Stem cell technology for drug discovery and development

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Stem cells have enormous potential to revolutionise the drug discovery process at all stages, from target identification through to toxicology studies. Their ability to generate physiologically relevant cells in limitless supply makes them an attractive alternative to currently used recombinant cell lines or primary cells. However, realisation of the full potential of stem cells is currently hampered by the difficulty in routinely directing stem cell differentiation to reproducibly and cost effectively generate pure populations of specific cell types. In this article we discuss how stem cells have already been used in the drug discovery process and how novel technologies, particularly in relation to stem cell differentiation, can be applied to attain widespread adoption of stem cell technology by the pharmaceutical industry.

Introduction

Stem cells are extraordinary cells, capable of self-renewal and differentiation to mature somatic cell types *in vivo* and *in vitro*. Different types of stem cells exist that differ in their longevity in culture and in the variety of mature cell types they can generate (Fig. 1). Pluripotent stem cells, either embryonic or induced, are the most potent stem cells [1]. They are capable of infinite self-renewal *in vitro* and can generate all somatic cell types. By contrast, adult stem cells are restricted in their differentiation potential [2], e.g. haemopoietic stem cells (HSCs) can only generate cells of the blood system. The ability of stem cells to generate physiologically relevant cells, such as cardiomyocytes, hepatocytes and neurons in limitless supply make them attractive for many biopharmaceutical applications, such as cell replacement therapies, drug discovery, disease modelling and toxicology studies. The most well-established stem cell therapy, practiced for over 40 years, is bone marrow transplantation [3]. HSCs are present in bone marrow at a very low frequency but are capable of reconstituting the entire blood system of recipient patients [4]. More recently, other stem cell treatments have progressed to the clinic, e.g. Geron's human embryonic stem (hES) cell derived neural cells for spinal cord injury [5] and ReNeuron's neural stem cells for treatment of Stroke [6]. However, the high cost of manufacture of these treatments along with a complicated and poorly understood regulatory pathway is hampering the widespread development of stem cell therapies. In addition, the use of pluripotent stem cells to generate cell therapies brings a risk of transplantation of undifferentiated cells and potential malignant transformation. An alternative therapeutic application of stem cells is their use in the discovery of conventional small molecule drugs for which the regulatory and manufacturing pathways are well established. Stem cell derived somatic cells are a promising alternative to currently used recombinant cell lines or primary cells for high throughput or secondary screens. Differentiation to functional hepatocytes and cardiomyocytes also opens the opportunity for the use of stem cells further down the drug development pathway, in critical toxicology studies. The emerging field of induced pluripotent stem (iPS) cells [7] has increased interest in stem cell technology, with the ability to develop disease-specific stem cells and to rapidly generate panels of stem cells with a range of genetic phenotypes thus enabling more accurate prediction of how a drug will behave across a mixed population. A further, fascinating application of stem cells is in the discovery of regenerative drugs to promote endogenous cells to repair lost or diseased tissue in conditions such as stroke and heart failure. Such drugs would promote the body to regenerate itself, overcoming the need of cell replacement therapies in some cases.

At present, realisation of the full potential of stem cells is hampered by the difficulty in routinely directing stem cell differentiation *in vitro* to generate fully functional, specific cell types of choice. The ability of stem cells to differentiate to multiple mature

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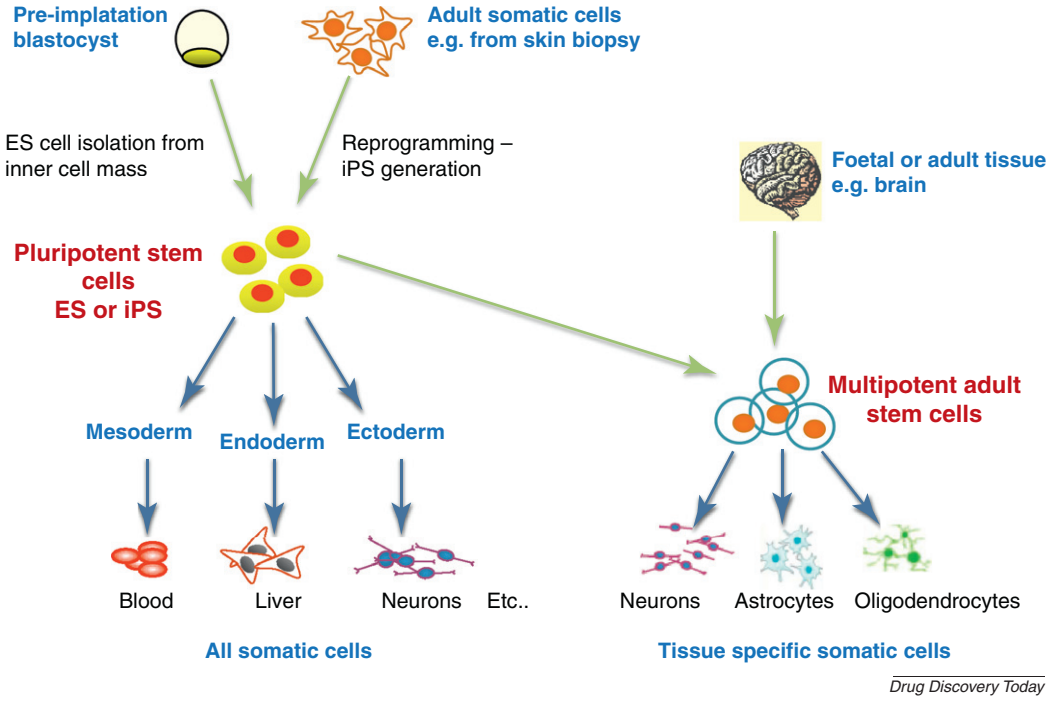

FIGURE 1

Stem cell sources and their differentiation potential. Different types of stem cells exist which differ in their longevity in culture and in the variety of mature cell types they can generate. Pluripotent stem cells, either embryonic or induced, are the most potent stem cells and are capable of infinite self-renewal *in vitro* and can generate all somatic cell types. Embryonic stem cells are isolated from the inner cell mass of blastocysts, whereas induced pluripotent stem cells are generated by reprogramming somatic cells. Adult, or tissue specific, stem cells are more restricted in their differentiation potential, typically only being able to generate cells of the tissue from which they were isolated.

cells can be problematic in terms of obtaining high yield and pure populations of a particular cell type. Being able to do this at large scale in a reproducible and cost effective manner is even more of a challenge. In this article, we describe some of the emerging technologies that are helping to overcome these issues and push stem cells to the forefront of drug discovery and development.

Stem cells

Pluripotent stem cells

Pluripotent stem cells are the most potent of all stem cells, being able to self-renew indefinitely *in vitro* and differentiate into all somatic cell types *in vivo* and many *in vitro* [e.g. haemopoietic, cardiac, neuronal, epithelial and pancreatic (Fig. 1)]. There are two types of pluripotent stem cells. Embryonic stem (ES) cells [8] are derived from the inner cell mass of preimplantation embryos. ES cells were originally isolated from mice and subsequently from many species, including of particular interest to the pharmaceutical industry, humans [9], monkeys [10], and rats [11]. iPS cells are generated by reprogramming adult somatic cells to a pluripotent state through expression of a combination of genes or reprogramming factors [1]. Reprogramming technology was also originally developed using mouse cells [7] but this was quickly followed by demonstration of the system using a variety of human somatic cell types [1]. iPS cells share many of the characteristics of ES cells, although there is speculation as to the true similarity of the cells, particularly in relation to the epigenetic state of their DNA [12]. In addition, it has been discovered that reprogramming of somatic cells can induce genomic alterations, such as copy number variations and point mutations [13,14]. Reprogramming technology has generated enormous interest as it raises the possibility of patient-specific therapies and the ability to generate disease-specific stem cells for *in vitro* studies by reprogramming somatic cells from patients. A further valuable feature of pluripotent stem cells is the ability to transiently and stably genetically modify them, enhancing their use in gene identification and function studies (cf. section 'Application of stem cells to drug discovery').

Adult stem cells

Adult stem cells, or tissue-specific stem cells, have more restricted differentiation potential than pluripotent stem cells, typically limited to generation of cell types of the tissue from which they were isolated [e.g. neural stem cells under normal circumstances are only capable of differentiating into the three neural lineages of neurons, astrocytes and oligodendrocytes [15] (Fig. 1)]. Adult stem cells typically also have limited *in vitro* self-renewal capacity, although there are some exceptions. For example, infinitely self-renewing neural stem cells have been isolated from foetal and adult brain [16]. Adult stem cells can be isolated from many adult and foetal tissues (e.g. haemopoietic, neural, mesenchymal and muscle [2]). Additionally, in some cases stable proliferating adult stem cells can be generated from pluripotent stem cells *in vitro* [16,17].

Application of stem cells to drug discovery

Stem cells have application in all stages of the drug discovery pathway from target identification through to toxicology studies.

Target identification

Through directed differentiation of stem cells to particular lineages *in vitro*, it is possible to study gene expression patterns and dissect the molecular mechanisms of lineage commitment, thereby identifying possible candidate proteins for therapeutic intervention. Coupling this with genetic modification has greatly aided gene function studies. In particular, gene targeting through homologous recombination has revolutionised the study of many biological systems [18,19]. Mouse ES (mES) cells and mice with a variety of modifications, such as null and point mutations, chromosomal rearrangements, large deletions and inserted reporter genes have enabled detailed analysis of gene function *in vitro* and *in vivo*. Although possible, gene targeting has proved more difficult in hES cells [20,21]. However, recent advances in technology, such as the use of zinc finger nucleases [22] will hopefully mean that the generation of knockout hES cells will soon be routinely possible. The ability to generate disease specific iPS cells extends target identification studies to the study of disease progression and pathology *in vitro* [23].

High-throughput screening

Current methods of drug screening rely largely on the use of recombinant transformed cell lines that express the target of interest (e.g. a G protein-coupled receptor) but otherwise are not directly relevant to the disease being studied. The use of primary cells is desirable since they are physiologically relevant. However, they are relatively difficult to obtain and since they can typically only be passaged a few times before senescence or death it is technically challenging to obtain cells in sufficient numbers for high-throughput screening (HTS) applications. Additionally, variability between donors and in preparation of cell batches can lead to inconsistent results. Stem cells offer an attractive alternative to primary cells and recombinant cell lines as they can be propagated for prolonged periods of time, can be cryopreserved and can differentiate to physiologically relevant cell types. Therefore, large batches of undifferentiated or differentiated cells can be generated for use in a series of experiments or screens. Furthermore, iPS cells now offer the opportunity to generate disease specific somatic cells and to rapidly generate panels of stem cells with a range of genetic phenotypes, enabling genetic effects on drug performance to be studied (Table 1).

While stem cell derived somatic cells have been used for several proof-of-concept studies with a small number of compounds [24,25] there are few reports of true HTS campaigns using stem cells. One such screen was carried out by Pfizer (http://www.pfizer.co.uk/default.aspx) [26], in which mES cells were differentiated into neuronal cells that express 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA) receptors and are pharmacologically responsive to standard AMPA potentiation compounds. Cells were formatted into HTS compatible 384-well plates and a library of 2.4×10^6 compounds screened. Novel chemical hits for AMPA potentiation were identified, followed by validation of chemical leads in secondary assays using hES cell derived neurons. A further interesting feature of this screen was that it made use of lineage selection technology. This is a method whereby a drug resistance gene is inserted under the control of a lineage specific gene promoter, in this case Sox-1 which is specific for the neural lineage. As the ES cells differentiate, drug selection can be applied such that all cells not expressing the resistance gene die. This is a powerful technique for selecting the lineage of choice from a heterogeneous mix of differentiated cells.

Recently there has been increasing evidence that the large pharmaceutical companies are seriously contemplating the use of stem cells for drug discovery purposes. For example, Roche (http://www.roche.com/index.htm) has invested US$20 million in a deal with Harvard University to use cell lines and protocols to screen for drugs to treat cardiovascular and other diseases. GlaxoSmithKline (http://www.gsk.com/) has signed a similar deal worth US$25 million.

Disease modelling

Stem cells can also be used to generate disease specific somatic cells or *in vivo* models of a particular disease. These can then be used in drug screening campaigns or for studying how a drug may behave in a particular disease context. Until the advent of reprogramming technology, diseased cells and animal models were generated through genetic modification of ES cells, followed by differentiation to the lineage in question or generation of a mouse model [19]. However, it is now possible to generate iPS cells from patients with a variety of diseases. These can then be differentiated to specific lineages to generate disease and patient-specific somatic cells. An example of this is the generation of iPS cells from patients with a K+ channel mutation found in congenital long QT syndrome associated with cardiac arrhythmias [27]. These iPS cells were differentiated to functional cardiomyocytes, which were found to recapitulate the longer action potentials observed in the patients. Small molecules were screened against these cells to see which could correct the underlying electrophysiological defect. iPS cells have been generated from patients with many other diseases, e.g. Huntingdon's, amyotrophic lateral sclerosis

TABLE 1

Comparison of desirable attributes of different cell sources for cell based drug discovery screens

	Recombinant cell line	Primary cells	Pluripotent stem cells	Adult stem cells
Physiological relevance	x	✓	✓	✓
Availability	✓	x	✓	✓
Ability to generate stable genetic modifications	✓	x	✓	x
Reproducibility	✓	x	✓	✓
Scalability	✓	x	✓	✓
Disease modelling	x	x	✓	x

(ALS), severe combined immunodeficiency, juvenile diabetes and spinal muscular atrophy (SMA) [23]. One of the major challenges of this approach, especially when studying complex neural diseases, such as PD or Alzheimer's is whether it will be possible to mimic such complex disease progression *in vitro* and whether enough diversity in the differentiated population can be reliably generated to recapitulate the mix of cells types that is present and affected *in vivo*. Consequently, modelling of monogenic diseases, affecting only one cell type, will likely benefit most from this approach. Ipierian (http://www.ipierian.com/) is one pharmaceutical company focusing on the use of disease-specific iPS cells in drug discovery, generating iPS cell panels from patients with several neurological and cardiological defects, such as SMA, ALS and Parkinson's.

Regenerative drugs

In addition to using stem cells and their derivatives for more traditional drug screening they also have potential for discovering novel drugs or factors to promote endogenous cells to repopulate lost or diseased cells in conditions such as stroke. Regenerative drugs are already available, such as erythropoietin and the small molecule eltrombopag [28] (Promacta/Revolade), which stimulate the production of red blood cells and platelets respectively, from haemopoietic progenitor cells. Eltrombopag, a thrombopoietin (TPO) receptor agonist, was discovered in a traditional drug screen using a recombinant cell line expressing the TPO receptor [29]. This approach relies on a knowledge of the receptors and cytokines to target for regeneration of a particular tissue. For most tissues this information is not known. An alternative target for screening is the endogenous stem and progenitor cells that will themselves effect regeneration. Many adult tissues contain stem cells [2] and in the cases where it is possible to isolate them, they provide a good target for regenerative drug screening. For example, mesenchymal stem cells (MSCs) were used to screen a library of just over 1000 molecules for their ability to promote osteogenic differentiation. A total of 36 compounds were found to promote osteogenesis and one lead compound has been studied in combination with an osteoconductive polymer for potential application *in vivo* to promote bone regrowth [30]. However, not all tissues contain stem cells that are well characterised or easily isolated in large enough numbers for this type of screen. Although ES/iPS cells themselves are not a good target, having no counterpart in the adult body, another approach is to generate adult stem or progenitor cells *in vitro* from pluripotent stem cells. This is the basis of Progenitor Labs' ProScreen technology [31], which generates physiologically relevant progenitor cells through differentiation of stem cells *in vitro*. These cells are used for screening of small molecules that can effect the terminal differentiation of the progenitors to cell types lost as a result of disease or injury. Fate Therapeutics (http://www.fatetherapeutics.com/) is another company that is pursuing discovery of regenerative small molecule drugs to stimulate adult stem cells to differentiate *in vivo*.

Toxicology

Approximately 30% of drugs that fail in early stage clinical trials do so because of toxicity issues, primarily hepatic and cardiac toxicity. This costs drug developers billions of dollars a year and demonstrates that current preclinical toxicology models are ineffective. Primary hepatocytes and cardiomyocytes are currently utilised for *in vitro* toxicity testing, along with transformed cell lines and animal models. However, primary cells are expensive to manufacture, are in short supply and vary significantly from donor to donor, while transformed cell lines and animal models are not as physiologically relevant to human liver function. Pluripotent stem cells could provide a limitless, consistent alternative resource of human hepatocytes and cardiomyocytes for toxicity studies and greatly reduce the need for animal testing. iPS cells hold particular value for this application since they can be readily derived from many different individuals [32,33]. They could therefore provide an efficient system for generation of cell panels to test the effects of drugs on different genetic populations. Several groups have differentiated hES cells into hepatocyte-like cells having a typical phenotype. However, stable high-level expression of key cytochrome P450 enzymes has yet to be demonstrated, limiting their use for toxicity studies [34,35]. There is considerable interest from the pharmaceutical industry in achieving this goal, for example, collaborations between Geron (http://www.geron.com) and GE Healthcare (http://www.gehealthcare.com/worldwide.html), AZ and Cellartis (http://www.cellartis.com/) and the industry sponsored Stem Cells for Safer Medicines initiaitve. More success has been achieved with the generation of functional cardiomyocytes from hES and iPS cells and Roche is already using iPS derived cardiomyocytes [36] [supplied by Cellular Dynamics International (http://www.cellulardynamics.com/)] in their drug discovery and toxicity processes.

Stem cell differentiation

A fundamental requirement for all the above applications of stem cells is the ability to reliably, robustly and reproducibly direct their differentiation to functional specific cell types in high yield and purity. This is technically extremely challenging and one of the primary reasons that stem cells are not more widely adopted for drug discovery applications.

Differentiation of stem cells to a mature cell type typically requires serial cell culture steps with sequential addition of growth and patterning factors, which essentially mimic processes that occur *in vivo* during development [37–39]. Furthermore, the microenvironment in which stem cells are cultured also needs to be considered, as the extracellular matrix (ECM) substrate and spatial configuration of the cells can have an enormous effect on their fate [40,41]. Testing a significant number of these variables is very labour intensive and time consuming, limiting the development of optimised differentiation protocols. Additionally, for large scale applications of stem cell differentiation, such as drug discovery, conditions need to be reproducible and standardised. Ideally this means serum-free, feeder cell free and fully defined. The use of small molecules in place of standard growth factors and cytokines would further ensure reproducibility and cost-effectiveness.

Novel methods for identifying optimal stem cell differentiation protocols would greatly accelerate the widespread use of stem cells in industrial applications and here we describe some of the innovative techniques which are being developed to overcome the hurdles described above.

Robotic high throughput platforms

Several groups have taken the approach of using automated cell culture systems to screen multiple differentiation conditions in multiwell format. These are typically coupled with an automated

screening readout, such as high content analysis platforms. In particular, the focus has been on the screen of small molecule libraries for their effect on stem cell differentiation [42–44]. For example, Studer's group performed an automated screen of over 2900 compounds for their effects on hES cell self-renewal and differentiation. Following compound treatment, cells were assessed by automated immunostaining and high content analysis for Oct-4 expression. Ten compounds were identified that resulted in early differentiation of hES cells. Interestingly, five of these were cardiac glycosides, a significant overrepresentation compared with the library as a whole. Four of the compounds (retinoic acid, selegiline, cymarin and sarmentogenin) were studied in more

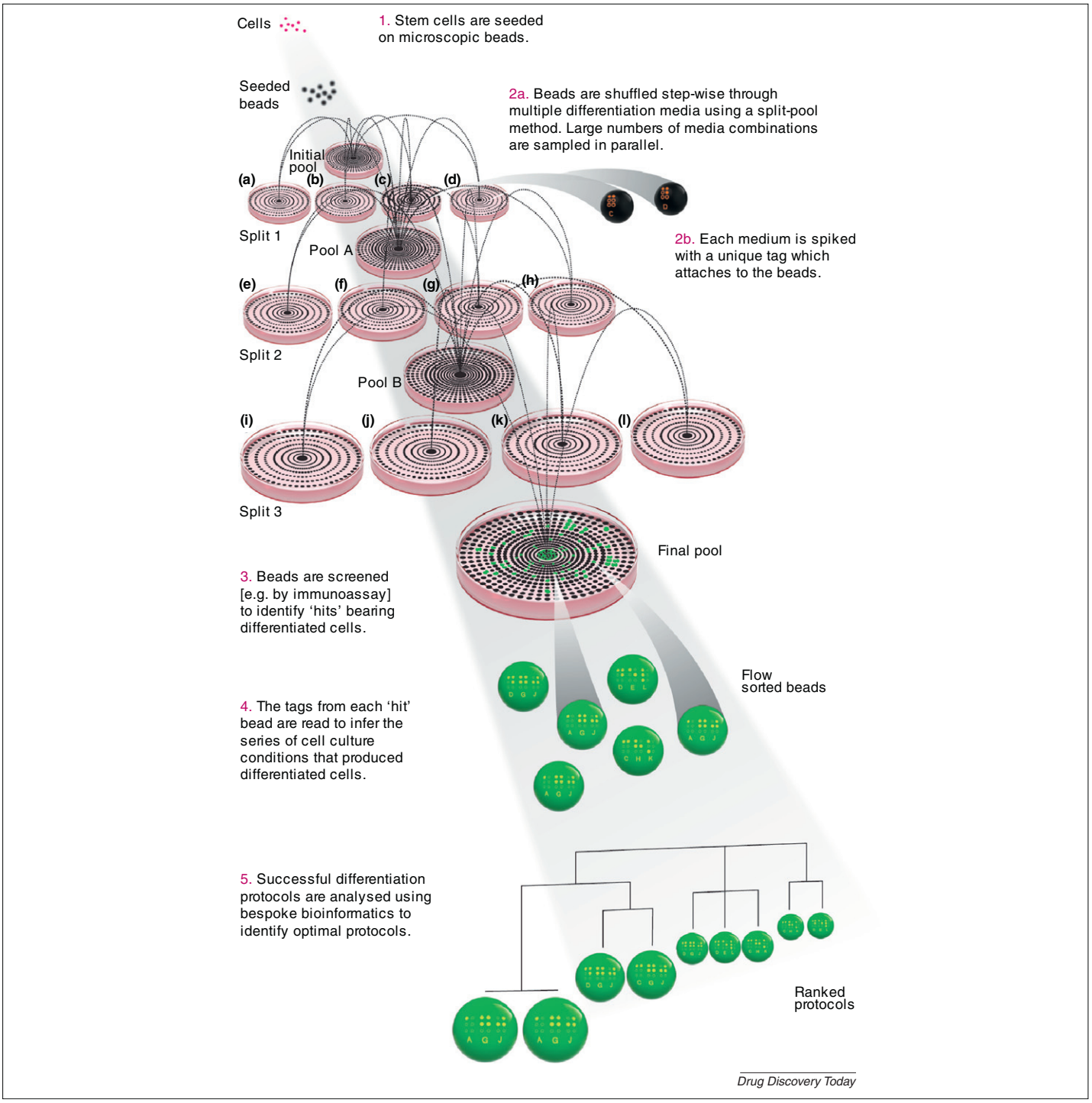

FIGURE 2

Combinatorial cell culture. Combicult™ is a high throughput platform for the rapid identification of stem cell differentiation protocols. Stem cells on beads are exposed to multiple combinations of media, containing active agents, such as growth factors or small molecules, using a split-pool technique. The optimal combinations for effective differentiation can be deduced rapidly and cost-effectively.

detail and found to induce differentiation to different lineages (i.e. trophectoderm, mesendoderm and neurectoderm [45]). Becton Dickinson (http://www.bd.com/uk/) provides a service for rapid discovery of stem cell culture conditions through the use of their robotic BD Discovery Platform. The platform integrates proprietary bioinformatics tools and automation to enable rapid rational screening of large numbers of combinations of biological factors. The culture medium, media supplements, cell adhesion surface and culture vessel can all be optimised.

Microarray/microfluidics systems

Another approach is minaturisation of cell culture using microfabrication techniques. This enables many experiments to be carried out in parallel, giving savings on expensive reagents and provides the ability to study single cell behaviour in a high throughput manner [46]. In particular, this approach has been used to study the interplay between stem cell fate and the microenvironment. Different ECM and cell adhesion factors can be robotically spotted onto microarrays in various combinations, enabling screens of tens to hundreds of putative microenvironments. La Flaim *et al.* used this technique to study the effect of different ECM proteins on ES cell hepatocyte differentiation, showing approximately 140-fold difference in efficiency of differentiation between the best and the worst protein combinations [47]. This technology has since been extended to probe interactions of ECM components in combination with soluble growth factors [48]. A multiwell microarray platform that enables 1200 simultaneous experiments on 240 unique signaling environments was developed. Each well contains 100 (20 mixtures in five replicates) ECM spots in the context of a different media composition, 12 of which were tested. A reporter ES cell line (green fluorescent protein under the control of the αMHC promoter) was used to monitor cardiac differentiation using a confocal microarray scanner. The results were consistent with what has previously been published, providing proof-of-concept for this approach.

The above platforms expose stem cells to various stimuli in a constant static manner. Microfluidics technology can also be used to elucidate time dependent processes enabling rapid medium exchange and culture condition switching at desired time points. It also opens the opportunity of studying continuous concentration gradients of biomolecules; such gradients have a key role in embryonic development and tissue regeneration [46]. Furthermore, microfluidic techniques have been used to study the effects of 3D culture on stem cell fate. Fernandes *et al.* utilised a microarray spotter to deposit cells onto a modified glass surface to yield an array consisting of cells encapsulated in alginate spots in volumes as low as 60 nl [49]. Different small molecules and growth factors were added, to study their effects in a more physiologically relevant 3D culture environment. mES self-renewal and neural differentiation were assessed, revealing effects of cell density on differentiation and demonstrating that known neural inducing factors could regulate neural differentiation in this system.

Combinatorial cell culture

The temporal, sequential nature of stem cell differentiation lends itself to a combinatorial approach to protocol discovery. Plasticell (http://www.plasticell.co.uk/) has developed a high throughput platform that uses combinatorial cell culture (Combicult™) technology to screen tens of thousands of protocols in one experiment [50]. Combicult™ combines miniaturisation of cell culture on microcarriers, a pooling/splitting protocol and a unique tagging system to enable multiplexing of experiments. Stem cells grown on microcarrier beads are shuffled randomly, stepwise through multiple differentiation media using a split-pool method. The iterative process of splitting, culturing and pooling, systematically samples all possible combinations of media in a predetermined matrix (Fig. 2). Each medium is spiked with a unique fluorescent tag that attaches to the beads. At the end of the differentiation process beads bearing differentiated cells are identified by a screening assay (e.g. immunostaining or reporter gene expression) and individual positive beads are isolated using an automated large particle sorter. The cell culture history of each positive bead is then deduced by analysis of the fluorescent tags attached to the bead. Typically 100 or more positive differentiation protocols are discovered in each screen. These are analysed using bespoke bioinformatics software which uses criteria, such as hierarchical clustering and probability analysis to select the optimal protocols for further validation.

The system has been successfully used to discover novel differentiation protocols for many different starting stem cell types and differentiated progeny (e.g. hepatocytes, neurons, cardiomyocytes and osteoblasts from hES, mES and hMSCs). Since large numbers of conditions can be tested in each screen it is possible to efficiently discover optimised protocols that have advantages over more traditional cell culture methods (e.g. are serum-free, use only small molecules or exclude other variable and expensive products). For example, a screen of 10,000 protocols identified serum-free, feeder cell-free protocols for the generation of megakaryocytes (platelet precursor cells) from hES cells. In several of these protocols growth factors were replaced with small bioactive molecules.

Concluding remarks

The unique properties of stem cells offer enormous potential to many biopharmaceutical applications. In the area of drug discovery and development discussed in this article, stem cells are already being used to some degree, particularly in target identification and toxicology testing. However, widespread adoption of stem cell technology in all aspects of the drug discovery process will be reliant on the development of robust, reproducible methods to culture stem cells and in particular to direct their differentiation to specific lineages. Discovery and optimisation of stem cell differentiation protocols is technically challenging owing to the large number of variables to consider; i.e. which growth factors or small molecules to add, at what time, which cell substrate to use and the optimal 3D configuration of the cells. We have described some of the technologies that are being developed to increase the efficiency of differentiation protocol discovery by testing variables in a high throughput manner. Adoption of these techniques and their further development, in particular to study the effects of culturing cells in different 3D biomaterial structures, effectively generating mini-tissues, will greatly accelerate efforts to industrialise stem cell differentiation and promote the widespread utilisation of stem cells by the pharmaceutical industry.

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