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Modeling Niemann Pick type C1 using human embryonic and induced pluripotent stem cells

M. Paulina Ordonez^{1,2,*} and John W. Steele¹

¹Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, CA 92037

²Department of Pediatric Gastroenterology, Hepatology, and Nutrition, University of California, San Diego, La Jolla, CA 92037

Abstract

Data generated in Niemann Pick type C1 (NPC1) human embryonic and human induced pluripotent stem cell derived neurons complement on-going studies in animal models and provide the first example, in disease-relevant human cells, of processes that underlie preferential neuronal defects in a NPC1. Our work and that of other investigators in human neurons derived from stem cells highlight the importance of performing rigorous mechanistic studies in relevant cell types to guide drug discovery and therapeutic development, alongside of existing animal models. Through the use of human stem cell-derived models of disease, we can identify and discover or repurpose drugs that revert early events that lead to neuronal failure in NPC1. Together with the study of disease pathogenesis and efficacy of therapies in animal models, these strategies will fulfill the promise of stem cell technology in the development of new treatments for human diseases.

Keywords

Niemann Pick type C1; human embryonic stem cells; human induced pluripotent stem cells; neurodegeneration; models of disease; drug screening; autophagy

2. Background and Significance

Niemann Pick disease type C1 (NPC1) is a rare but devastating lysosomal storage disease that leads to severe neuronal degeneration, imposing a burden on patients, families, caretakers, and society that is disproportionate to its relative infrequency. NPC1 is caused by loss of function mutations of the cholesterol transporter NPC1, a transmembrane protein that resides on the membrane of the late endosomal/lysosomal compartment (Liscum, 2006; Sturley et al, 2004). The disease is clinically and genetically heterogeneous, with more than 350 described mutations that result in severe loss of function of NPC1 (McKay Bounford

*Please direct correspondence to Paulina Ordonez at pordonez@ucsd.edu.

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and Gissen, 2014). The resulting cellular phenotype is the significant accumulation of unesterified cholesterol and other lipids, particularly sphingolipids and gangliosides, in the lysosome. An interesting feature of the disease is that mutations of NPC1 lead to selective neuronal failure, despite the ubiquitous nature of the lipid trafficking pathway that they affect (Ordóñez, 2012).

The clinical spectrum of NPC1 ranges from a neonatal rapidly fatal disorder to an adult-onset chronic neurodegenerative disease. In its most aggressive form, affected children present as toddlers and die during childhood or early adolescence. Although hepatomegaly usually precedes onset of neurological symptoms, liver involvement typically does not progress to liver failure. Spleen and bone marrow abnormalities can also occur but their contribution to morbidity and mortality are also small in comparison to central nervous system pathology and dysfunction. Neurodegeneration and neuronal failure are the primary contributors to lethality in NPC1, which has a variable clinical phenotype (Klein et al, 2014; Vanier, 2010). Patients with classic childhood onset usually appear normal for 1 or 2 years, with symptoms appearing between 2 and 4 years of age. These patients gradually develop ataxia, dystonia, dysphagia and loss of previously learned speech, reflective of the preferential susceptibility of cerebellar Purkinje neurons to mutations of NPC1. Spasticity is striking and seizures, particularly myoclonic jerks, are common. Other features include vertical supranuclear gaze palsy, dementia, and psychiatric manifestations. In the childhood-onset form, death usually occurs between age 5 to 15 years. More recently, late-onset disease has been increasingly recognized as the biochemical diagnosis of NPC1 is more widely applied in adult neurology clinics (Imrie et al, 2006). Evaluation of common NPC1 variants extracted from large exome sequencing data sets suggests that a late-onset NPC1 phenotype may have a markedly higher incidence than that described for classic NPC1 disease (Wassif et al, 2015).

Interestingly, neurodegeneration caused by mutations of NPC1 shares many clinical and pathologic features with Alzheimer's disease (AD) (Liu et al, 2010; Malnar et al, 2014). Indeed neurofibrillary tangles, a lesion associated with AD and related tauopathies, is also typical of NPC1, especially in cases with a prolonged course of disease (Mattson et al; Maulik et al, 2012). Other histopathological similarities between NPC1 and AD include axonal spheroids, β -amyloid deposition, and dystrophic neurites. Furthermore, a specific polymorphism of apolipoprotein E (apoE4), the main extracellular carrier of cholesterol in the brain, is the strongest known risk factor for the development of sporadic AD, and carries a worse prognosis in NPC1 (Fu et al, 2012; Vance et al, 2005). Added to these similarities are findings that some neuronal populations in NPC1 develop abnormalities of endosomes resembling those seen at the earliest stages of AD, and that aberrant cholesterol trafficking is associated with the potentiation of toxic AD-like processing of the amyloid precursor protein (APP) (Borbon and Erickson, 2011; Grimm et al, 2005). Therefore, NPC1 and AD may share common mechanism(s) related to onset or progression of disease in neurons, and strategies that reverse neuronal dysfunction in NPC1 could potentially be extrapolated to develop therapies for AD, a disease that affects over 5 million people in the U.S. alone with significant human and economic burdens (Brookmeyer et al, 2007).

3. Current NPC1 Model Systems

There are currently no FDA-approved therapies for the treatment of NPC1 and efforts to develop new therapeutic strategies to combat neurological deficits in NPC1 are urgently needed. Much of the mechanistic work to date has utilized mouse models or cultured non-neuronal cells (e.g. patient fibroblasts), and these studies predominantly focus on a direct contribution of lysosomal lipid accumulation to neuronal failure and death. Two commonly used and independently derived mutant mouse colonies have played an essential role in delineating the biochemical basis of NPC1 and have been widely used for disease modeling. One is a BALB/c mouse presenting clinical and biochemical features of NPC1 (Pentchev et al, 1984); the other is the C57BL/Ks mouse characterized as a sphingomyelinosis because of attenuated sphingomyelinase activity and excess sphingomyelin accumulation (Miyawaki et al, 1986). More recently, a knock-in mouse model expressing the most common human mutation of NPC1 (I1061T) was generated and characterized (Praggastis et al, 2015). Compared with the null NPC1 mouse, this model displays a less severe, delayed form of NPC1 disease with respect to weight loss, decreased motor coordination, cerebellar Purkinje neuron death, lipid storage, and premature death, and this model it is suitable to test the effect of proteostatic therapeutic interventions (Gelsthorpe et al, 2008). In addition, a novel mouse model carrying a single nucleotide change (D1005G-Npc1) that is comparable to commonly observed human mutations has been reported. Analysis of this mouse model revealed a more slowly developing phenotype than in mice with null mutations, which may offer advantages to model late-onset, slowly progressive forms of NPC1 that comprise a large number of human cases (Maue et al, 2012).

Although NPC1 may have a common fundamental role in lipid trafficking in mice and humans, it is unclear whether the pathological consequences of NPC1 dysfunction are the same for both species due to biochemical and physiological differences between mouse and human neurons. Specifically in NPC1 mouse models, tau and β -amyloid proteins do not readily form neurofibrillary tangles or plaques, respectively, which do form in human mutant NPC1 neurons and may contribute to neurodegeneration (Walkley and Suzuki, 2004). In addition, mice lack the ApoE genotypes found in human subjects, which can also influence disease course in humans. Neurons in mammalian models of NPC1 exhibit many cellular changes that are remarkably similar to those in humans such as endolysosomal storage, Golgi fragmentation and cerebellar/cortical neurodegeneration (Sarna et al, 2003; Lopez et al, 2011). However, neuroaxonal pathology and growth of ectopic dendrites are not obvious in NPC1 mice. These changes, typical of human NPC1 pathology, are more accurately replicated in NPC1 cat pyramidal neurons. For these reasons and due to advantages attributable to scale, the naturally occurring cat model of NPC1 may be better suited to test proposed therapies for NPC1 (Brown et al, 1994; Somers et al, 2003). Although these animal models are important tools for understanding the disease biology and for in vivo therapeutic development, the use of animal models is prohibitively expensive and frequently inadequate for high throughput drug discovery programs (Lopez and Scott, 2013).

4. Drug Discovery and Clinical Development

Strategies using animal models of NPC1 have not yet revealed how mutations in the *NPC1* gene cause neuronal failure in humans and have not yielded an FDA-approved therapy for NPC1 disease. However, animal models have been critical to the development of current therapeutic efforts, leading to the approval of misglustat in Europe, and setting the foundation for current Phase I and Phase 2/3 trials using cyclodextrins. Substrate reduction therapy with miglustat, an iminosugar that inhibits glycosphingolipid synthesis, was proposed to treat NPC1 based on evidence of slower disease progression and prolonged survival in animal models (Zervas et al, 2001). Miglustat has shown limited efficacy based on a controlled study and a series of case reports (Patterson et al, 2009; Chien et al, 2013) and has not yet been approved by the FDA to treat NPC1.

Another approach under consideration is the use of cyclodextrins, a family of cyclic oligosaccharides that have been shown to solubilize cholesterol from NPC1 cells. Although 2-hydroxypropyl- β -cyclodextrin (HP β CD) appears to reduce cholesterol accumulation and prolong survival in NPC1 animal models (Liu et al, 2008 and Davidson et al, 2009), the need for intrathecal infusion and adverse side effects (i.e. accelerated hearing loss; ototoxicity) highlights the necessity for improved therapeutics and new therapeutic targets. A novel and recent therapeutic approach for NPC1 involves histone deacetylase (HDAC) inhibitors, a family of small molecule compounds traditionally used for antineoplastic purposes. HDAC inhibitors, such as Vorinostat (SAHA), have been shown to stabilize NPC1 protein and decrease cholesterol accumulation in NPC1 fibroblasts (Pipalia et al, 2011). HDAC inhibitors presumptively work by post-translational stabilization of the NPC1 protein, allowing it to be transported out of the endoplasmic reticulum (Maceyka et al, 2013). Interestingly, these compounds are effective only for some, but not all, mutations of *NPC1*, and toxicity is still an important consideration, especially given the promiscuity of these epigenetic modifiers and a fundamental lack of understanding of mechanism of action in disease-susceptible cell types. Further mechanistic understanding in disease models harboring HDAC-amenable NPC1 mutations is necessary to develop a better understanding of how HDAC inhibitors function in NPC1 disease.

Clinical trials are currently being planned for Vorinostat and cyclodextrin. Vorinostat is an FDA-approved HDAC inhibitor labeled for the treatment of cutaneous T-cell lymphoma and is known to cross the blood brain barrier. Patient recruitment is undergoing for a Phase I, non-randomized, open-label, single-center study using Vorinostat for the repurposing treatment of NPC1. The rare disease drug development company *Vtesse* is also planning a Phase 2b/3 clinical trial using a proprietary form of HP β CD based on promising preclinical and Phase 1 results (Ottinger et al, 2014). The recruitment goal for this trial is 51 juvenile patients, which underscores the enormous challenges of performing clinical trials for very rare diseases in regards to recruiting patients, defining and measuring outcomes, and monitoring for potential adverse events of candidate compounds. In this setting, stem cell-derived platforms of human neurons for drug testing, especially when screening for drug-amenable mutations (e.g. with HDAC inhibitors), can greatly accelerate therapeutic development for NPC1 and related neurodegenerative diseases, and has the potential to improve the likelihood of successful treatment by targeting amenable patient populations.

Fast and cost-effective methods of hiPSC generation and neuronal differentiation make it possible to generate neuronal lines from multiple patients and patient-derived cells saved in repositories, providing expanded sets for drug screening. Additionally, current genome editing technologies allow for rapid development and characterization of isogenic hiPSC lines for screening of known *NPC1* mutations. Careful selection and rigorous analysis of disease phenotypes in human neurons, in combination with animal models, has the capacity to advance drug discovery and development efforts at an unprecedented pace.

Drug screening efforts in non-neuronal human cells (e.g. patient fibroblasts) currently focus on ameliorating accumulation of cholesterol in NPC1, and this strategy may overlook potential targets in neurons, which are highly specific post-mitotic cells. In addition, recent attention has been brought to alternative pathways that may contribute to slow cholesterol release in the absence of NPC1 function (Ouimet et al, 2011; Lange et al, 2012). In this context, lysosomal cholesterol accumulation, or its absence in sites outside of the lysosome, may not be the primary mechanism leading to neuronal failure in NPC1. The significant gap in our knowledge of NPC1 can be attributed in part to two major factors: 1) prior lack of a facile human neuronal model of NPC1 that has forced the field to rely mainly on extrapolation of findings from animal studies to model human disease, and, 2) lack of human neuronal cultures suitable for high throughput drug screening programs. Therefore, mechanistic studies and development of additional effective therapeutic candidates is likely to require analysis of cellular phenotypes in human NPC1 neurons, and these studies will be supported by on-going efforts in the animal models described above.

5. Considerations in the Modeling of NPC1 with Human Stem Cells

The development and accessibility of human embryonic stem cell (hESC), human induced pluripotent stem cell (hiPSC), and genome editing technologies offer the unique opportunity to study NPC1 in disease-relevant human cell types, to explore the contribution of early pathogenic phenotypes to NPC1 disease onset and progression, and to develop disease-relevant models for high throughput drug screening efforts. The use of live human NPC1 neuronal cultures is crucial to understand the early events that trigger neuronal failure in NPC1 and bypass the obvious difficulties associated with obtaining viable human brain tissue for research purposes. Since neuronal dysfunction must precede neuronal death, identifying early events affecting neuronal health is key for the development of new therapies that may prevent or slow the progression of neurodegeneration in NPC1 and related disorders before significant cell death has occurred.

Two independent groups have reported developing human neurons derived from stem cells with decreased function of NPC1 (Ordóñez et al, 2012; Maetzel et al, 2014). Human stem cell-derived neurons have typical morphology, express lineage specific markers, and are electrophysiologically active. NPC1 stem cell-derived neurons replicate hallmark NPC1 phenotypes such as accumulation of cholesterol and presence of large acidic vesicles consistent with aberrant lysosomes. However, modeling NPC1 neurodegeneration with hESC and hiPSC is not free of challenges. Whereas generation of purified populations of neurons from control and mutant stem cells has become a relatively straightforward procedure (Pang et al, 2011; Yuan et al, 2011), directed differentiation to the most sensitive

subpopulation of cerebellar Purkinje neurons remains difficult. Generation of cerebellar neurons from hESC and iPSC has been reported (Erceg et al, 2012; Wang et al, 2015), however this has proven to be resource-heavy and low throughput in its current form. Interestingly, phenotypes typical of NPC1, such as cholesterol accumulation and lysosomal dysfunction have been documented in non-Purkinje iPSC-derived neurons, providing proof-of-principle that existing hESC and iPSC-derived models of NPC1 replicate many features of the disease. This is consistent with the ubiquitous nature of the cholesterol trafficking defects induced by mutations of NPC1, and with the fact that cortical and thalamic involvement are also important features of human NPC1 disease progression. Whereas these somewhat heterogeneous populations of stem cell-derived neurons do exhibit differences in survival over time (Maetzel et al, 2014), probing the contribution of NPC1 mutations to viability of the most susceptible population of cerebellar neurons remains an important goal that can provide essential mechanistic data and offer an attractive platform for drug screening programs.

Another issue in the modeling of NPC1 that can be further clarified using a stem cell platform is the contribution of non-neuronal cells (i.e. glia) to disease pathogenesis. Whereas it is generally well established that neuronal failure in NPC1 is a cell autonomous event (Ko et al, 2005; Lopez and Scott, 2013), it is possible that mutations of NPC1 in glia may disrupt cholesterol trafficking between glial and neuronal cells (Erickson, 2013). Experiments designed to address this question have thus far used co-culturing of NPC1 mouse neurons with wild type glia that do not share the same genetic background. The possibility of generating isogenic glial cells from stem cells opens an avenue to extrapolate these studies to a human system, but importantly, to utilize neuronal and glial cells with the same genetic makeup. This approach greatly simplifies assessment of the direct contribution of NPC1 mutations in glia to neuronal health by cholesterol dependent and independent mechanisms. Further studies using a combined approach with existing animal models will be essential to confirm findings and to advance the mechanistic knowledge obtained from human stem cell models to preclinical and clinical stages.

6. Mechanistic Studies of NPC1 Obtained Using hESC and iPSC- Derived Neurons

6.1. NPC1 hESC-derived model

The importance of modeling NPC1 in human neurons is highlighted by our discovery of a cholesterol-independent mechanism of neuronal failure in hESC-derived neurons engineered to mimic loss of function of NPC1 (Ordonez et al, 2012). Our group was the first to report a human stem cell derived model of NPC1 generated by shRNA-mediated knock down (KD) of *NPC1* in the hESC line HuES9. *NPC1*-KD hESC-derived neurons replicate the key pathology seen in NPC1, specifically showing accumulation of cholesterol and enlarged LAMP2-immunoreactive acidic vesicles consistent with late endosomes/lysosomes. These changes are similar to those observed with the compound U18666A, an inhibitor of intracellular cholesterol trafficking that mimics cellular phenotypes of NPC1 (Cenedella, 2009). Interestingly, we found that human *NPC1*-KD neurons, or neurons treated with U18666A have both a strong spontaneous activation of autophagy and impaired clearance of

autophagic substrates such as LC3-II and p62 (Ordonez et al, 2012). The autophagy pathway is a ubiquitous pathway that is required by neurons for the turnover of long-lived proteins, protein aggregates, and damaged organelles (Menzies et al, 2015). Induction of autophagy would therefore be expected to increase turnover of various cellular products, including mitochondria, due to its essential role in neuronal maintenance and homeostasis. However, using high resolution imaging of fluorescently labeled mitochondria, we noted an accumulation of mitochondrial fragments in *NPC1*-KD neurons as well as in neurons treated with U18666A (Ordonez et al, 2012). Our observations suggest that aberrant autophagy is the primary defect underlying accumulation of mitochondrial fragments in NPC1 KD neurons. Indeed, although autophagy is traditionally thought to be mitoprotective, induction of autophagy does not rescue, but rather aggravates mitochondrial fragmentation in NPC1 neurons. This is further supported by our observation that autophagy flow is relatively blocked in NPC1 KD neurons (Ordonez et al, 2012). Accumulation of mitochondrial fragments appears to be a neuron-specific phenotype that is not observed in NPC1 patient fibroblasts at baseline. This finding may be reflective of the inability of post-mitotic cells (e.g. neurons) to dilute the load of mitochondrial fragments by cell division. Normal autophagy is essential to neuronal homeostasis (Lim and Yue, 2015), and autophagy dysfunction is a proposed contributor to various neurodegenerative disorders (Tanaka and Matsuda, 2014). Because mature neurons are reliant on efficient autophagy, this pathway is an attractive candidate to explain why neurons are particularly susceptible to mutations of NPC1. Prior studies have implicated autophagy activation in the pathogenesis of NPC1 in mouse neurons and postmortem human brain slices (Ishibashi et al, 2009; Ko et al, 2005), however, the dynamics of autophagy had not been analyzed in live human neurons prior to our initial study in hESC-derived neurons. More recently, cellular reprogramming technology has made it possible to revert adult somatic cells to a pluripotent state (iPSCs) that is induced by activation of key transcription factors (Takahashi et al, 2007). Human iPSC are very similar to hESC and make disease- modeling possible without the potential off-target effects of genetic manipulation or the ethical concerns and regulations inherent to hESC-derived models.

6.2. NPC1 hiPSC-derived model

Abnormalities of autophagy have also been reported in NPC1 hiPSC-derived neurons. Jaenisch et al generated NPC1 hiPSC lines from patient fibroblasts using CRE-excisable lentiviral vectors, and differentiated these lines to successfully generate neuronal cultures (Maetzel et al, 2014). This hiPSC-derived platform has been utilized to perform elegant studies of autophagy activation and its contribution to disease pathogenesis. These investigators previously showed evidence in non-neuronal NPC1 cells that a defect in amphisome maturation, caused by a deficient formation of SNARE complexes, is thought to produce a relative delay in autophagic progression (Sarkar et al, 2013). In contrast to our hESC data, work by these investigators suggest that induction of autophagy by itself or in combination with low dose HP β CD can rescue autophagic progression as measured by clearance of LC3-II and p62 in hiPSC-derived neurons. This group therefore advocates for combined therapy with small molecules that induce autophagy and HP β CD to mobilize lysosomal cholesterol as a potential therapy for NPC1. However, our mechanistic data showing autophagy-dependent mitochondrial fragmentation in human hESC-derived NPC1

neurons raises a complication to the use of autophagy induction as a therapeutic approach. The proposed combination therapy with cyclodextrin may enhance autophagic flow by decreasing the burden of lysosomal cholesterol, however cyclodextrin therapy is not ideal for reasons listed previously.

Organelle-specific autophagy is now considered to be a more selective process than originally thought (Baumann, 2015; Sica et al, 2015), and therefore clearance of traditional markers (LC3, p62) may not accurately reflect mitochondrial turnover by autophagy. Jaenisch et al recently showed that NPC1 hPSC-derived neurons exhibit decreased viability over extended periods in culture as compared to control neurons. In our hands, NPC1 hPSC-derived neurons are also more sensitive to the mitochondrial depolarizing effects of the mitochondrial uncoupler FCCP (unpublished data). Interestingly, the Jaenisch group found that viability of NPC1 hPSC-derived neurons can be rescued by autophagy induction. This may be consistent with an effect of autophagy on the mobilization of lysosomal cholesterol (Liu and Czaja, 2013). In this context, induction of autophagy may have a short-term effect of enhanced cell viability, but a long-term effect of slow neuronal decay due to progressive mitochondrial dysfunction. A possible solution to this problem is to identify strategies that specifically modulate mitophagy, while preserving the neuroprotective function of bulk autophagy. These and other therapeutic strategies, as well as any potential side effects, remain to be fully tested in human NPC1 neurons. As a relatively new field, the combination of highly accessible genome editing (La Russa and Qi, 2015) and human iPSC technologies will provide a much needed and rapid path forward for the rapid development and characterization of isogenic hPSC lines to study disease-associated *NPC1* mutations and for the development of mutation-targeted drug discovery programs in human neurons.

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