High-content screening of small compounds on human embryonic stem cells

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Abstract

Human ES (embryonic stem) cells and iPS (induced pluripotent stem) cells have been heralded as a source of differentiated cells that could be used in the treatment of degenerative diseases, such as Parkinson’s disease or diabetes. Despite the great potential for their use in regenerative therapy, the challenge remains to understand the basic biology of these remarkable cells, in order to differentiate them into any functional cell type. Given the scale of the task, high-throughput screening of agents and culture conditions offers one way to accelerate these studies. The screening of small-compound libraries is particularly amenable to such high-throughput methods. Coupled with high-content screening technology that enables simultaneous assessment of multiple cellular features in an automated and quantitative way, this approach is proving powerful in identifying both small molecules as tools for manipulating stem cell fates and novel mechanisms of differentiation not previously associated with stem cell biology. Such screens performed on human ES cells also demonstrate the usefulness of human ES/iPS cells as cellular models for pharmacological testing of drug efficacy and toxicity, possibly a more imminent use of these cells than in regenerative medicine.

Introduction

ES (embryonic stem) cells, obtained from a blastocyst stage embryo [1], and iPS (induced pluripotent stem) cells, derived by somatic cell reprogramming [2,3], have the ability to self-renew in culture while remaining pluripotent. These features make human ES/iPS cells a potential renewable source of all somatic cell types for use in regenerative therapies. However, the clinical potential of ES cells is hampered by many obstacles, such as the difficulties in achieving directed differentiation of ES cells in an effective and efficient manner [4]. Indeed, during embryonic development, stem cells reside in complex microenvironments that present them with a plethora of signals to instruct them on their developmental pathways. Deciphering these signals and mimicking them in vitro to drive the differentiation of human ES cells to a desired cell type presents an imposing challenge.

Chemical genetics approach in studying stem cell biology

The complementary approach to classical genetics, where small molecules are exploited to probe biological functions, is termed ‘chemical genetics’ [8,9]. Whereas classical genetics establishes genotype–phenotype relationships through means of genetic manipulation, chemical genetics uses small molecules that intervene in signalling pathways through direct interaction with proteins to unravel relationships between proteins and phenotypes. By analogy to classical genetics, small compounds in chemical genetics are equivalent to mutations in classical genetics. In fact, given that chemicals can be removed from cells by simple washing, they are analogous to conditional mutations.

Although small molecules allow reversible temporal and dose-dependent control of protein function, one of their major limitations is that a single compound often affects multiple proteins and, ipso facto, multiple pathways simultaneously [10,11]. The promiscuous binding of a compound to a range of proteins seriously impedes efforts to ascertain its true molecular targets and mechanism of action. However, as long as the binding properties of compounds are known, their pleiotropic nature can also be beneficial, for example, if a compound affects a combination of proteins such that the overall effect results in a phenotype of interest. In fact, genetic studies in various models have shown that inactivation of a single gene is often not enough to lead to a discernable phenotype, due to mechanisms of genetic robustness [12,13]. Thus treatment with a single small molecule can be analogous to simultaneous inactivating mutations in several genes.

Key words: drug discovery, embryonic stem cell, high-content screening, high-throughput screening, induced pluripotent stem cell.

Abbreviations used: ES, embryonic stem; iPS, induced pluripotent stem; Oct4, octamer-binding protein 4; SSEA3, stage-specific embryonic antigen 3.

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Using small molecules to influence and direct stem cell fates has a long history. All-trans-retinoic acid has been used for over 30 years to induce differentiation of both mouse and human embryonal carcinoma cells [14,15]. Other examples include hexamethylenebisacetamide and DMSO, which have been utilized to promote differentiation of embryonal carcinoma cells and Friend erythroleukaemia cells respectively [16,17].

**High-throughput chemical screens on ES cells**

Arguably the biggest advantage of using small compounds as opposed to producing genetic mutations to analyse cellular mechanisms is that small molecules can be easily applied to cells and are thus particularly amenable to high-throughput phenotype-driven screens. The phenotype-driven high-throughput approach involves treatment of cells with thousands of small compounds in an initial primary screen, the aim of which is to filter out compounds that show undesirable effects and identify the compounds that give rise to a phenotype of interest. Such compounds, termed ‘hits’, are tested again in a second confirmatory screen, and the confirmed hits are analysed in-depth for the mechanism of action and the structure–activity relationship, in which the chemical structure is correlated with its biological activity.

High-throughput screens on human ES cells were relatively slow to emerge in the field and most such screens are still being performed on mouse ES cells. The main obstacle to be overcome for the high-throughput approach on human ES cells is their normal propensity to grow in colonies and their poor survival after dissociation into single cells. This affects the reproducibility of plating cells in multi-well plates, which must be high if a high-throughput screen is to be robust. In fact, this shortcoming prompted Watanabe et al. [18] to initiate a screen to identify compounds that promote survival of human ES cells. The Rho-kinase inhibitor Y-27632, which was identified in this way, is now widely used to increase cloning efficiency of human ES cells [19,20].

Overall, chemical screens in the ES field can be broadly divided into screens that aim to identify compounds that: (i) promote human ES cell survival [18,21,22], (ii) promote ES self-renewal [21,23], (iii) promote differentiation of ES cells [21,22,24,25], and (iv) promote somatic cell reprogramming [26–29].

Given that the phenotype-driven approach makes no *a priori* predictions as to the function of the protein(s) involved, it provides a powerful strategy to identify proteins not previously associated with a certain phenotype or biological process. Indeed, discovery of the effect of Y-27632 [18] on survival of human ES cells was the first indication of Rho-kinase involvement in this process.

**High-content screening assay**

In addition to the appropriate choice of a cell line and a selection of compounds (chemical library) for screening, the design and quality of the screening assay are critical for a successful outcome. The assay must be designed to detect changes in the phenotype of interest and must be rigorously tested and validated for performance and robustness [30,31]. Reporter assays commonly employed in high-throughput screens read out the effects of small compounds on a single physiological feature or a single pathway [32,33]. Owing to a limited read-out, this approach does not detect unanticipated effects, neither does it record important indicators of altered cell physiology, such as those manifested as changes in cell morphology, potentially losing valuable information. Furthermore, the changes in high-throughput screens are usually assessed at a cell population level and so obscure detection of the response of an individual cell to treatment. Detection of responses on a cell-by-cell basis may become important due to the heterogeneity in stem cell cultures, which may be inherent to the cells themselves or a result of spontaneous differentiation. Such heterogeneity makes stem cells particularly prone to responding to signalling cues in a variable manner and necessitates establishment of screening methods that can disentangle this variability by analysing single-cell responses to signalling cues [34].

High-content screening addresses the need for better assessment of a compound’s activity by reporting on a variety of cellular features in the primary screen. It can be performed by automated imaging of high-resolution images of cells labelled with appropriate fluorescent probes, followed by automated image analysis. Information extracted from such images, regarding cell morphology, intracellular patterns, intensity of fluorescent signals and their spatial distribution, provides a quantitative, objective and sensitive output [35]. It is due to this large amount of information obtained that image-based screening is often termed ‘high content’.

**High-content screen set-up**

High-content screens commonly rely on the use of fluorescent reporters or fluorescent probes that can be analysed *in situ* in the cells and report on changes in the intensity as well as the localization of the fluorescent signal. Multiplexing several different reporters/probes enhances the power of the assay to profile the compound’s activity on cells, particularly when they read out changes in distinct pathways. In our screen for compounds that affect human ES cell proliferation and/or pluripotency, we utilized the cell-surface pluripotency marker TRA-1-60 to score the undifferentiated status of the cells and the DNA intercalation dye Hoechst 33342 to visualize the nuclei [22]. Our screening protocol consisted of plating Shef4 human ES cells in optical-bottom 96-well plates in the presence of test compounds. After 5 days, the cells were fixed and stained for TRA-1-60 (detected using a FITC-labelled secondary antibody) and Hoechst 33342 (Figure 1). Plates were imaged using an automated epifluorescent microscopy system (InCell Analyzer 1000; GE Healthcare) (Figure 1a). The resulting image stacks were imported into Developer Toolbox™ (GE Healthcare) software for image analysis.
Figure 1 | Outline of the high-content screening assay for detecting compounds affecting human ES cell survival and/or pluripotency

(a) Imaging ES cell colonies. A field imaged in a well of a 96-well plate after staining with Hoechst 33342 (left-hand panel) and TRA-1-60 (right-hand panel) using the InCell Analyzer microscopy system (GE Healthcare). (b) Image analysis and data output from the imaging. Nuclei stained for Hoechst 33342 were segmented (upper panel). Segmentation of human ES colonies in the image (lower panel) was achieved using image analysis algorithms that ‘dilated’ segmented nuclei, causing closely positioned nuclei of human ES cells in colonies to merge, marking accurately the colony borders. Size filtering enabled exclusion of feeder cells from further analysis. Images were analysed for a variety of measures at three different levels: single cell, colony and population level.

Image analysis

Image analysis starts by segmentation of the objects of interest in the image, i.e. recognizing individual cells. The use of a DNA-intercalating dye, Hoechst 33342, enabled us to identify each individual nucleus in the image (Figure 1b). The segmentation of the nuclei (and, as a result, of the cells) then enabled the interrogation of each ‘cell’ for its FITC fluorescent signal (TRA-1-60).

One of the defining features of human ES cells is their growth in colonies, and colony morphology is an important indicator of cell state. For example, increased spacing between the nuclei within a colony is associated with non-neural differentiation, whereas neuronal differentiation is characterized by the formation of neural rosettes [36]. These morphological changes may precede changes in marker expression. To extract measurements regarding colony morphology, image analysis in our screening assay included identification of individual colonies in the image. This was achieved by applying a ‘dilation’ function, i.e. expanding a shape a fixed distance in all directions from each of the previously segmented nuclei. This has the effect of merging the image of the nuclei and, as the amount of cytosol in ES cells is small and the cell margins are close to the nucleus, the shape of colonies is accurately demarcated in the image (Figure 1b).

Measurements

The segmentation of individual cells as well as colonies enabled analysis at several levels to extract information about the effects of drugs on the system (Figure 1b). At the highest level, information was extracted well-by-well, and measures included colony number, size and shape changes, fluorescent intensity values of stained markers, and total counts of nuclei to detect changes in the ES cell phenotype (Figures 2a and 2b). A further refinement was evaluating results on a colony basis (Figure 2c). Analysis of colony parameters is particularly useful when reporting the results from experiments that utilize single cells as their starting point. For instance, a higher number of colonies upon treatment with a compound indicates better attachment and/or survival of cells at the time of seeding, whereas bigger colonies reflect a higher proliferative capacity of the cells.

Ultimately, the power of high-content analysis comes from its ability to analyse cell cultures on a cell-by-cell basis (Figure 3). The co-localization of markers can be probed further for associated morphological parameters (cell size and shape) as well as spatial distribution of the markers. Given the highly spatial nature of tissue differentiation (e.g. dorsal horn neurons are phenotypically distinct from ventral horn neurons), this ability could prove important when interrogating, for example, small-molecule effects on the efficiency of neural differentiation.

Although the presence or absence of a certain marker [e.g. SSEA3 (stage-specific embryonic antigen 3) or Oct4 (octamer-binding protein 4); officially termed POU5F1 (POU class 5 homeobox 1)] on the cell can be easily interpreted, the biological meaning of some of the cellular measurements that can be obtained by high-content image analysis (e.g. nuclear shape and size) is less clear. However, these measurements can still be used to detect deviation from the normal cell phenotype. By measuring multiple cellular features simultaneously, Loo et al. [37] showed that compounds from the same chemical category affect cell phenotype in a similar way and that it was possible to streamline assignment of a compound to a chemical category on the basis of measured cellular features.
**Future prospects**

Modern drug discovery is limited by the lack of suitable models for primary screens. The paradigm of using chemical genetics to uncover fundamental aspects of stem cell biology exemplifies the tractability of human ES cells for high-throughput screens in drug discovery. The humanized *in vitro* models that have been used by the pharmaceutical industry mostly involve primary cells that can only be cultured for a limited time or transformed cell lines that often differ significantly from the cells of the original tissue. In contrast, human ES cells can self-renew in culture indefinitely, allowing their production on a large scale. Unlike transformed cell lines, ES cells generally have a normal genetic composition and, as they are pluripotent, they can be induced to differentiate into most somatic cell types, including otherwise experimentally inaccessible cells of the central nervous system. The developing availability of iPS cells is particularly promising for drug discovery and validation of drugs using patient-specific cells, which may carry specific disease-associated mutations [38–40].

High-content screening maximizes the information obtained from a chemical screen as it measures multiple parameters simultaneously not only on a well-by-well population level, but also on a single-cell basis. This makes it a very powerful and sensitive method for detection of compounds with distinct effect on cell phenotypes and enables better understanding of complex interactions of signalling pathways governing stem cell biology. In pharmacological screens, parallel assessment of multiple cellular features should enhance the hit prediction and aid in decreasing attrition rates (compounds that fail at later stages of drug development due to factors such as toxicity). Thus convergence of use of human ES/iPS cells and high-content screening technologies should provide a powerful tool for future drug discovery efforts.

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**References**


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