MINIREVIEW

Small Molecule Screening in Human Induced Pluripotent Stem Cell-derived Terminal Cell Types

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A need for better clinical outcomes has heightened interest in the use of physiologically relevant human cells in the drug discovery process. Patient-specific human induced pluripotent stem cells may offer a relevant, robust, scalable, and cost-effective model of human disease physiology. Small molecule high throughput screening in human induced pluripotent stem cell-derived cells with the intent of identifying novel therapeutic compounds is starting to influence the drug discovery process; however, the use of these cells presents many high throughput screening development challenges. This technology has the potential to transform the way drug discovery is performed.

Despite substantial investment in research and development, the drug discovery industry has struggled in recent years to deliver safe and efficacious new medicines to address significant unmet clinical needs. Numerous reasons for this lack of productivity have been proposed, and the drug discovery industry has been intensely scrutinizing how it identifies potential new medicines (1–3). Because clinical outcomes are predicated on choosing the right target(s) and molecular entity for the right disease (4), much of the attention has been focused on the earliest stages of drug discovery.

Two major approaches to the discovery of small molecule drugs are generally being pursued: target-based and phenotypic. With the first, a link between a disease state and modulation of a specific molecular target is postulated, thus allowing for a more focused search (5). With the latter, researchers aim to discover chemical entities affecting a given phenotype using a biological system (cell, organ, animal) thought to be representative of the disease (6, 7). With either approach, this process usually begins with an in vitro high throughput screen (HTS)2 in which a large number of chemical compounds are tested in a biochemical or cell-based assay (Fig. 1) (8). It is critical to note that the ultimate success of the drug discovery process depends in large part on whether the target or phenotype assayed in the HTS is relevant to the disease indication being pursued (9).

Mounting evidence suggests that capturing the biological complexity of the disease state in the earliest in vitro assays results in better clinical translation (10). This has driven a renewed interest in the use of physiologically relevant models in the drug discovery process (11–13). Historically, such models consisted primarily of animal disease models (in vitro and in vivo) as well as patient-derived primary human cells. Clear limitations are associated with these models, including the increasingly documented lack of human disease relevance of the former and the very limited availability of the latter, with only few specific cell types being easily accessible from patients in significant numbers (14, 15).

Patient-specific human induced pluripotent stem cells (hiPSCs) offer a novel source of human cells for drug discovery. These cells are derived from postnatal somatic cells through transient ectopic expression of pluripotency-associated transcription factors, which establishes an epigenetic state able to maintain unlimited proliferation capacity in the undifferentiated state while retaining the ability to differentiate into any somatic cell type under the appropriate culture conditions (16, 17). Importantly, because hiPSCs can be generated from postnatal cells, it is possible to make human pluripotent stem cells from specifically selected patients with relevant thoroughly sequenced genotypes, well characterized disease, and/or known positive/negative response to drug(s). This makes it possible to investigate the genotype-phenotype-drug relationship in a controlled and systematic manner. Panels of patient-specific hiPSCs representing the spectrum of the disease and/or drug response can be generated and differentiated into relevant cell type(s). Because the hiPSC-derived cells initiate from a renewable source, unlike primary human cells, they can be delivered on a predictable timetable over the 2–5 years necessary to run a drug discovery program, thus facilitating the pharmacological screening process. If longitudinal clinical data associated with the hiPSC line are collected and coupled with accumulating data from different assays and drug screens over time, the opportunity to translate in vitro preclinical response into clinical outcome becomes unprecedented.

A wide variety of small molecule screens using mouse or human pluripotent stem cells have been described in the literature. Initial screens focused on identifying factors promoting pluripotent stem cell survival and/or proliferation (18–22) and, more recently, inhibition of pluripotency to increase the potential safety of transplanted stem cell-derived cells (24). With the advent of hiPSCs, multiple screens were performed to identify small molecule enhancers of reprogramming (25–27). Additional screens have focused on identifying factors that enhance differentiation to a specific cell lineage or that increase the functional phenotype of a stem cell-differentiated cell type (28–32). Specific stem cell-derived cell types, particularly cardiomyocytes and hepatocytes, are increasingly being used in toxicology testing of compounds with approaches sharing many of the features of small molecule efficacy screens (33–37).

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1 To whom correspondence should be addressed. E-mail: sandra.engle@pfizer.com.
2 The abbreviations used are: HTS, high throughput screen; hiPSC, human induced pluripotent stem cell; qPCR, quantitative PCR; IVD, in vitro differentiation.
In 2010, McNeish et al. (38) described a >2-million compound-calcium flux HTS in wild-type mouse stem cell-derived neurons to identify positive allosteric modulators of the AMPA receptor in what was arguably the first HTS utilizing stem cell-derived cells. They confirmed human translatability by showing a similar rank order in hit potency using human stem cell-derived neurons. More recently, Yang et al. (39) described a much smaller image-based screen (~5000 compounds) in motor neurons derived from wild-type and SOD1G93A mutant mouse embryonic stem cells to identify neuroprotective compounds for amyotrophic lateral sclerosis. Additionally, a growing body of literature has reported two- to five-compound hypothesis testing experiments in studies focused on delineating phenotypes associated with patient-specific human stem cell-derived cells (40–45). In a slightly larger screen using presumed normal human stem cell-derived dopaminergic neurons, Peng et al. (46) evaluated 44 compounds previously documented to be neuroprotective in rodent or human cell line models or in vivo rodent models in a 1-methyl-4-phenylpyridinium neuroprotection assay with follow-up screening in a rotenone neuroprotection assay. Interestingly, they found that only 16 of the 44 compounds showed efficacy in their initial 1-methyl-4-phenylpyridinium assay. They attributed their findings to shortcomings in the earlier assays to reflect the human disease because positive and negative findings in this assay correlated with positive and negative outcomes in human clinical trials. Collectively, these experiments have helped lay the conceptual groundwork for small molecule screens in hiPSC-derived cells with direct applicability to identifying novel compounds with symptom- or disease-modifying properties.

To date, only a handful of drug efficacy screens evaluating from hundreds to thousands of small molecules in hiPSC-derived cells have been reported (Table 1). The vast majority of these screens focus on stem cell-derived neural progenitors or neurons, arguably the most well developed and extensively explored human stem cell-derived differentiation pathway (47–51). Choi et al. (52) have provided a notable exception in using stem cell-derived hepatocyte-like cells generated from 1-antitrypsin-deficient patient hiPSCs in an immunofluorescence screen to detect inhibition of mutant 1-antitrypsin accumulation. Most of the researchers generated the terminal cell types themselves, although Xu et al. (53) described using commercially available, presumed normal, predominantly forebrain GABAergic and glutamatergic neurons to screen for inhibitors of amyloid-β(1–42) toxicity by measuring neurite outgrowth. Although a majority of the screens reported use high content imaging, Lee et al. (54) used quantitative PCR (qPCR) to detect changes in transcript levels of the target gene (IKBKAP), and Charbord et al. (55) introduced a luciferase-based reporter plasmid to detect changes in target (REST) activity. The compound libraries chosen for screening generally contained known bioactive molecules (56), some with clinically relevant information. Although these screens differ on specific details, when taken together, they serve to highlight the many parameters that must be addressed at both the cell and assay
level to develop and execute a successful stem cell-derived drug efficacy screen.

**HTS Considerations Using Human Stem Cell-derived Cells**

To realize the drug discovery potential of small molecule screening assays based on in vitro differentiated, patient-specific hiPSC-derived cells, it is necessary to develop robust, reproducible, and relevant assays with reasonable throughput that have been rigorously validated. Although these characteristics are essential to all small molecule screening assays, the use of patient-specific stem cell-derived cells presents unique challenges. At its core, a HTS represents the industrialization (scale-up) of a compound testing experiment already performed on a limited scale. Despite this conceptual simplicity, a number of hurdles are introduced while increasing the number of experiments being conducted by 3–6 orders of magnitude depending on the size of the compound library used. Here, we aim to detail these hurdles as well as define guideposts and suggest solutions awkward to obtain a set of valid hit compounds following the HTS.

**Cell Robustness and Reproducibility**—The generation of in vitro differentiated, hiPSC-derived cells is a complex multistep process originating from terminally differentiated cells to pluripotent cells and back to a potentially new type of terminally differentiated cell. As with any multistep process, variability introduced at each stage can accumulate to the point of limiting or obscuring any meaningful results in the final evaluation. Considerable care must be taken at each stage of the process to mitigate variation.

For example, reports have suggested that the method of reprogramming, the tissue from which the hiPSCs were generated and somatic mosaicism can contribute variation to the quality of hiPSCs generated and their resulting differentiation potential (4, 57–60). As a result, non-integrating reprogramming methods (modified mRNA, Sendai virus, episomal vectors, small molecules, proteins, etc.) that are more efficient at establishing a true pluripotent state have been developed and are rapidly becoming the standard (61–65). Similarly, protocols for hiPSC generation from peripheral blood, a cell source more patient friendly and less susceptible to mosaicism, are rapidly becoming the norm (66–68). Furthermore, a consensus is developing that thorough evaluation of hiPSCs to ensure high quality before use is essential and that the minimum character-

**TABLE 1**

| Ref. Year | Disease | Differentiated cell type | Assay format | Readout(s) | Compound no. | Library |
|-----------|---------|--------------------------|--------------|------------|--------------|---------|
| Lee et al. (54) 2012 | Familial dysautonomia (IKBKAP) | Neural crest cells | qRT-PCR | WT IKBKAP | 6912 | Bioactive compounds |
| Xu et al. (53) 2013 | Alzheimer disease (Aβ) | Neurons | Luminescence | Cell viability | >100 | Proprietary compounds |
| Makhortova et al. (23) 2011 | Spinal muscular atrophy (SMN1/2) | Motor neurons | Immunofluorescence | Cytoplasmic and nuclear SMN protein levels | 3500 | Annotated compounds |
| Burkhardt et al. (56) 2013 | Amyotrophic lateral sclerosis (TDP-43) | Motor neurons | Immunofluorescence | ISLET1 and TDP-43 aggregates | 1757 | Bioactive compounds |
| Choi et al. (52) 2013 | AAT deficiency | Hepatocytes | Immunofluorescence | Total AAT protein/well | 3131 | Clinical ready drug library |
| Charbord et al. (55) 2013 | Huntington disease (REST) | Neural stem cells | Luminescence | Derepression of reporter | 6984 | Purchased compound libraries |

**TABLE 1** Small molecule HTS conducted in hiPSC-derived cells

Aβ, amyloid-β; AAT, α1-antitrypsin.
Reasonable Throughput—As mentioned previously, the identification of small molecule drugs generally involves a HTS, followed by iterative rounds of chemical analog synthesis along with secondary and tertiary screens to impart on a compound the properties required for clinical testing. The availability of cells must match the anticipated number of compounds to be screened at each stage. Human pluripotent stem cells were originally cultured using labor-intensive techniques such as co-culture with mouse or human fibroblasts and manual subculturing (16, 17). This limited the number of cells that could be produced in both the undifferentiated and differentiated states and created significant culture-to-culture variability (82). The development of single cell dissociation and improved feeder-free culture conditions has facilitated the bulk production of undifferentiated cells through reduced manual handling, automation, or bioreactors and enabled the subsequent generation of larger quantities of differentiated cells (78, 83–85). Efforts have also been made to identify proliferative committed progenitor cells for each in vitro differentiated cell type that can self-renew, be generated in bulk, and be frozen in large quality-controlled batches (79, 86, 87). Enabling a HTS requires diligent attention to assay logistics because dozens to more than a thousand assay plates will likely be required. In general, simpler work flows are highly preferable both to lower labor requirements and to avoid introducing additional assay variability. To this end, freezing of progenitor or differentiated cells and their subsequent plating prior to the HTS are a valuable option, when achievable, as opposed to conducting a lengthy cell differentiation in assay plates due to the often observed variability resulting from this process.

The type and size of the compound library used for a HTS depend on both the goal of the work and the limitations imposed by the cells and assay format used. For target-based drug discovery, large (10⁵–10⁶ compounds) and chemically diverse libraries are routinely screened to maximize the number of different chemical series identified. For those studies described in Table 1, a phenotypic screening strategy with libraries containing only a few thousand compounds was employed instead. Rather than covering chemical space, these smaller libraries are collections of biologically active molecules, allowing researchers to survey the relevance of numerous biological mechanisms and signaling pathways to their phenotype of interest. A challenging cell supply can thus limit the options available for screening.

Relevance—Because physiological and disease relevance provides the most compelling rationale for the use of hiPSC-derived cells, it is essential to identify a robust measurable phenotype to assay. Many examples have now been reported of in vitro differentiated hiPSC-derived cells from patients with monogenic disorders recapitulating cell autonomous disease phenotypes in vitro (88), and elegant studies using genomic engineering to return the mutation to the wild-type version have shown that the observed phenotype is directly associated with the mutation. Additionally, there are some data to suggest that cells derived from sporadic diseases may have observable phenotypes that can be quantified regardless of the underlying mechanism (40, 89). However, not all diseases can be effectively modeled. For example, IVD protocols do not currently exist for all cell types of disease relevance, and for those IVD protocols that do exist, the cells must be characterized to ensure that they are expressing the relevant pathways to a level that will allow a readout to raise above the proposed assay background. Cells from Fanconi anemia patients are difficult to reprogram to hiPSCs, thus eliminating this option for obtaining patient-specific cells (90, 91). Likewise, X chromosome reactivation and subsequent inactivation in fragile X and Rhett syndrome hiPSCs can be inconsistent and problematic (92–95). Additionally, weak, low penetrant, or environmentally induced phenotypes may not be successfully modeled in a reproducible and quantifiable manner in vitro. Some phenotypes, particularly those associated with late onset disorders, may be elicited only under conditions that mimic cellular aging or stress. Determining what is an appropriate and physiologically relevant in vitro equivalent of in vivo aging and stress has not been well defined.

Identifying the appropriate cellular control is also problematic. As noted, variation may come from many sources unrelated to the genetics of the patient material, and the vast genetic diversity of the human population means that a significant range of “normal” can exist for any phenotype. In general, this type of variability has been managed by increasing the sample size to reach an average value. In the case of a stem cell-derived, cell-based HTS, this could quickly become cost-prohibitive. Realistically, the HTS would be performed on cells representing one patient sample. Although this need to characterize cells from multiple donors is often presented as a drawback of primary or hiPSC-derived cells, it is well worth pointing out that the cell lines used historically in biomedical research are mostly derived from an individual patient tumor. As follow-up, hit compounds would need to be tested in a variety of cells, including those from additional patients with the same mutation/disease, samples from patients with the same disease but potentially different or unknown mutations or mechanisms of disease development, and presumed normal cells or cells in which the mutation has been returned to the wild-type version by genomic engineering. It may also be useful to understand the effect of the compounds in stem cell-derived cells from healthy donors or patients with a different disease presumably affecting the same cell type to rule out any response due to the cells being generated by IVD.

For screening, the preferred and most relevant assay formats are those that do not require introduction of artificial systems or gene expression because cellular functions and behavior may be perturbed in unpredictable ways. Image-based screens provide non-invasive assay readouts with the added benefit of being compatible with heterogeneous cell populations when appropriate markers are available. For example, by monitoring both increased cell viability and cell morphology (neurites), Yang et al. (39) were able to use a cell population containing only 30–50% motor neurons to carry out a compound library screen and identify GSK3β inhibition as a mechanism of potential therapeutic interest for amyotrophic lateral sclerosis. High throughput flow cytometry may also be used to evaluate heterogeneous cell populations and may be particularly applicable if the terminal cell type is non-adherent or the target of interest is expressed on the cell surface (96). High throughput qPCR as used by Lee et al. (54) and more traditional assay readouts such
as ELISA and homogeneous time-resolved fluorescence technology offer a similarly direct readout but are much more susceptible to variation associated with a heterogeneous cell population. Impedance and microelectrode array technologies, which are sensitive to changes in cell shape and electrical activity, respectively, can be particularly effective direct measurements of drug-induced cellular responses when the cells intrinsically rely on these phenotypes. When necessary to facilitate detection of specific cellular events, genetically encoded reporters, biosensors, or structural tags may be introduced into stem cells and their derivatives, although validation of the hits in non-engineered cells will be necessary after the screen.

Assay Characteristics—A minimal set of requirements need to be met for an assay to be successfully used for a HTS. First, a positive control is necessary to properly evaluate the dynamic range and signal-to-background window of the assay. Care must be taken to use a positive control with a mechanism of action that is relevant to the types of hits one wishes to identify. For example, using a cell-lysing detergent to provide complete cell death in a cell viability assay may lead to the erroneous assumption that a short compound incubation with the cells would be sufficient to reveal most cytotoxic agents. A biologically (as opposed to physicochemically) driven cell toxicant such as puromycin would likely be a better choice in that instance. Although significant emphasis is usually placed during assay development on increasing the signal to background, it is only one component of assay robustness, with the main other being variability of signal (standard deviation). The Z' factor is a statistical measure of assay quality bringing these two parameters together; assays with $Z' > 0.5$ are considered highly suitable for conducting a HTS (97). Such a bar can be difficult to reach when working with complex cellular systems or assay readouts such as those associated with hiPSC-derived cells. Nonetheless, Lee et al. (54) were able to obtain $Z'$ values of 0.78 in their screen of stem cell-derived neural crest precursors, and Charbord et al. (55) were able to obtain a value of 0.5 in their screen of neural stem cells. Options do exist to overcome assay variability and enable a successful screen. Simply stated, the level of statistical confidence provided in hit identification by $Z' > 0.5$ is quite high due to its mathematical definition, and screens (especially smaller ones with $10^3$-$10^4$ compounds) can be run with $Z' > 0.2$ (97), although one may need to lower the hit selection threshold (percent effect required for declaring a hit) and retest more putative hits in confirmation assays. Alternatively, given the statistical nature of the hit identification process, testing each compound in duplicate or triplicate rather than singlicate will lead to a large increase in $Z'$ for the averaged values and a correspondingly higher level of confidence in the assay results (54). Finally, use of higher throughput screening (1536-well format) can allow compound screening in dose-response mode, thereby also providing greater statistical confidence in hit identification for assays with low $Z'$ values (98). Prior to conducting the screen itself, assaying a small subset of compound plates at several different compound concentrations (e.g. 1, 3, and 10 μM) is recommended to validate the HTS logistical process and to provide an estimate of the hit rate to inform the choice of the final testing concentration (54). Importantly, multiple concentrations of compounds can be used for the primary screen with smaller libraries to account for possible biphasic behaviors (23, 39). Following the screen itself and retesting at a single concentration for hit confirmation, compound ranking is best obtained from freshly solubilized compounds through the determination of IC$_{50}$ values using dose-response curves (52, 54). Here, ensuring that IC$_{50}$ values can reproduce within ±0.5 log units can be seen as a minimal requirement for proper hit ranking.

Screening Cascade Design—Designing a well thought-out screening funnel is a critical step in the HTS process. It should be considered at the earliest stages of the project, as multiple assays will usually be required to successfully validate hits and/or biological mechanisms (Fig. 1) (23). Assays should be fit for purpose and take into account the availability of cellular material, the complexity of the assay, and the translational value of the data derived from the assay. To that end, these secondary assays should take into account the intrinsic weaknesses of the primary assay. A common hurdle for phenotypic screens lies in their readouts being often “negative” such as decreased gene or protein expression (52, 54). In these instances, cytotoxic compounds will often represent a majority of the HTS hits, and hit validation will be significantly more complex. Although it is preferable to have a “positive” readout setup for the primary assay, a well designed cytotoxicity assay will be required if this cannot be accomplished. A number of formats are available for this purpose, although minimal assay attributes will normally include using the same cellular system and a compound incubation at least equal to 24 h to allow most cytotoxic agents to be uncovered (76). Moreover, assay format artifacts can often be observed and, depending on their frequency, may need to be accounted for by using the appropriate counterscreen. Additionally, hit and/or mechanism validation will necessitate the use of orthogonal functional assays such as a protein ELISA and Western blotting to validate the increased gene expression observed with qPCR or a reporter gene system, for example (54). Once hits have been properly validated, it is useful to obtain structure-activity relationship information by testing closely related internal or commercially available compounds to rank chemical series for follow-up. Correspondingly, validation of a biological mechanism will also require the testing of additional compounds targeting the same mechanism. Preference will be given to compounds based on a different chemical scaffold for added confidence, as the activity of the hit may not originate from its annotated biological mechanism but from an off-target effect instead. Yang et al. (39) used this strategy, in combination with genetic knockdown, to demonstrate that the activity of kenpaullone was truly mediated through its inhibition of GSK3β. Furthermore, biological mechanisms can usually be prioritized for follow-up based on a comparison of the potency displayed by hits in the phenotypic screen and against the annotated target, with large unexplainable discrepancies being a significant red flag during the validation of a given mechanism (23). Finally, both novel hits and mechanisms identified should be confirmed in cells derived from additional donors as discussed above to ensure relevance across a desired patient population (52–54).
Concluding Remarks

hiPSC-derived cell-based assays are poised to significantly impact the drug discovery process by providing in vitro cellular systems more accurately representing human disease. An increasing number of high quality, well characterized patient-specific hiPSC lines are becoming available, issues with robust and reproducible production of terminal cell types are rapidly being addressed, and it is now possible to recapitulate many disease relevant phenotypes in vitro. Although additional technical hurdles may apply to the conduct of a HTS employing these cells, recently published examples as described in this minireview illustrate that these can be successfully addressed. Through these pioneering studies, researchers are gaining a realistic understanding of the benefits and limitations of the technology. This will allow them to develop fit-for-purpose assays that better capture the complexity of human biology such that safer, more efficacious medicines make their way to the patient.

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