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Concise Review: Induced Pluripotent Stem Cell-Based Drug Discovery for Mitochondrial Disease

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ABSTRACT

High attrition rates and loss of capital plague the drug discovery process. This is particularly evident for mitochondrial disease that typically involves neurological manifestations and is caused by nuclear or mitochondrial DNA defects. This group of heterogeneous disorders is difficult to target because of the variability of the symptoms among individual patients and the lack of viable modeling systems. The use of induced pluripotent stem cells (iPSCs) might significantly improve the search for effective therapies for mitochondrial disease. iPSCs can be used to generate patient-specific neural cell models in which innovative compounds can be identified or validated. Here we discuss the promises and challenges of iPSC-based drug discovery for mitochondrial disease with a specific focus on neurological conditions. We anticipate that a proper use of the potent iPSC technology will provide critical support for the development of innovative therapies against these untreatable and detrimental disorders. STEM CELLS *2017;35:1655–1662*

SIGNIFICANCE STATEMENT

Mitochondrial disease is an untreatable condition caused by mutations in nuclear or mitochondrial DNA. This review describes the application of patient-derived induced pluripotent stem cells (iPSCs) in the drug discovery process of mitochondrial disease. iPSCs allow the development of innovative and effective cellular model systems in a personalized approach. Their use may significantly benefit the search for treatments against debilitating mitochondrial disease.

TARGET-BASED AND PHENOTYPE-BASED DRUG DISCOVERY

Drug discovery is a long and costly process. Pursuing the most efficient strategy is therefore of utmost importance. The conventional target-centered drug discovery system has been the focus of the pharmaceutical industry since the advent of modern genome and molecular biology [1]. This approach allows the establishment of controlled and optimized high-throughput (HT) compound screenings against a defined target. Unfortunately, druggable targets considered to be relevant for disease pathogenesis are not always available. Targets that can be efficiently modulated in cell-free systems may also not be easily accessible within complex cellular contexts, where compensatory mechanisms and cell type-specific feedback pathways may be in place [2]. The consequences of a target modulation might therefore vary with the epigenetic background (e.g., different cell types, distinct individuals). This can result in drugs with reduced efficacy and with off-target effects that are individual-specific and hard to predict.

In recent years, the downsides of target-centered drug discovery have become evident, as indicated by its attrition rate estimated to be at 96% [1]. This has led to the revival of phenotypic screenings. Phenotypic drug discovery requires the identification of a disease-specific trait (phenotype) that can be modulated within the physiological environment of a cell or organism [2]. The phenotypic readout may be close to an actual bio-molecular target or very far from it. In any case, the underlying target does not need to be known "a priori." Therefore, lengthy and costly target identification and validation are not required before performing the screenings. Phenotypic drug discovery has the advantage of identifying compounds that show an effect in complex biological environments in the absence of negative consequences on cellular or organismal functionality. This requires the presence of a robust disease-relevant phenotype that can be efficiently modulated using a detection

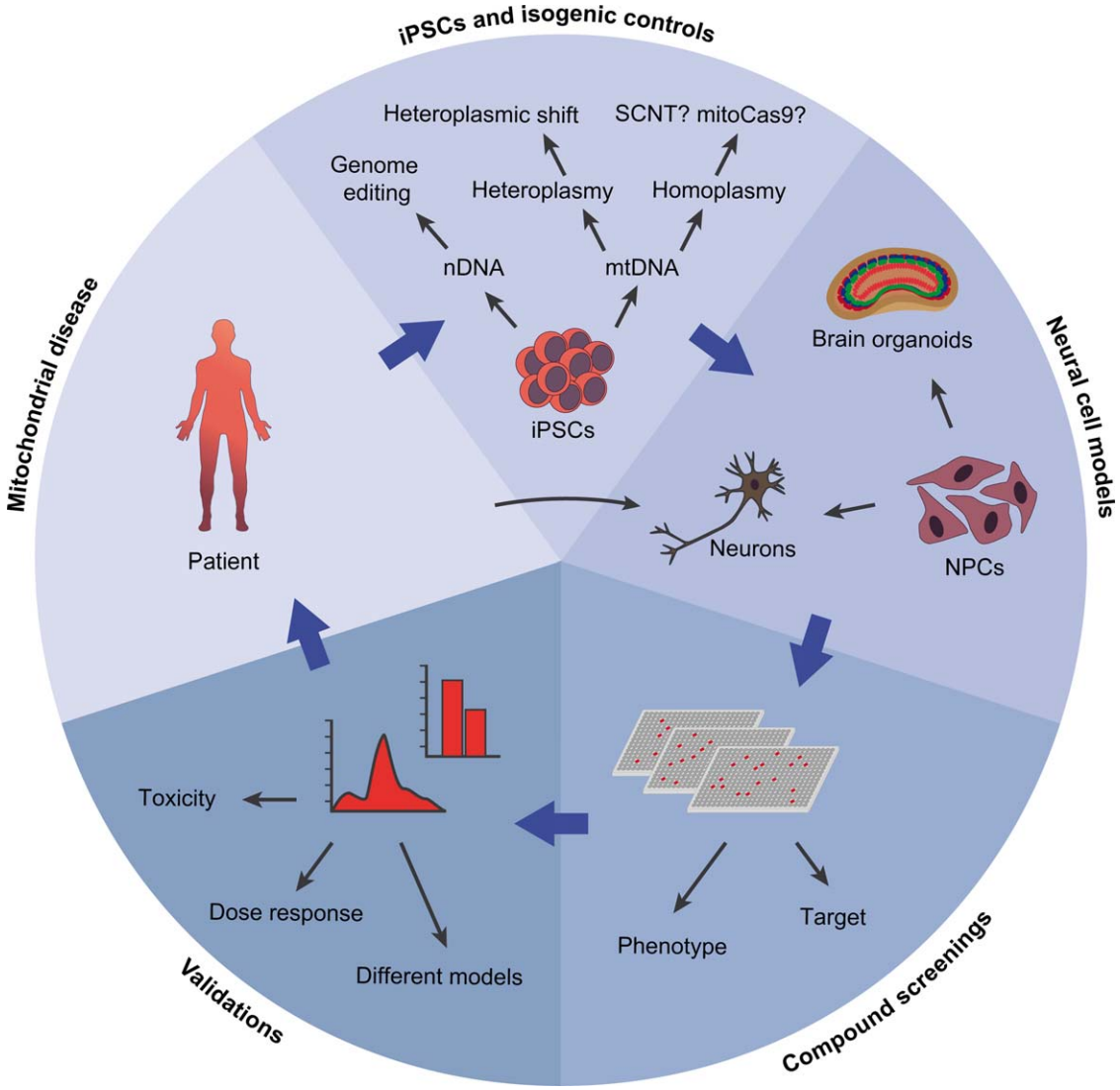

Figure 1. A personalized strategy for drug discovery of neurological mitochondrial disease. Once iPSCs have been derived from mitochondrial patients, isogenic controls can be generated using genome editing technologies. Correcting the mutations is, however, currently very challenging in the case of mtDNA. The choice of an effective neural cell model is critical for enabling the establishment of high-throughput compound screenings. The screenings can be developed based on targets that are already known or on cellular phenotypes that are identified using the iPSC-derived neural cells. After the screenings, several validation steps using various methods and model systems are necessary before being able to propose any compound as a treatment for mitochondrial disease (see text for details). Abbreviations: iPSC, induced pluripotent stem cells; mtDNA, mitochondrial DNA; NPC, neural progenitor cells; nDNA, nuclear DNA; SCNT, somatic cell nuclear transfer.

method that is reliable, quantitative, and amenable to automation.

In the past, phenotype-based assays were limited by the lack of HT techniques, the weakness of disease-associated read-outs, and the scarce access to biological material. This is now radically changed following the discovery of induced pluripotent stem cells (iPSCs). Patient-derived iPSCs can be differentiated into disease-relevant cell types, including those that are commonly not available, such as neurons and glia. Screenings conducted on such differentiated cells could be based on highly significant cellular phenotypes that become apparent within patient-specific disease-relevant cellular contexts [2, 3]. Target-centered screenings can also be performed in iPSC derivatives, where the effects of the target modulation can already be monitored within the correct cellular environment. All these approaches might enable the identification of drug candidates that have a higher chance of success rate and the potential to be applied in a personalized manner (Fig. 1).

Here, we discuss the promises and challenges of iPSC-based drug development for mitochondrial disease and its neurological manifestations.

Mitochondrial Disease

Mitochondrial disease describes a group of disorders caused by defects in components of the mitochondrial respiratory chain (RC) that can be nuclear or mitochondrially encoded [4, 5]. Mitochondria are double membrane organelles that contain their own genome, which is maternally inherited and

present in multiple copies in all nucleated eukaryotic cells. The main function of mitochondria is the generation of energy in the forms of ATP via the RC through the process of oxidative phosphorylation (OXPHOS). The RC consists of five complexes and is embedded in the mitochondrial inner membrane (MIM), which divides the mitochondrial matrix from the intermembrane space (IMS). Complexes I-IV of the RC transfer electrons and expel protons into the IMS thereby generating a proton gradient across the MIM that is known as mitochondrial membrane potential (MMP). The energy stored in this gradient is used by Complex V to produce ATP upon oxygen consumption by allowing the reentry of protons into the mitochondrial matrix [4, 5].

Mitochondrial disease often presents itself with neurological features [6, 7]. This may be due to the high bioenergetic requirements of neuronal cells. Brain energy metabolism relies on complex and delicate neuronal-glia interactions, which may be disrupted by RC defects. Mitochondrial dysfunction may also affect mitochondrial transport and dynamics that are particularly important for the physiology of polarized cells like neurons. Given the importance of mitochondria in calcium handling [8], mitochondrial impairment might have higher detrimental consequences in excitable cells like neurons, which are dependent on tight calcium regulation for their functionality [9].

The genetic cause of mitochondrial disease can reside in either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). In fact, the OXPHOS pathway is under dual control of the two genomes. Over 150 RC genes have been associated with mitochondrial disease. Although individual mutations are rare, mitochondrial disease represents the first cause of inborn error of metabolism in humans, with an overall prevalence of 1 over 4,300 live births in the general population [10].

mtDNA mutations can occur in a homoplasmic state, where all mtDNA molecules carry the same mutation, or heteroplasmic state, where only a proportion of mtDNA molecules carries the mutation. Diseases due to heteroplasmic mutations include mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), neurogenic weakness, ataxia, and retinitis pigmentosa (NARP), and Leigh syndrome (LS), whereas Leber's hereditary optic neuropathy is associated with homoplasmic mtDNA mutations [4].

Cellular dysfunctions and clinical manifestations are believed to occur only after a certain threshold of mutation load has been exceeded. This concept has been recently challenged, as exemplified by the fact that the same level of *MT-ATP6* mutations can cause both mild and severe neurological syndromes [11]. The phenotypic variability cannot be entirely explained by the mtDNA mutation load and has been attributed to the patient-specific nuclear background [12]. The individual match between nDNA and mtDNA should therefore play an important role in the disease pathogenesis of mitochondrial disease.

Challenges for Mitochondrial Disease Drug Discovery

There have been significant breakthroughs in the field of reproductive medicine for mitochondrial disease due to mtDNA mutations. Mitochondrial replacement therapy may prevent the transmission of pathogenic mtDNA mutations [13]. In addition, mouse studies showed that particular heteroplasmic mtDNA mutations can be targeted in germ cells and eliminated using mitochondrial-targeted restriction endonucleases (RE) [14]. These exciting technological advances might provide important strategies allowing women carrying pathogenic mtDNA mutations to have healthy children. Moreover, there have been major advances in dissecting the genetic causes of mitochondrial disease and the respective potential pathophysiological mechanisms [4, 5]. However, these successes failed to translate into improved patient care [15]. Up to now, therapeutic options for mitochondrial patients are mainly limited to supportive measures that do not modify the primary defects [6, 16].

One of the major challenges in drug development of mitochondrial disease is their extreme clinical variability [15]. Similar mtDNA mutations associate with various distinct disorders and the same clinical condition may rise from several RC defects. For example, LS, a progressive encephalopathy with basal ganglia involvement, can be caused by nDNA mutations in Complex I (like *NDUFS4*), nDNA mutations in Complex IV assembly factors (like *SURF1*), or mtDNA mutations in Complex V genes (like *MT-ATP6*) [17].

Another important hurdle in the discovery of mitochondrial disease therapies is the lack of effective modeling systems. Notably, not all nDNA mutations can be recapitulated in animal models. This is the case for LS due to mutations in the gene *SURF1*, where *Surf1*-knock-out animals show extended lifespan and no neurological defects. At the bases of this diversity may be a high dependency on SURF1 activity for Complex IV assembly that is specific for human cells [18].

The lack of modeling systems is particularly evident for mitochondrial disease due to mtDNA mutations, where the difficulty in genome engineering has strongly hindered the development of animal models [19]. Cytoplasmic hybrids (cybrids), obtained by fusing immortalized human cell lines depleted of mtDNA with patient-derived enucleated cells, have been used to dissect the cellular impact of mtDNA mutations. However, cybrids disrupt the patient-specific interplay between nDNA and mtDNA, as all nDNA-encoded mitochondrial proteins in cybrids come from the immortalized cell line. Moreover, cybrids and other immortalized cell lines that are currently used in drug discovery pipelines primarily rely on glycolysis for energy generation [20–22]. This may result into milder responses upon mitochondrial inhibition. A recent HT screening identified drugs improving the growth rate and glycolytic flux of cybrids with Complex V mutations [23]. However, these parameters may not be of essential importance in the physiology of human neurons, which do not proliferate and do not depend on glycolytic metabolism [7]. In conclusion, appropriate cellular models of mitochondrial disease should reproduce the individual-specific mitochondrial/nuclear genome match and the metabolic and functional features of the cell types actually affected in the patients.

iPSCs for Mitochondrial Disease

iPSCs are obtained from somatic cells through a process known as "cellular reprogramming." During this conversion, there is a profound restructuring of the metabolic program toward glycolysis and mitochondria undergo changes in number, morphology, and functionality [24, 25]. This metabolic reconfiguration appears as an early reprogramming event, preceding the expression of genes controlling pluripotency

and self-renewal [26, 27], suggesting a potential role for metabolic restructuring in the epigenetic remodeling required for cell fate conversion.

What is highly relevant for the generation of iPSC models of mitochondrial disease is whether mtDNA changes may occur during the induction of pluripotency. The potential presence of genomic alterations in individual iPSCs constitutes a major obstacle for medical applications. The presence of mtDNA mutations has been detected in iPSCs derived from healthy individuals [24, 28, 29] with an increased mutation load in iPSCs obtained from aged subjects [30]. The integrity of mtDNA has been therefore suggested as an important parameter to assess during iPSC characterization. In fact, the differentiation ability of stem cells can be influenced by the mtDNA mutation load [31] and by specific mtDNA haplotypes [32].

The presence of mtDNA mutations within iPSCs does not appear to be due to reprogramming-induced genome alterations, but rather to clonal expansion of mutations already present at low levels within the parental fibroblast population [29, 30]. Accordingly, iPSCs are able to retain the same mtDNA profile of the original fibroblasts, including the hypervariable region of the D-loop, which is a highly heterogeneous marker often used as fingerprinting for forensic purposes [33]. It was previously suggested that human embryonic stem cells (hESCs) harbor large mtDNA deletions [34]. However, recent data show that iPSCs and commonly used hESC lines (H1 and H9) do not carry mtDNA deletions [33]. Hence, pluripotent stem cells have the potential to be used as a faithful model of mitochondrial disease, once their nDNA and mtDNA profile has been thoroughly analyzed at the single cell level.

Several recent works demonstrated the establishment of iPSCs from patients affected by mitochondrial disease due to heteroplasmic mtDNA mutations. mtDNA mutations commonly associated with MELAS have been used, including mutation m.3243A > G in the *MTTL1* gene of Complex I [28, 35–38], mutation m.13513G > A in the *MT-NT5* gene of Complex I [39], and mutation m.5541C > T in the *MT-TW* gene of Complex IV [40]. iPSCs were also generated from patients affected by Pearson syndrome carrying heteroplasmic mtDNA deletions [41]. All these studies show that the mutation load may vary greatly among different iPSC lines from the same individual, but remain constant upon differentiation. Therefore, following in depth mtDNA genotyping, iPSC lines derived from patients carrying heteroplasmic mtDNA mutations can be used for differentiation and downstream applications.

Patient iPSCs carrying homoplasmic mtDNA mutations associated with LS have been also generated, including m.8993T > G in the *MT-ATP6* gene of Complex V [28], m.13513G > A in the *MT-NT5* gene of Complex I [28], and m.9185T > G in the *MT-ATP6* gene of Complex V [33]. In all these cases, no changes in mtDNA mutation load were observed in patient iPSCs that could thus be used for modeling the disease pathogenesis.

iPSC-Derived Neural Cell Models for Mitochondrial Disease Drug Discovery

Patient iPSC-derived neurons appear as an ideal cell type for dissecting the pathogenetic mechanisms of neurological mitochondrial diseases. They represent the disease-affected cell type with the correct OXPHOS-dependent metabolism and carry the patient-specific nDNA and mtDNA match. Using these cells, it may be possible to identify meaningful cellular phenotypes that can be used to test therapeutic compounds.

iPSC-derived neurons have been successfully used to investigate neurological diseases in which mitochondrial impairment is known to play a pivotal role. This was the case for Friedreich ataxia (FRDA), a neurodegenerative disorder caused by mutations in the gene *FXN* encoding for frataxin, a mitochondrial protein part of the iron-sulfur cluster. Neurons from FRDA patients showed increased frataxin protein levels after treatment with a specific histone deacetylase inhibitor [42]. iPSC-based neuronal modeling was also applied to spinal muscular atrophy (SMA), where the existence of differences between mice and humans may limit the translation of basic findings into clinical trials. Using this approach, drugs inhibiting endoplasmic reticulum (ER) stress were found to enhance the survival of SMA neurons [43]. Similarly, the effect of compounds modulating γ-secretase was addressed using neurons obtained from sporadic Alzheimer's disease (AD) [44] and familial Alzheimer's disease (FAD) [45]. Interestingly, the concentrations of γ-secretase modulators needed to elicit a response in human neurons needed to be higher than those used on conventional non-neuronal cell models [46], highlighting the need for using the appropriate model system for neuronal drug development. Collectively, these findings indicate that it is possible to use iPSC-derived neurons to dissect the specific neuronal consequences of known or novel drugs. Nonetheless, despite these successes, performing HT compound screenings in iPSC-derived neurons still remains challenging.

The differentiation of iPSCs to neuron-like cells in a dish is very time-consuming and often results in heterogeneous cultures with batch-to-batch variability [47]. Recent studies indicate that it may be possible to substantially shorten the derivation of neurons of the central nervous system even in the absence of glia coculture [48]. However, even if iPSC-derived neurons show signs of electrophysiological activity, their gene expression pattern may still be immature in comparison to neurons of the human brain [49]. Neurons can also be generated directly from patient fibroblasts by-passing the iPSC state. This approach dramatically reduces the time needed to produce patient neurons and may preserve aging-associated epigenetic signatures [50]. Still, this strategy may not be easily applicable for HT screenings because of the paucity of material due to the lack of a self-renewable cell source.

The lack of homogeneity could represent a major drawback for the development of HT screenings with iPSC-derived cells, given the importance of the robustness of the cell model used for ensuring reproducibility. Furthermore, a large number of cells are required to test large libraries of compounds. In order to obtain high numbers of pure neurons, enrichment technologies need to be applied, including sorting for cell surface markers or purification according to neuronal-specific promoters [3].

A more physiological approach would involve the use of three-dimensional (3D) organoids, which are complex brain region-specific structures containing both glial and neuronal cells [51]. Cerebral organoids can be derived from human PSCs through spontaneous differentiation and allow modeling

whole brain development and related diseases such as microcephaly [52]. 3D human neural cell cultures have been also used to study the effect of known drugs for FAD [53]. In addition, it is possible to use patterning factors to derive brain-region-specific organoids. These structures enable the investigation of individual brain regions and have been used to demonstrate a direct connection between Zika virus and human microcephaly [54]. Nevertheless, critical issues still remain to be solved in order to allow the use of organoids for the development of therapies against neurological diseases due to mitochondrial impairment.

Human PSC-derived cerebral organoids still do not show the signs of functional maturation of post-natal and adult human brain. Increased maturity of the 3D structures may potentially be attained by introducing mesenchymal-derived microglia-like cells [55]. Additionally, the use of biomaterial and engineered vasculature may help addressing the issue of nutrient delivery into the organoids [55]. The scalability still represents an important obstacle; although the use of miniaturized spinning bioreactors may significantly improve the future applications of cerebral organoids for HT screenings [54]. Finally, the reproducibility should be critically improved to reach a state in which the derived 3D structures consistently exhibit similar traits—shape, size, and cellular composition- under the same experimental conditions [55].

We recently suggested iPSC-derived neural progenitor cells (NPCs) as an effective cellular model system for drug discovery of mitochondrial disease [33]. NPCs can be rapidly obtained from iPSCs using different strategies, including the formation of embryoid bodies (EBs) followed by manual isolation of neural rosettes [56, 57], or the small molecule-based direct induction under adherent culture conditions [58, 59]. Importantly NPCs show relatively homogeneous features and a mild proliferative state. Their cultivation is cost-effective and rather simple, which makes them well suited for scalability and HT experiments. Moreover, iPSC-derived NPCs can retain a gene expression profile similar to that of human brain-derived NPCs [33].

Previous studies showed that iPSC-derived NPCs from neurological patients exhibit disease-associated phenotypes that may be used for phenotypic drug screenings. iPSC-derived NPCs from patients affected by Huntington's disease (HD) have been found to exhibit bioenergetic defects [60]. HD-specific NPCs were also used to carry out a screening based on a predefined target measuring REST activity, resulting in the discovery of a compound effective on REST degradation [61].

In order to use iPSC-derived NPCs for mitochondrial disease drug discovery, these cells should retain the genetic features of the patients and exhibit a metabolic state dependent on mitochondrial respiration. Accordingly, mitochondria within NPCs derived from mouse iPSCs [62] or human iPSCs [33] acquire an elongated morphology with defined cristae and dense matrices. This is accompanied by a metabolic shift toward OXPHOS metabolism that resembles that of differentiated iPSC-derived neurons [33]. In accordance, proliferating neural progenitors in adult mouse hippocampus have been found to be critically dependent on OXPHOS metabolism [63]. In addition, the mtDNA sequence of iPSC-derived NPCs entirely mirrors that of the original fibroblasts from which the iPSCs were generated, both in the case of control individuals and patients carrying a pathogenic mtDNA mutation [33].

iPSC-derived NPCs have been obtained from mitochondrial patients carrying homoplasmic mutations in the *MT-ATP6* gene m.8993T > G and m.9185T > C [28, 33]. Both mutations caused defects in NPC bioenergetics, although to a milder extent in the case of m.9185T > C [33]. m.9185T > C NPCs showed abnormally increased MMP and altered mitochondrial calcium homeostasis that could also be observed in iPSC-derived neurons but not in patient fibroblasts or cybrids carrying the same mutations [33]. Therefore, NPCs may represent an attractive cell type for the establishment of HT screenings, while purified differentiated neurons may be still used to carry out the refined and detailed validations that are needed to support phenotype-based drug discovery.

iPSC-Based Approaches for Neurological Drug Discovery

Once the correct iPSC-derived neural cell model has been selected, several technological platforms may be applied for compound screenings. One of the most commonly used techniques is the cellular image-based high-content screening (HCS) analysis [64]. HCS can be used to quantify the amount of a defined protein in the derived neural cells [2]. This approach was used for phenotypic screenings with food and drug administration (FDA)-approved compounds in iPSC-derived motor neurons from patients affected by amyotrophic lateral sclerosis (ALS) carrying a *SOD1* mutation [65]. HCS-based assessment of MMP was used to conduct a phenotypic screening of FDA-approved compounds in iPSC-derived NPCs carrying a *MT-ATP6* mutation [33]. In the latter study, the phosphodiesterase type 5 inhibitor avanafil was found capable of partially rescuing the MMP and calcium defects in both patient NPCs and patient neurons. HCS-based platforms may also enable monitoring several mitochondrial parameters at the same time, which would be extremely interesting for mitochondrial disease drug discovery. This multiplexed approach was recently demonstrated using somatic fibroblasts [66], but it may also be applied in the future to iPSC-derived neural cells.

HT related quantitative reverse transcription polymerase chain reaction represents another detection technology available for iPSC-based drug discovery as it allows accurate measuring of the transcriptional responses of selected genes in a multi-well format [2]. This approach was used in neural crest precursors derived from iPSCs from individuals with familial dysautonomia to screen for compounds able to rescue the expression of I-κ-B kinase complex-associated protein [67]. Another HT option used for iPSC-derived neural cells involves the use of luciferase-based reporter plasmids [61].

HT screenings addressing functional parameters may also be particularly useful. This is the case for multi-electrode arrays that enable large-scale parallel recordings of neuronal activity. This method was applied on iPSC-derived motor neurons from ALS patients that displayed hyperexcitability [68]. Importantly, the drug identified to be capable to normalize the firing of iPSC-derived ALS neurons has been successfully used to set up a clinical trial [69].

Conclusion and Outlook

The use of patient iPSC-derived neural model systems capable of recapitulating the genetic and metabolic features of the

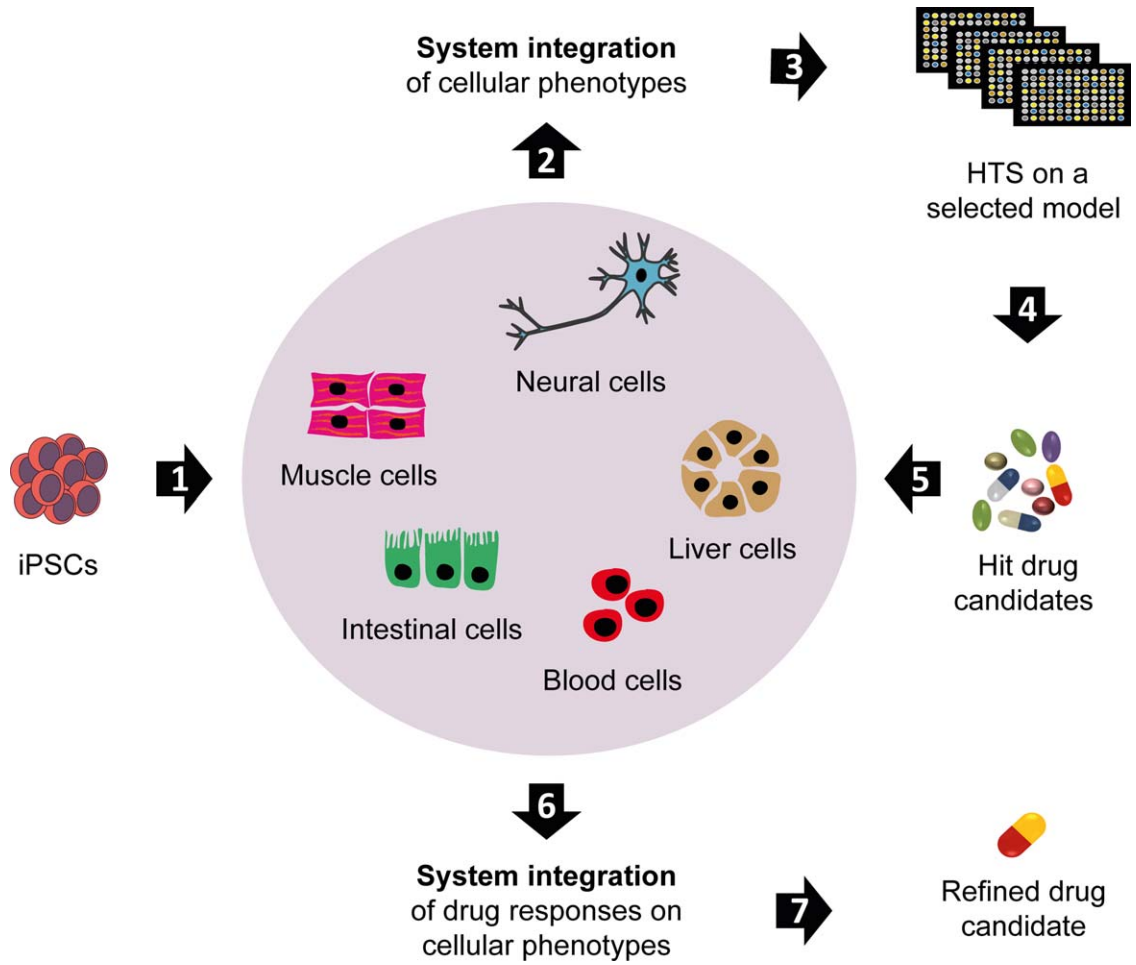

Figure 2. "Patient-in-a-dish" approach for mitochondrial disease drug discovery. iPSCs can be differentiated into several cell types that are known to be affected in the individual patient. Using these cell types, it may be possible to identify disease phenotypes that are patient-specific and/or cellular-specific. One of the analyzed cell types can then be used as a model for carrying out HTS. The obtained hit drug candidates can be applied on all the iPSC-derived cell types to analyze the efficacy and toxicity of the compounds within different patient-specific cellular systems. The integration of these responses may allow identifying with increased predictive power those drugs that have a higher chance to succeed once administered to the individual patient. Abbreviations: HTS, high-throughput screenings; iPSC, induced pluripotent stem cells.

cells affected in mitochondrial patients represents an important advance for the development of disease-modifying treatments for mitochondrial disease. A critical point to consider is the nature of the control group used for the identification and validation of the targetable cellular phenotype [3]. Isogenic control iPSC lines can be generated through genetic correction of mutated gene variants for mitochondrial disease due to nDNA mutations. Gene editing of nuclear mutations in patient iPSCs is now routinely performed using designer nucleases, such as TAL effector nucleases (TALENs) or the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system [3].

Critical challenges exist for mitochondrial disease due to mtDNA mutations given the multicopy nature of mtDNA. For specific heteroplasmic mtDNA mutation, it is possible to induce a heteroplasmic shift through selective degradation of the mutant mtDNA molecules using mitochondrial-targeted RE [70, 71]. The lack of appropriate RE for the vast majority of pathological mtDNA mutations limits the feasibility of this approach. Heteroplasmic shift may also be achieved via mitochondrial-targeted TALENs [71] or heterodimeric zinc finger nucleases [72].

Currently, the option available for homoplasmic mtDNA mutations is the mitochondrial replacement via somatic cell nuclear transfer. This procedure was used to correct the bioenergetic phenotype observed in NPCs carrying a pathogenic *MT-ATP6* mutation [28]. However, this approach leads to a mismatch between nDNA and mtDNA, which may alter the cellular phenotypes and would therefore not be ideal in disease modeling and drug discovery studies. Moreover, this method involves the use of human embryos, which is rather complex and ethically not allowed in several countries. Recently, mitochondrial-targeted Cas9 has been described, which may potentially be applied to edit mtDNA using gRNA expression vectors without affecting nDNA [73]. Further works are needed to confirm this potentially very promising technology.

Additional challenges of iPSC-based drug discovery are inherent to all in vitro cellular models, which may be dependent on the culture conditions and may not fully recapitulate the complex response of a whole organism [15]. One way to tackle this issue may be through a system-biology approach aiming at integrating the single cellular responses of distinct

cellular systems (e.g. neurons, cardiomyocytes, hepatocytes differentiated from the same iPSC lines) to particular compounds. This "patient-in-a-dish" approach has the potential to predict the effect of a drug within different disease-relevant biological environments (Fig. 2).

Overall, iPSC-derived cells appear as a potent model system which may allow conducting personalized drug discovery of complex mitochondrial disorders. It is our hope that this approach may ultimately enable the identification of curative treatments against these debilitating conditions.

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Author Contributions

G.I. and C.L.: manuscript writing and figure generation. P.L., A.Z., and B.M.: manuscript writing. A.P.: conception and design, manuscript writing, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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