted their beneficial effects mainly by stimulating surrounding striatal cells rather than by replacing dying neurons (Snyder et al., 2010). In a separate study, MSCs engineered to express BDNF were injected into the striatum of YAC-128 mice, which led to marked improvements in motor function and a reduction in striatal cell loss (Dey et al., 2010). These effects may be mediated by MSC-induced secretion of trophic factors and cytokines that induce angiogenesis and prevent apoptosis by promoting the generation of new neurons (Joyce et al., 2010). MSC-based therapies may circumvent overgrowth or abnormal proliferation in the brain caused by injection of neuronal progenitors. Another interesting approach has been to employ MSCs to deliver shRNAs that can stimulate the degradation of mHtt mRNAs (Olson et al., 2012). Although this approach has not yet been used in HD mouse models *in vivo*, it has been used to successfully knock down the expression of mHtt in neuron-like cell lines (Olson et al., 2012). Even if MSCs are not able to replace dead or dying cells, their ability to release factors that can support neurons and/or glia or block the effects of potentially harmful proteins is tantalizing. It will be interesting to see how these gene-deliverable, cell-based therapies develop.

Concluding remarks

Modeling HD using iPSCs is well underway. Because iPSCs come directly from affected patients, they are likely to represent the most molecularly and genetically precise cell model of the disease. Thus, using iPSCs may bridge the gaps in knowledge gleaned from rodent models. Furthermore, this technology holds great promise for elucidating the mechanisms of disease progression, in light of the growing body of evidence showing that many molecular changes take place in cells well before the onset of clinical symptoms, such as during development and neurogenesis. iPSCs may therefore be key to helping unravel the underlying pathogenic mechanisms of mHtt early in its course that eventually culminate in the symptoms of HD. Finally, these cells offer great hope as a potentially invaluable drug-screening platform to uncover and devise therapies targeting the disease course well before cell death and clinical symptoms emerge, which could lead to greater success in clinical trials.

Acknowledgments

This work was supported by U24 NS078370-01 and from CIRM fellowship TG2-01160 with additional support from the Taube-Koret Center, the Hellman Family Foundation, and the Roddenberry Stem Cell Center. We would like to thank Anna-Lisa Lucido, Gary Howard, John Carroll, Gaia Skibinski , Kelly Haston, Sami Barmada, Ian Kratter, Matt Campioni and Cory Nicholas for help and comments on the manuscript. We also thank members of the Finkbeiner lab.

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