Review

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iPS cell derived neuronal cells for drug discovery

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Owing to the inherent disconnect between drug pharmacology in heterologous cellular models and drug efficacy *in vivo*, the quest for more predictive *in vitro* systems is one of the most urgent challenges of modern drug discovery. An improved pharmacological *in vitro* profiling would employ primary samples of the proper drug-targeted human tissue or the *bona fide* human disease-relevant cells. With the advent of induced pluripotent stem (iPS) cell technology the facilitated access to a variety of disease-relevant target cells is now held out in prospect. In this review, we focus on the use of human iPS cell derived neurons for high throughput pharmaceutical drug screening, employing detection technologies that are sufficiently sensitive to measure signaling in cells with physiological target protein expression levels.

Pharmacological screening and profiling '*in vivitro*': a new level of *in vitro–in vivo* translation

The pharmaceutical industry is facing vast challenges in addressing reduced healthcare reimbursements in the public sector, augmented hurdles to have a new medical entity (NME) registered, and the increasing costs of drug discovery [1,2]. The situation is aggravated by the fact that the low hanging fruits of straightforward symptomatic drugs have largely been harvested and that discovery of currently desired disease-modifying drugs is much more demanding. The ultimate plague of pharmaceutical companies is, however, the late stage attrition of drug candidates in costly clinical studies.

One reason for the failure of many drug candidates in the human system is the poor predictivity of the preclinical biological models. Although target-directed drug discovery has been outstandingly successful in producing nano- and picomolar binders of a hypothesized disease-relevant protein *in vitro*, the drug candidates frequently display poor efficacy in clinical settings. What are the underlying causes for these costly letdowns in late-stage drug development? One probable reason is the fact that early drug discovery largely employs heterologous recombinant systems overexpressing a target of interest in a disease-unrelated host cell line. Likewise, drug candidate profiling based upon primary animal cells or *in vivo* animal models is frequently flawed due to significant species-specific differences in disease pathology.

Therefore, an ideal pharmacological profiling would rely on testing the drug candidate against the human disease target tissue or — in a more minimalistic setting — against the human disease-relevant cell. Whereas some primary human cell types such as blood cells or adipocytes are available at relative ease, other human cells such as primary neurons or primary pancreatic β cells are much more difficult to access. The latter dilemma may prospectively be resolved by the advent of a novel technology: in 2006, Takahashi and Yamanaka [3] discovered that adult somatic cells can be reprogrammed to a state of virtual pluripotency much similar to that of embryonic stem cells (Box 1).

The products of this reprogramming process are referred to as induced pluripotent stem (iPS) cells. Human iPS cells may, for instance, be reprogrammed from fibroblasts that are accessible through the skin biopsy of an adult donor. These human iPS cells can be amplified to well-nigh infinite numbers using adapted standard cell culturing equipment, and can then be differentiated towards a cell type of interest [4]. To underline the high *in vitro–in vivo* translational potency of iPS cell derived target tissue modeling cells, we suggest the merged term '*in vivitro*'. For the time being, however, there are still many challenges including, for example, (i) the maintenance of the reprogrammed iPS cells in amplification culture while preventing their uncontrolled differentiation or (ii) the establishment of differentiation protocols that lead to the *bona fide* disease-relevant model cells of interest.

This review analyzes the recent paradigm shift in early pharmaceutical drug discovery towards more physiological disease modeling cells with a higher potential of predicting *in vivo* drug efficacy. A further focus of this paper is the emerging applicability of iPS cell derived model cells to pharmaceutical high throughput screening (HTS) [5,6]. Although many published reports establish proof of

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Box 1. Induced pluripotent stem cells

In 1998, the isolation of hES cells [56] was hailed by some as the dawn to a new age of regenerative medicine, but was condemned by others due to the ethical issues associated with the use of human blastocyst stage embryos for research purposes. Surmounting the latter concern, Takahashi and Yamanaka [3] demonstrated that a cocktail of only four transcription factors, *OCT4*, *SOX2*, *KLF4*, and *c-MYC*, sufficed to 'reprogram' adult-derived fibroblasts into pluripotent cells resembling ES cells [3]. In subsequent studies, all of these factors, except for *OCT4*, have been replaced or omitted in the reprogramming process towards human iPS cells [57–62].

Practical applications of human stem cell derived cells for various therapeutic approaches have been worked on for some years, with some success, for example, in the area of macular degeneration [63]. In addition, the utilization of these cells in pharmaceutical drug discovery has more recently gained much momentum. For instance, the Innovative Medicine Initiative StemBANCC (http://stembancc.org) aims at developing iPS cell based platform technologies to profile the safety and efficacy of pharmaceutical compounds. Modeling a disease using human iPS cells offers many opportunities to be included into a drug-screening platform. In the case of modeling a monogenic disease, cells differentiated from an iPS cell line of a genetically affected subject may be compared with genome-corrected isogenic controls. However, the challenge is to identify an *in vitro* assay endpoint that is robust, consistent, and connected to the respective disease phenotype. Recently, this approach was successfully used in the context of Alzheimer's disease (AD). Neurons differentiated from iPS cells generated from patients with monogenic AD showed increased levels of amyloid-β and phospho-tau compared with wild type controls [64,65].

If there are diverse monogenic forms of a disease, then iPS cell based models could be generated from patients with different monogenic disease causes. This approach may provide clues to unveil individual themes for the genetically less understood, potentially 'polygenic' disease conditions. Thus, in the polygenic disease phenotype, multiple signaling pathways may be implicated in the pathology. Even when there is no 'known' genetic mutation associated with disease pathology, an iPS cell based disease model can be used to unmask disease-disturbed signaling events of genetic origin and reveal novel targets by exposing iPS cell derived cells to diverse pharmacological challenges addressing various intracellular signaling pathways.

concept for iPS cell based disease modeling, these studies are almost all small scale and are not ideally suited for drug discovery. We review recent technological breakthroughs in the areas of ultrasensitive cellular detection techniques as well as iPS cell derived neuronal precursor cells that, together, enable large-scale HTS campaigns using iPS cell derived disease models (Figure 1).

Looping back to the phenotypic roots of drug discovery

In the mid-nineteenth century, the pharmaceutical industry typically based its research around a historically known pharmacophore of interest with an acknowledged disease-mitigating profile in humans. In relatively slow, restricted cycles of chemical variation and subsequent 'phenotypic' measurement in animal models, the drug candidate could be improved without any deeper understanding of the biomolecular mechanism of action of the compound, as may be exemplified by the history of Aspirin™ [7]. This stratagem profited from the fact that all activities were based upon a well-known and experience-verified link between the pharmacophore and the respective human disease. Then – with the advent of molecular biology – it became customary to restrain the focus to a bimolecular interaction using techniques to recombinantly overexpress the target protein in solution or on the surface of disease-unrelated host cells. Although the latter approach may provide a better insight to the direct interaction of the test compound with the molecular target, it bears the disadvantage of ripping the cellular target out of its stoichiometric balance with other physiologically interacting proteins and metabolites.

In the early 1990s, the concept of *random* testing developed gradually as an attempt to capitalize on the large collection of chemical compounds, which had been assembled and retained within most long-standing pharmaceutical companies. Because a large number of test compounds — typically several hundred thousand — is inevitably required for random testing, the feasibility of a cellular HTS approach depends on the possibility of upscaling of upscaling the model cells to numbers in the range of a few billions. To meet these demands, it was initially very tempting to employ robust and easily fermentable host systems such as Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells for heterologous target expression. At the same time, the recombinant overexpression of the biomolecular target of interest facilitated the establishment of robust assays with high signal-to-noise ratios. On the downside, the employment of disease-unrelated host cells and the use of heterologous target overexpression have generated rather artificial *in vitro* screening systems.

Given a simple disease biology depending on a single molecular target, the above-described recombinant cellular systems have successfully been employed to identify valuable, meanwhile marketed, drugs. In recent years, however, the frustration with inefficacious drug candidates that had been produced in the above-described target-focused systems has led to a revival of the phenotypic screening concept [2,8]. The phenotypic testing of compounds positions the assay endpoint on a pathophysiologically relevant event while allowing for a varying multitude of known and unknown upstream targets. The obvious advantage of the phenotypic approach is the closer link between the measured effect and the targeted disease. Two obvious downsides are the initial ignorance of the direct biomolecular drug target and the increased risk that the drug candidate modulates a disease-unrelated upstream process (e.g., possesses a cell toxic effect).

What is defined as a phenotypic screen varies considerably. On the one hand, a phenotypic assay may monitor the endpoint of a well-characterized signaling chain, allowing for a relatively straightforward deconvolution of the biomolecular target afterwards. On the other hand, the phenotypic readout could be a cell morphological change, such as neurite outgrowth from a regenerating neuron, rendering the subsequent target deconvolution much more of a challenge. An ideal phenotypic screen should employ model cells that closely correspond to the disease target cells to render the phenotypic readout as close to the native target tissue response as possible. In addition, we argue that the cellular context is likewise a concern in a target-focused approached, as detailed below.

New drug target concept: target-focused screening in iPS cell derived model cells

Let us consider a scenario in which we are screening for test compounds supposed to modulate the activity of a

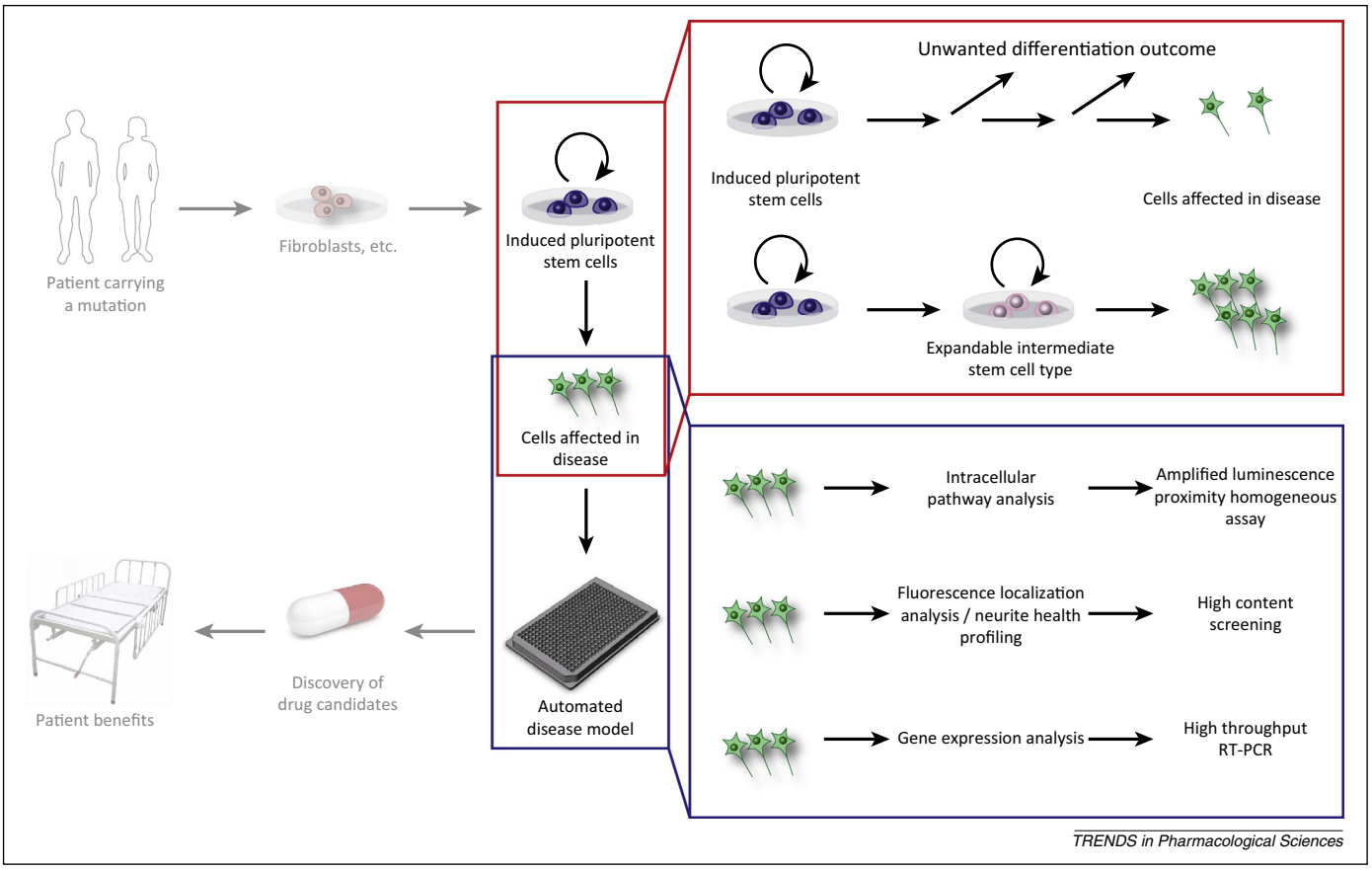

Figure 1. Overview. Induced pluripotent stem (iPS) cells are generated from primary somatic cells, such as fibroblasts, that are biopsied and cultured from patients with an observable phenotype and known genotype. iPS cells can then be expanded and differentiated to generate theoretically limitless numbers of specialized cells that should recapitulate pathogenic mechanisms in patients. In principle, these models can be incorporated into high throughput screening (HTS) campaigns to identify novel therapeutic compounds that can be developed into new drugs. Although many iPS cell based disease models have been published, there are at least two challenges that need to be addressed in order to use these models for HTS. First, as outlined in red, iPS cell differentiation requires up to several months and gives heterogeneous results (top). We review the use of expandable intermediates as a possible solution (bottom). Second, as shown in the blue box, disease models have to be converted into HTS-compatible assays. We review the three possible HTS-compatible platform technologies that can be applied to iPS cell based disease models: amplified luminescence proximity homogenous assay (top), high content screening (middle), high throughput reverse transcriptase polymerase chain reaction (RT-PCR) (bottom).

single biomolecular target such as a G-protein-coupled receptor (GPCR). A test compound that acts as a full GPCR agonist in a recombinant system with an amplified assay readout may easily turn out to be a partial agonist or even a completely ineffective compound in a screening system that relies on a pathophysiological assay readout in a disease-relevant cell type. Why is that so? The portion of receptors required to be stimulated to achieve the maximal response of the analyzed receptor-modulated bioactivity may be significantly lower in the recombinant receptor overexpressing system, leaving a large percentage of spare receptors that are pharmacologically referred to as the *receptor reserve*.

However, the extent of the receptor reserve for a given agonist is not only determined by the receptor numbers per cell, but also by the signal amplification between the activated receptor and the ultimately measured cellular response. Thus, also the receptor reserve in a native tissue environment with a low receptor density can be considerable if the ultimately measured event is sufficiently amplified through the signaling pathway: for example, the maximal smooth muscle contraction of guinea pig ileum may be elicited by only activating 2% of the present histamine receptors with histamine [9,10]. These facts argue in favor of employing a model cell that contains physiological receptor numbers and that mirrors the disease-relevant signaling pathway.

For instance, GPCR-driven cellular signal amplification is implemented as a cascade of succeeding levels, involving heterotrimeric G proteins, GPCR kinases (GRKs), scaffolding proteins, and other signaling factors [11]. For all steps of the signaling cascade regulated by the receptor of interest, the amplitude of the response, and thereby the virtual efficacy of the agonist, critically depends on the expression levels and isotype composition of all proteins, lipids, or other biomolecules that influence the signal progression. The GPCR example illustrates how advantageous it is to employ model cells that reflect the disease relevant drug-targeted tissue as closely as possible not only in a phenotypic, but also in a 'target-focused' screening campaign.

The importance of assessing compound efficacy in the appropriate human cell type even for a target-focused approach was further underlined by a recently published study in which clinically failed γ-secretase modulators (GSMs) were re-evaluated in iPS cell derived model cells [12]. Although these presumed GSMs were clearly efficacious in amyloid precursor protein (APP)-overexpressing, non-neuronal cell models, they failed to elicit any effect on

Aβ42/Aβ40 ratios in the iPS cell derived human neurons at therapeutic concentrations.

Although permitting the pharmacological profiling of drug candidates in a more physiological environment, the employment of iPS cell derived model cells issues a challenge to the sensitivity of the assay detection technologies, as elaborated upon below.

Higher sensitivity for physiological signaling with an improved assortment of enabling tools

Whereas a target-driven pathway and concurring detection signals may be forcefully enhanced in a target-overexpressing recombinant system, a feeble endogenous signaling pathway may be rather difficult to measure. This is probably one of the reasons why so far only 'medium' throughput screening campaigns have been carried out with iPS cell derived neuronal precursors or differentiated neurons [5,6]. A pharmaceutical HTS campaign covering a larger collection of several hundred thousand compounds requires a robust signal-to-noise ratio, which is of course more easily established under conditions of target overexpression and concomitant signal enhancement. Fortunately, however, drug discovery–suitable detection techniques have advanced considerably over recent years, enabling the robust and reproducible measurement of endogenous signaling events under more physiological conditions. We briefly discuss three particularly suited techniques below, the amplified luminescent proximity homogeneous assay (Alpha^{TM}) format, the high content screening (HCS) format, and the high throughput reverse transcriptase polymerase chain reaction (RT-PCR).

Amplified luminescent proximity homogeneous assay (Alpha^{TM})

An exceedingly sensitive technique based upon luminescent oxygen channeling [13] has been commercialized under the trade name Alpha^{TM}. The Alpha^{TM} technology relies on monitoring the formation of pairs between two types of latex particles, which are referred to as donor and acceptor beads.

How can this technique be applied to monitor an intracellular signaling pathway? In one biological application, a sandwich of antibodies is employed, one attached to the donor bead and one to the acceptor bead. To monitor a signaling cascade, for example, involving a phosphorylation step, one of the sandwich antibodies may be directed against a nonphosphorylated epitope of the protein substrate and the second sandwich antibody could detect the phosphorylated substrate only. By this means, a signaling assay may be established by stimulating iPS cell derived model cells in a microplate well and adding the antibody-conjugated donor and acceptor beads in a cellular lysis buffer for detection of the phosphorylation. In an exemplary application (Figure 2), iPS cell derived iCell^{TM} neurons (Cellular Dynamics International, Madison, WI, USA) were stimulated with brain-derived neurotrophic factor (BDNF), and BDNF-induced phosphorylation of tropomyosin receptor kinase B (TrkB), extracellular signal-regulated kinase (ERK), and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) were monitored after cellular lysis using respective Alpha^{TM} detection reagents. In a drug discovery project attempting to identify positive modulators of TrkB, the Alpha^{TM} assay on TrkB phosphorylation directly demonstrates TrkB target engagement for a drug candidate. The ERK assay and the CREB assay may then secondarily establish that the TrkB-engaging drug candidate also modulates both pathophysiologically relevant downstream signaling pathways of TrkB.

Although the Alpha^{TM} technique is well suited to quantify the mean pharmacological response of a cell population, the Alpha^{TM} format does not allow for the distinction of cellular subpopulations within a single microplate well and it does not enable the investigation of, for instance, drug-induced intracellular protein transport or morphological change. The latter areas of pharmacological analysis where

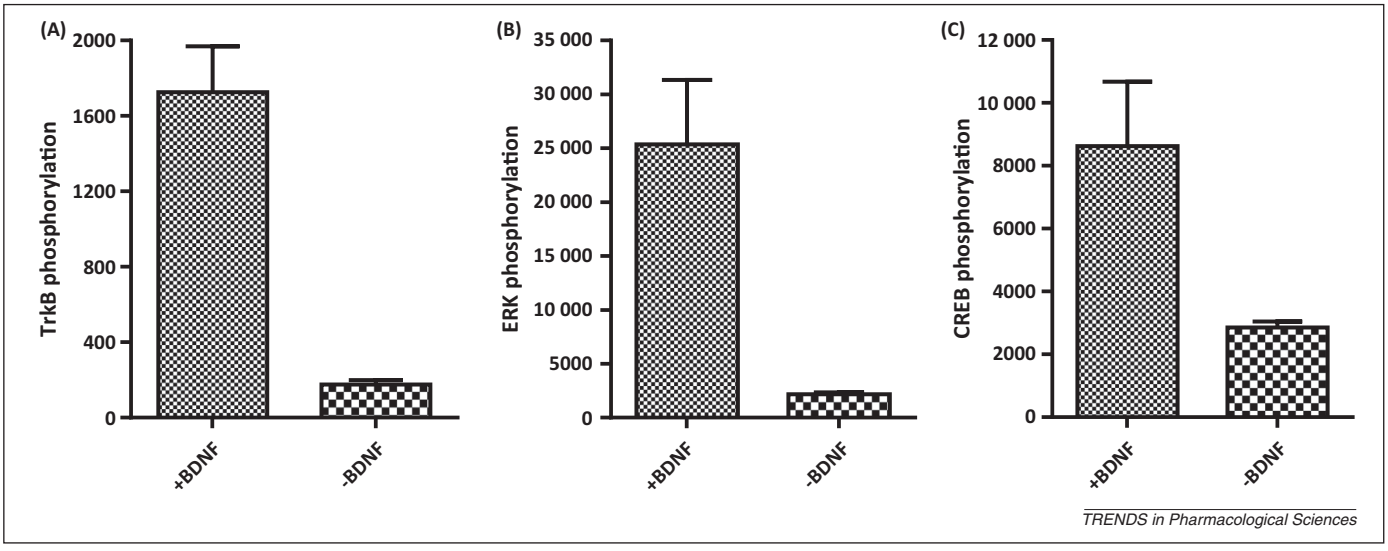

Figure 2. Alpha^{TM} measurement of phosphorylation events in induced pluripotent stem (iPS) cell derived iCell^{TM} neurons. iPS cell derived iCell^{TM} neurons (Cellular Dynamics International, Madison, WI, USA) were cultured for 7 days in 384-well microplates. Cells were stimulated with brain-derived neurotrophic factor (BDNF), then lysed, and analyzed for intracellular phosphorylation events. BDNF-induced signaling was monitored using the respective Alpha^{TM} detection reagents for phosphorylation **(A)** of Tyr706 in tropomyosin receptor kinase B (TrkB), **(B)** of the Thr-X-Tyr motif in extracellular signal-regulated kinases 1/2 (ERK1/2), and **(C)** of Ser133 in cAMP response element-binding protein (CREB).

the Alpha™ format fails are suitably covered by HCS, as described in the following section.

High content screening (HCS)

Apart from measuring a signaling event in the cellular lysate as described above, cellular signaling may also be monitored in the intact living cell or at least in the framework of the formaldehyde-fixed cellular protein skeleton, for example, by using a technique referred to as HCS. HCS is typically defined as automated fluorescence microscopic imaging combined with automated image analysis [14,15]. In HCS technology, an adherent cell layer at the bottom of a microplate well is labeled with several fluorophores, and the biomolecules of interest are observed by fluorescence microscopy in parallel at different wavelengths. Although the above-described Alpha™ technique only allows for measuring the concentration, phosphorylation state, or degradation condition of proteins of interest in the cellular lysate, HCS additionally enables the localization of the target protein in cellular substructures of the model cells. Therefore, HCS may also be utilized as a nondestructive and sensitive technique to monitor protein translocations with respect to subcellular organelles and structures. In addition, HCS may serve to detect single cellular responses that only occur in a subpopulation of the examined cells. However, the added information does not come without an extra cost: although the detecting antibodies may be identical to the ones that are used for the Alpha™ approach, HCS sample preparation frequently employs cumbersome cellular fixation, permeabilization and washing steps that are not required in the homogeneous Alpha™ protocols. Along the same lines, it is much more difficult to measure nonadherent cell types by HCS than by Alpha™ because HCS devices typically focus on an optical plane adjacent to the bottom of the microplate well. Furthermore, HCS fluorescence emission detection is typically not as sensitive as the singlet oxygen-amplified Alpha™ format. And finally, the interpretation of a HCS experiment necessitates the onerous design of an appropriate image analysis algorithm. For these reasons, it should be assessed on a case-by-case basis, whether the supplementary information of intracellular protein localization or the potential elucidation of a cellular subpopulation response as provided by the HCS format is worthwhile given the added experimental effort and the concomitant loss of sensitivity.

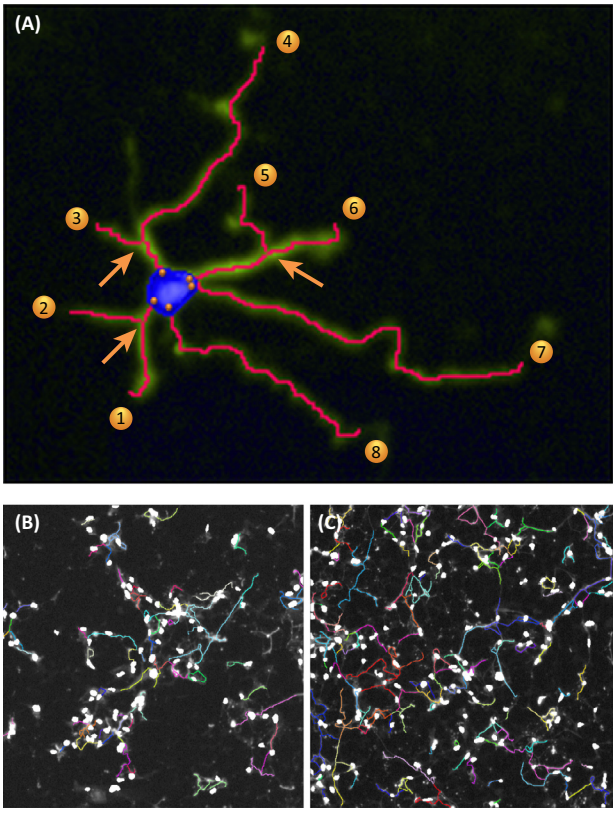

Figure 3. Quantification of neurite arborization using high content screening (HCS). Induced pluripotent stem (iPS) cell derived dopaminergic neurons were fixed using formaldehyde, and then permeabilized. Nuclei were fluorescently labeled using 4′,6-diamidino-2-phenylindole (DAPI), and neuron-specific class III β-tubulin (Tuj1) was immunostained with an appropriate antibody. The fluorescence microscopic images from a high throughput HCS device were analyzed for neurite arborization using a dedicated algorithm of the Acapella™ software (kindly supported by Dr Jacob Tesdorpf, PerkinElmer, Hamburg, Germany). **(A)** Image with example neuron from the PerkinElmer archive to illustrate the image analysis algorithm: briefly, the algorithm segmented the cells into nucleus-adjacent neuronal soma (blue) and neurite traces (red). Furthermore, it identified budding sites (orange dots in the somatic region) of neurites in the soma, it determined neurite branching nodes (marked by arrows), and it numbered neurite endpoints (marked by numbered orange circles). Same numbers per area of iPS cell derived dopaminergic neurons were **(B)** treated with rotenone alone or **(C)** with rotenone in combination with the leucine-rich repeat kinase 2 (LRRK2) inhibitor CZC-25146. Various facets of neuronal plasticity were quantified: cell number per area, number and length of neurites per neuron, and the number of neurite branching nodes per neuron. CZC-25146 was found to reduce the rotenone-driven cellular toxicity while leaving the various facets of neurite arborization untouched.

Figure 3A gives an example of how HCS can be applied to an iPS cell derived disease model. Parkinson's disease (PD) is primarily characterized by the specific degeneration of midbrain dopaminergic neurons. The G2019S mutation in *leucine-rich repeat kinase 2* (*LRRK2*) is the most common known cause of familial (PD) and is thought to increase the risk a patient will develop PD by increasing LRRK2 kinase activity [16]. iPS cell derived dopaminergic neurons can be used to model the contribution of LRRK2 kinase activity to the risk of developing PD by adding stressors such as rotenone, which chemically induces PD-like symptoms *in vitro* and *in vivo* [17]. Neurite arborization can be used as a measure of neuronal health and function. In our example, the neurite arborization of a stem cell derived dopaminergic neuron was quantified using an adequate HCS image analysis algorithm, which enabled measuring the number and length of neurites, and the number of neurite branching nodes (Figure 3B). This analysis can be repeated for cultures of iPS cell derived dopaminergic neurons treated with different conditions: for example, with rotenone alone or with rotenone in combination with the LRRK2 kinase inhibitor CZC-25146 [18]. In this example, inhibition of LRRK2 kinase activity is thought to be a possible strategy to treat PD, suggesting that this approach could be used to evaluate novel drug candidates being developed [19]. Indeed, CZC-25146 reduced rotenone-driven neuronal apoptosis while leaving the neurite plasticity unscathed in the employed experimental approach. Alternatively, a genetic approach can be used to genetically correct the G2019S mutation in iPS cells and compare the phenotype in differentiated dopaminergic neurons using

HCS [20]. Additional advantages of HCS are the ability to evaluate a large number of samples in combination with automation as well as the ability, through immunostaining for specific markers, to focus only on the cells of interest (in this case midbrain dopaminergic neurons), even in heterogeneous cultures.

In summary, the spotlight of HCS is on assigning effects of drug candidates to subcellular protein translocations or morphological features and on providing cell population statistics, for example, identifying a drug-responsive subpopulation of cells. Although the below-described high throughput RT-PCR cannot cover these facets of the drug response, the latter technology distinguishes itself as a tool of extraordinary sensitivity down to the single molecule mRNA level.

High throughput reverse transcriptase polymerase chain reaction (RT-PCR)

PCR was introduced approximately 30 years ago [21]. Since then, dedicated devices have been developed that act as PCR-driving thermocyclers and at the same time monitor the PCR-based amplification process using a fluorescent signal. To employ PCR for the quantification of messenger RNA (mRNA), PCR must be combined with a reverse transcription (RT) reaction. Two fairly recent technological leaps now enable the application of the RT-PCR principle to high throughput drug discovery. Firstly, a device introduced by Roche Applied Science in 2009, the LightCycler™ 1536, implemented the RT and PCR reactions in the 1536-well microplate format [22]. Secondly, Roche Applied Science and LabCyte Inc. jointly demonstrated that the lysate of drug-treated cells may directly be transferred into a combined RT-PCR bioreagent mixture, reducing the processing time to approximately 45 min per 1536-well plate and thereby catapulting RT-PCR into the realm of high throughput technology [23].

In preparation of an RT-PCR approach, next-generation sequencing (NGS) technology [24] lends itself ideally to measure the transcriptome changes of disease-modeling cells in response to, for instance, the positive or negative modulation of a particular disease-relevant signaling pathway. Although the details of NGS technology vary with different commercial vendors [24], all transcriptional applications of NGS employ highly parallelized sequencing of cDNA. Therefore, NGS technology can also monitor the modulation of previously unknown transcripts or splicing variants, thereby covering a broader transcriptome space than microarray-based techniques.

One of the NGS-identified, disease-driven marker transcripts is then selected for the RT-PCR assay and appropriate PCR primers are designed. To raise the specificity of monitoring the selected transcript only, a fluorogenic oligonucleotide probe with complementarity to the transcript may be included in the amplification reaction [25,26]. Fluorogenicity originates from labeling the probe with a fluorophore at the 5′ end and an appropriate quencher at the 3′ end, so that the 5′–3′ exonuclease activity of the PCR-driving *Thermus aquaticus* DNA (*Taq*) polymerase releases both labels during the PCR reaction in an amount that is linearly correlated to the amount of the targeted PCR product.

In the given exemplary application (Figure 4), iPS cell derived iCell™ neurons were stimulated with BDNF for 6 h. By NGS, VGF (nonacronymic) had been identified as a BDNF-dependent marker transcript. Accordingly, the cellular lysates were analyzed for a BDNF-modulated change of VGF transcription using a LightCycler™ device. VGF transcription was increased approximately 4-fold after BDNF stimulation.

In summary, all three above-described detection technologies, Alpha™, HCS, and RT-PCR, possess some particular advantages and present some specific challenges. However, the information that can be obtained from all three technologies is partially complementary, and the combined use of all three methods may disclose diverse facets of a cellular drug effect. Based upon their sensitivity, all three techniques are amenable to extreme miniaturization, which, for example, opens the route towards the 1536-well microplate format, thereby reducing the required

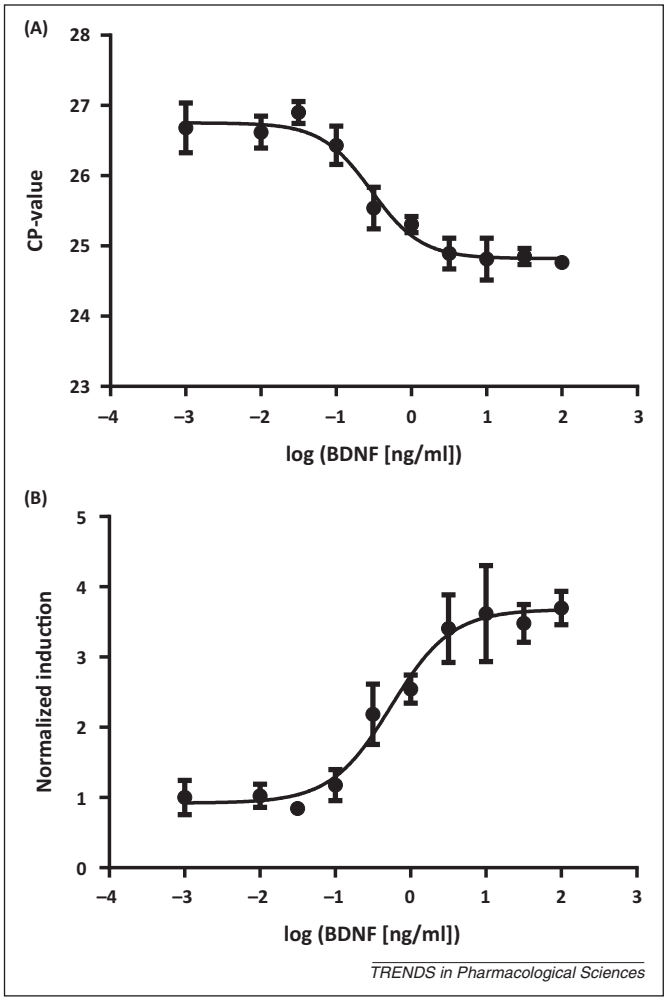

Figure 4. High throughput reverse transcriptase polymerase chain reaction (RT-PCR) to analyze brain-derived neurotrophic factor (BDNF)–modulated transcriptional changes. Induced pluripotent stem (iPS) cell derived iCell™ neurons (Cellular Dynamics International, Madison, WI, USA) were cultured for 7 days in 384-well microplates. Cells were stimulated with BDNF, and then lysed. Without prior RNA purification, the lysate was directly subjected to RT-PCR in the LightCycler™ device, using a primer-probe set for VGF. **(A)** When observing the increase of the fluorescence signal versus the number of thermal PCR cycles, VGF transcription was measurable at a crossing point (Cp) [66] value of 26.8 with vehicle stimulation, and at a Cp value of 24.8 with saturating BDNF stimulation. **(B)** Accordingly, VGF transcription increased approximately 4-fold with saturating BDNF stimulation compared with vehicle stimulation.

number of model cells per well significantly. Another route forward with respect to the generation of sufficiently high cell numbers for a large-scale screening campaign is to facilitate the cell amplification process itself, as described in the following section.

Expandable neural progenitors as a means of upscaling iPS cell based neuron generation to resource primary screening

Because embryonic stem (ES) and iPS cells are pluripotent, protocols aimed at directing their differentiation into specific lineages including neuronal subtypes frequently result in heterogeneous cultures with inefficient yields and large batch-to-batch variability [27–29]. Although these characteristics may be compatible with proof-of-concept studies, they prohibit HTS campaigns. One attractive alternative is to differentiate pluripotent stem cells into an expandable intermediate cell population that is multipotent and committed to the lineage of interest, such as the central nervous system (CNS). For example, neural stem (NS) cells are multipotent stem cells that are found in the CNS of various animal species [30–35] and even human brain tissue [36,37]. NS cells may be cultured *in vitro* as immortal cell lines and have the potential to differentiate into the three major CNS lineages: neurons, astrocytes, and oligodendrocytes [36]. Consequently, the derivation of cells that are similar to NS cells from pluripotent stem cells would allow for amplification to cell numbers that could suffice as a resource for a primary screening campaign and minimize the heterogeneity that typically results from the direct differentiation of pluripotent stem cells. Table 1 summarizes the characteristics of existing expandable neural progenitors.

One such expandable population is 'neural rosette' stage cells, which are radially organized, columnar epithelial cells formed by human ES (hES) cells as an intermediate stage during neuronal differentiation [38–41]. These 'neural rosette' cells have the potential to differentiate into every neuronal subtype in the CNS including cortical neurons, striatal neurons, midbrain dopaminergic neurons, and spinal motor neurons [40,42–48]. Initially, it was only possible to cultivate rosette cells for very few passages before they lost this broad CNS potential [43]. Subsequently, using fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), which direct the self-renewal of *in vivo* derived NS cells [31], it became possible to derive the rosette-like, so-called 'long-term neuroepithelial stem' (lt-NES) cells from both hES and iPS cells [38,49]. These lt-NES cells retained their multipotency even after an extended proliferation period and preserved their responsiveness to instructive regionalization cues, for example, towards ventral midbrain-type tyrosine hydroxylase-positive cells or towards motor neurons. The utility of lt-NES cells has been demonstrated through their use in disease modeling [12,50,51], and even automated screening [6]. However, the respective differentiation efficacies are still to some extent limited, which is probably a reflection of the use of FGF2 and EGF for self-renewal [52]. lt-NES cell derived neurons have been successfully used to generate at least two disease models. One report used lt-NES cell derived neurons to model Alzheimer's disease (AD) [12,51]. It was shown that AD phenotypes using lt-NES cell derived neurons were not ameliorated by nonsteroidal anti-inflammatory drugs similar to AD patients. lt-NES cell derived neurons have also been used to establish an *in vitro* disease model for Machado–Joseph disease by demonstrating that disease-associated accumulation of ATXN3 protein is induced by glutamate excitotoxicity [50]. Unfortunately, lt-NES cells require splitting three or more times per week at low ratios, which makes expansion to billions of cells for HTS doable [6], but rather tedious and cumbersome. As a result, the one published HTS campaign using lt-NES cells is for compounds that modulate their proliferation and viability [6].

Recently, primitive NS cells (pNS cells) were derived and expanded from hES and iPS cells [53]. pNS cells are dependent on inhibition of glycogen synthase kinase 3 (GSK3), transforming growth factor-β (TGF-β), and Notch signaling pathways in combination with recombinant purified leukemia inhibitory factor (LIF), and can be efficiently differentiated into dopaminergic neurons. One study has generated a model of mutant LRRK2-induced PD using pNS cells [54]. It was shown that pNS cells with *LRRK2* G2019S had aberrant nuclear envelope morphology and exhibited deficits in differentiation. These phenotypes could help to understand the hippocampal lesions that can be observed in patients. Nevertheless, the use of LIF makes large-scale use of pNS cells for HTS impractical and expensive.

Table 1. Overview of existing expandable neural progenitor cell types

		Cultured cell type			
		NS cells	lt-NES cells	pNS cells	smNP cells
	Origin	Fetal/adult brain	Human PS cells	Human PS cells	Human PS cells
	Immortal self-renewal?	Yes	Yes	Yes	Yes
	Self-renewal with only small molecules?	No	No	No	Yes
Differentiation potential	Peripheral neurons (neural crest-derived)	No	Not tested	No	Yes
	Mesenchymal cells (neural crest-derived lineage)	No	Not tested	No	Yes
	Neural rosettes (neural tube lineage)	No	Not tested	Yes	Yes
	Neural progenitors (neural tube lineage)	Yes	Yes	Yes	Yes
	Ventral neural tube (neural tube-derived lineage)	Mixture	Yes	Yes	Yes
	mDANs (ventral neural tube-derived lineage)	No/very few	Yes	Yes	Yes
	MNs (ventral neural tube-derived lineage)	No/very few	Yes	Yes	Yes
	Astrocytes	Yes	Yes	Yes	Yes
	Oligodendrocytes	Yes	Yes	Not reported	Yes

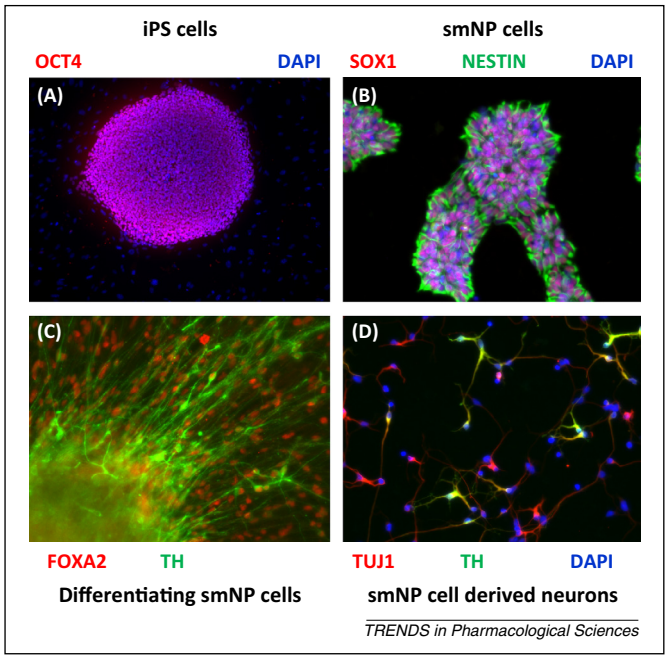

Figure 5. Derivation and dopaminergic differentiation of small molecule neural progenitor (smNP) cells. Induced pluripotent stem (iPS) cells were obtained by reprogramming dermal fibroblasts from human donors using retroviral vectors that encoded for OCT4, SOX2, KLF4, and c-MYC. **(A)** A derived iPS cell colony was immunostained with antibody raised against the pluripotency marker OCT4 (red); nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). **(B)** Colonies of smNP cells derived from iPS cells were immunostained with antibodies raised against neural progenitor markers SOX1 (red) and NESTIN (green); nuclei were stained with DAPI (blue). **(C)** smNP cell differentiation was monitored by immunostaining with antibodies raised against FOXA2 (red), a floor plate marker; and against tyrosine hydroxylase (TH; green), a marker of dopaminergic neurons. Nuclei were stained with DAPI (blue). **(D)** smNP cell derived dopaminergic neurons were immunostained with antibodies raised against neuronal marker Tubulin Beta Class III (TUBBIII/TUJ1; red); and against TH (green); nuclei were stained with DAPI (blue).

We have recently described [52] the derivation of so-called small molecule neural progenitor (smNP) cells (Figure 5) that have characteristics ideally suited to large-scale fermentation and subsequent microplate differentiation culture. smNP cells, which are derived from either hES or human iPS cells, provide a source of cells capable of robust and immortal clonal expansion without costly growth factors or cumbersome manual steps. In addition, the smNP cells may be efficiently differentiated into lineages such as midbrain dopaminergic and motor neurons. smNP cell derived dopaminergic neurons differentiated from patients with PD with *LRRK2* G2019S displayed an increased proportion of apoptotic cells under conditions of oxidative stress in comparison to neurons differentiated from iPS cells generated from age- and gender-matched controls [52]. These observations were consistent with results obtained with dopaminergic neurons that had been directly differentiated from iPS cells [20,28]. Hence, the properties of smNP cells are ideal for large-scale disease modeling including HTS.

Pilot scale screenings using expandable neural progenitor cells

Currently, there are no published reports of a HTS campaign using expandable neural progenitors. However, at least two papers have been published for pilot scale screenings using libraries of up to 1000 compounds. In one report the authors used an *in vitro* model of amyloid-β-induced neurotoxicity in stem cell derived neurons, which identified small molecules ameliorating the toxicity [5]. It should be noted however that neural progenitor cells used in this study were expanded only for two passages.

Another pilot scale screening used undifferentiated smNP cells directly [6]. In this study, the authors identified several compounds that enhanced survival and proliferation of undifferentiated lt-NES cells, including a Cdk2 modulator. These studies also demonstrate the scalability of these assays, as long as appropriate methods or read-out are implemented in the experiments.

Concluding remarks

One of the cardinal barriers during clinical development of pharmaceutical drug candidates is the divergence of drug efficacies in heterologous disease-modeling cells as compared with human patients. This translational dilemma is further aggravated by the limited predictivity of many animal disease models [55]. In this context, human iPS cell derived model cells may be better suited to extrapolate from *in vitro* disease-relevant human biology to *in vivo* clinical studies. In this review, we focused on iPS cell derived neurons and their applicability to large-scale HTS. The value of these model cells was demonstrated for both target-focused and phenotypic compound screening as well as drug profiling campaigns. In various examples, we verified that state-of-the-art detection technologies are available for the iPS cell derived cells that are adequately sensitive to monitor cellular signaling in the more physiological context of these model cells. Finally, we reviewed expandable neural progenitors as a promising approach to generating the large numbers of iPS cell derived neurons required for a HTS campaign. Several expandable progenitors have been reported including NS, pNS, lt-NES, as well as smNP cells, and smNP cells have properties best suited to HTS campaigns because (i) they could robustly be expanded to virtually unlimited numbers without costly growth factors and labor intensive maintenance cell culture protocols; (ii) they displayed a broad differentiation potential; and (iii) they could be differentiated with high efficiency to dopaminergic cells that displayed physiological characteristics with regard to, for instance, electrophysiology and protein composition.

In the following years, we expect to see a consolidation of the technologies covering the large-scale amplification of neuronal precursor cells at commercial vendor companies, thereby facilitating the high throughput application of iPS cell based model cells in drug discovery. Furthermore, we envision that the HTS facilities of larger neuropathology-focused pharmaceutical companies will enable automated microplate-based neuronal differentiation culture. Additionally, we anticipate further complexity in the design of drug discovery assays, for example, employing mixed model cell cultures such as mixtures of specific iPS cell derived neurons and astrocytes. In this context, it will be of fundamental importance that the differentiation protocols towards various neuronal subtypes should be established in a sufficiently robust and reproducible manner, so as to meet the requirements of automated large-scale cell culture.

Ultimately, a shift towards iPS cell based model cells for HTS will need to establish its added value through lower attrition rates of drug candidates in clinical trials.

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