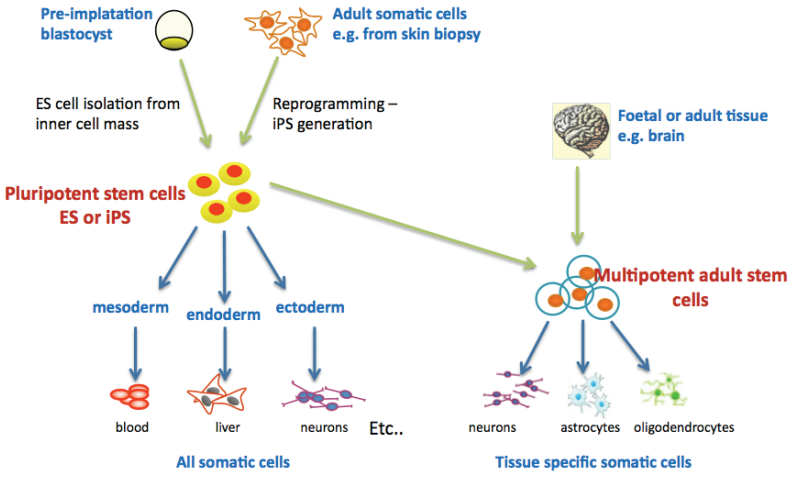
Stem Cells and Drug Discovery

Introduction:

Stem cells are extraordinary cells, having many features and advantages that could revolutionise drug development and healthcare applications. They are capable of both self-renewal and differentiation to mature somatic cells *in vivo* and *in vitro*[1,2] and as such offer a limitless, consistent supply of physiologically relevant cells for applications such as cell replacement therapies, drug development and disease modelling. The ground-breaking emerging field of induced pluripotent stem cells (iPS cells) in which somatic cells can be reprogrammed to a pluripotent stem cell state[1] has further increased interest in stem cell technology, as they present the opportunity to generate patient- and disease-specific cells for personalised medicine and disease modelling.

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.



Many different types of stem cell exist, of diverse origin and with differing potential for self-renewal and lineage differentiation. Pluripotent stem cells (embryonic stem (ES) and iPS) are the most potent of stem cells, being able to self-renew indefinitely and differentiate into all somatic cell types in vivo and many *in vitro*[1]. Of particular interest to the pharmaceutical industry, pluripotent stem cells have been used to generate human cardiac, hepatic and multiple neuronal (e.g. dopaminergic, GABAergic, motor neuron) cells in vitro. Multipotent, or adult, stem cells can be isolated from many foetal and adult tissues e.g. haemopoietic, neural, mesenchymal and muscle[2]. They have more restricted self-renewal and differentiation potential than pluripotent stem cells, typically limited to generation of cells 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[3] (Figure 1).

Stem cells have been utilised in cell replacement therapies for over 40 years in the form of bone marrow transplantation[4]. Haemopoietic stem cells (HSCs), although present in bone marrow at a very low frequency, are capable of reconstituting the entire blood system of recipient patients[5]. More recently, other stem cell treatments have progressed to the clinic, for example Mesoblast's adult stem cell RevascorTM therapy for congestive heart failure[6] and Advanced Cell Technology's human ES cell derived retinal pigmented epithelial cells for Stargardts disease[7]. However, the high cost of manufacture of these treatments along with a complicated and poorly understood regulatory pathway, particularly for pluripotent stem cell derived therapies, is impeding their widespread development. An alternative 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 cells have application in all stages of the drug discovery pathway from target identification, to high-throughput screening to toxicology studies. Here we will highlight examples of how stem cells are already being utilised in this process and describe innovative techniques which are helping improve performance and functionality *in vitro* in order to bring the application of stem cells to the forefront of the pharmaceutical industry.

Stem Cells and Drug Discovery:

High Throughput Screening: Current methods of drug screening rely largely on the use of recombinant transformed cell lines that express a target of interest, e.g. a GPCR receptor, but otherwise are not directly relevant to the disease being studied. More physiologically relevant primary cells are in short supply and batch variability limits their application. 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. 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. While stem cell derived somatic cells have been used for several proof of concept studies with a small number of compounds[8,9] there are few reports of true high-throughput screening (HTS) campaigns. Pfizer, however, have carried out one such screen, in which mES cells were

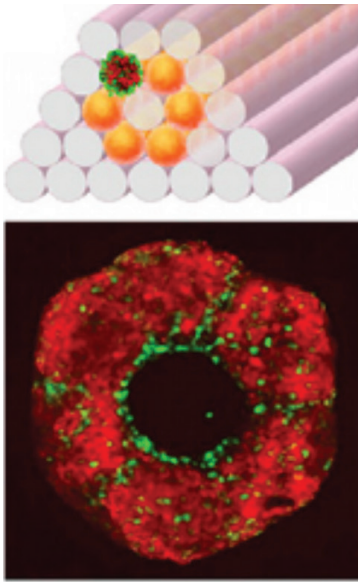
differentiated into pharmacologically responsive glutamatergic neurons and used to screen a library of 2.4x106 compounds[10]. Novel chemical hits for AMPA potentiation were identified and validated in secondary assays using hES cell derived neurons. There is increasing evidence that the large pharmaceutical companies are seriously contemplating the use of stem cells for drug discovery purposes. For example, Roche invested $20million in a deal with Harvard University to use cell lines and protocols to screen for drugs to treat cardiovascular and other diseases, and GSK have signed a similar deal worth $25m.

Adult stem cells, or progenitors derived from pluripotent stem cells, also have application in discovery of regenerative drugs that would promote their in vivo counterparts to repopulate lost or diseased cells in conditions such as stroke or heart failure. Regenerative drugs are already available – for example Eltrombopag[11] (Promacta/ Revolade), a TPO receptor agonist, which stimulates the production of platelets from haemopoietic progenitor cells. However, Eltrombopag was discovered in a traditional drug screen using a recombinant cell line expressing the TPO receptor[12], an approach which relies on knowledge of the receptors and cytokines to target for regeneration of a particular tissue. For most tissues this information is not known and in these cases the in vitro use of stem cells and their progeny would be very advantageous.

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 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 organ 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, therefore providing an efficient system for generation of cell panels to test the effects of drugs on different genetic populations[13,14]. Several proof of concept studies have been carried out to evaluate the use of pluripotent stem cell derived hepatocytes and cardiomyocytes to predict drug effects in humans, and Roche is already using iPS derived cardiomyocytes[15] (supplied by Cellular Dynamics International) in their drug discovery and toxicity studies.

Disease Modelling: The ability to genetically manipulate mouse and human ES cells has been used for many years in the generation of somatic cell and mouse models of human disease in which genes are 'knocked-out' or point mutations are engineered[16,17]. The advent of reprogramming technology now brings the ability to generate iPS cells, from patients with a variety of diseases, which can then be differentiated to specific lineages producing disease- and patient-specific somatic cells. For example, iPS cells have been generated from patients with a K+ channel mutation associated with cardiac arrhythmias[18]. Cardiomyocytes differentiated from these iPS cells were found to recapitulate the longer action potentials observed in the patients and used to discover small molecules that could correct the underlying electrophysiological defect. iPS cells have been generated from patients with a wide variety of diseases such as Huntingdon's, ALS, SCID, juvenile diabetes and spinal muscular atrophy (SMA)[19]. Although such studies on diseased cells are informative, in many cases it has been shown that cellular responses to drug candidates observed in 2D cultures are not applicable to in vivo response. Much effort is therefore being applied to generate more functional, physiologically relevant in vitro 3D disease models which contain multiple cell types in a relevant tissue architecture - i.e. tissue engineering. One approach is to seed cells on a biomimetic scaffold that guides cells to differentiate and form a 3D cell construct. By modifying the scaffold material, strength and structure different outcomes can be achieved, and by seeding different cell types or spatially organising developmental cues, a functional 3D structure can be generated which mimics organ architecture, cell-cell and cell-ECM interactions[20]. For example, in a very elegant study, 3D hydrogel scaffolds were generated in which Sonic Hedgehog and Ciliary Neurotrophic Factor were simultaneously immobilised in distinct patterns. These factors differentially affected the differentiation of neural progenitor cells[21], opening the possibility of generating 3D organ mimetics by spatially controlling the differentiation of stem and progenitor cells. Technologies such as bioprinting have also recently come to the fore. Bioprinting is a computer-controlled cell deposition technique that allows precise spatial resolution and control of 3D cell constructs[22]. For example, blood vessel substitutes of different diameters have been generated by printing mixtures of endothelial and smooth muscle cells in defined geometries and subsequently applying physiological signals such as shear flow[20] (Figure 2).

Figure 2. Bioprinted blood vessels
The top image shows a template to build a construct with spheroids composed of smooth muscle cells (red) and endothelial cells (green). A transversial section after fusion, bottom image, shows the lumen is predominantly composed of endothelial cells. From: Jakab, K., et al., Tissue engineering by self-assembly and bio-printing of living cells. Biofabrication, 2010. 2(2): p. 022001.

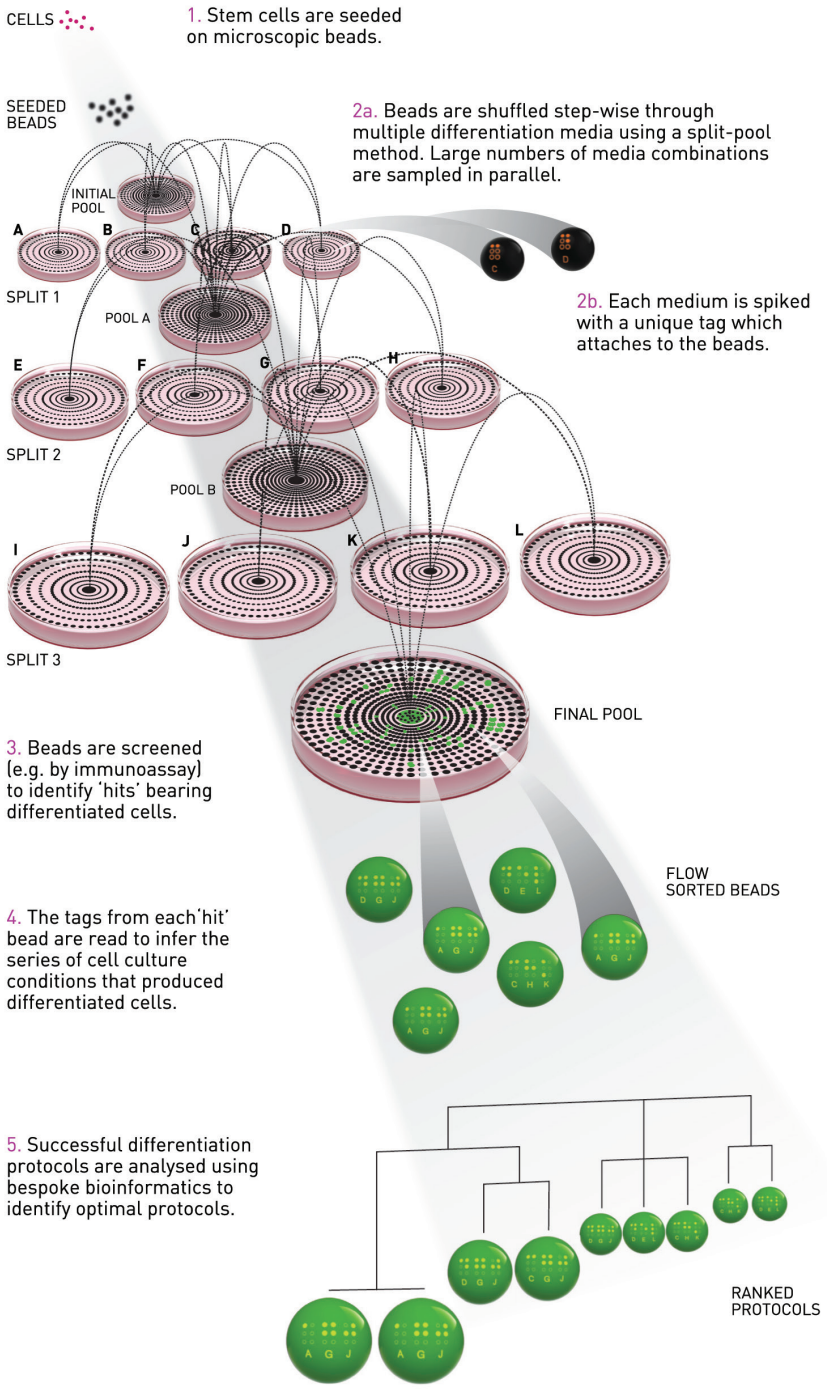


Controlling Stem Cell Differentiation

A fundamental requirement for all the above applications of stem cells is the ability to reliably and robustly direct their differentiation to functional specific cell types in high yield. This is technically extremely challenging, and generating cell batches at scale, in a cost-effective manner, as required for cell therapy and drug discovery applications, is even more demanding.

Many factors have to be considered when developing methods to differentiate stem cells. Typically the sequential addition of particular combinations of growth and patterning factors is required, essentially mimicking processes that occur *in vivo* during development[23]. The microenvironment in which cells are cultured also needs to be optimised, as the extracellular matrix (ECM) substrate and spatial configuration of stem cells can have an enormous effect on their fate[24]. Testing a significant number of such variables is very labour-intensive and time-consuming, limiting the development of optimal protocols. Here we describe some high-throughput techniques that are being applied to expedite discovery of methods to control stem self-renewal and differentiation.

Figure 3. Combinatorial Cell Culture (below)
CombicultTM 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.



Discovering optimal cell culture media

The addition of growth factors or small molecules that target particular signalling pathways is one of the principal methods researchers use in attempting to direct the differentiation of stem cells to a particular cell type. Selection of these factors is typically based on what is known of lineage development during embryogenesis or in the adult during tissue repair. For example, the differentiation of hES cells to pancreatic cells requires a series of four different culture media, each containing a combination of growth factors and/or small molecules which first induce stem cells to commit to definitive endoderm, then to pancreatic endoderm, to pancreatic endocrine/exocrine cells and finally to more mature islet cells[25]. To date, the development of such complicated protocols has been carried out empirically and involved much effort and resource.

The temporal, sequential nature of stem cell differentiation lends itself to a combinatorial approach to protocol discovery. Plasticell has developed a high-throughput platform that uses combinatorial cell culture (Combicult®) technology to screen tens of thousands of protocols in one experiment[26]. Combicult® combines miniaturisation of cell culture on microcarriers, a pooling/splitting protocol and a unique tagging system to allow multiplexing of experiments. Stem cells grown on microcarrier beads are shuffled randomly, stepwise through multiple differentiation media using a split-pool method,

systematically sampling all possible combinations of media in a predetermined matrix (Figure 2). The tagging system allows the cell culture history (i.e. differentiation protocol) of beads bearing cells of the desired lineage to be deduced. 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, 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.

Several groups have taken the approach of using automated robotic cell culture systems to screen multiple growth and differentiation conditions in multiwell format. These are typically coupled with an automated screening readout such as high content analysis platforms that enable simultaneous assessment of multiple cellular features in an automated and quantitative way. In particular, focus has been on the screening of small molecules for their effect on self-renewal and stem cell differentiation, as they have advantages in terms of reproducibility and cost-effectiveness. In one example, over 5000 compounds were screened for their effect on pancreatic differentiation of hES cells using high-content analysis of pdx-1 expression as a readout. One compound in particular was found to promote efficient generation and expansion of pancreatic progenitor cells[27].

Recreating the stem cell niche

Understanding the microenvironments in which stem cells reside and differentiate *in vivo* and trying to recapitulate these in vitro to further control stem cell differentiation has become an increasingly important area of stem cell research[28]. In particular, focus has been on the biochemical and mechanical influence of different ECM components, and how these and the 3D configuration of cells affects their fate. Innovative microfabrication techniques have been used to investigate these influences, allowing a high throughput and cost-effective way of discovering how different materials affect stem cell fate[29]. For example, different ECM and cell adhesion factors can be robotically spotted onto microarrays in various combinations, allowing screens of hundreds of putative microenvironments. La Flaim et al. used this technique to probe interactions of ECM components in combination with soluble growth factors[30]. A multiwell microarray platform that allows 1200 simultaneous experiments on 240 unique signalling environments was developed. A reporter ES cell line (GFP under the control of the MHC promoter) was used to monitor cardiac differentiation using a confocal microarray scanner.

The effect of mechanical forces on stem cell differentiation has also become a major topic of investigation. It is clear that applied mechanical forces can affect the activity and expression of transcription factors and chromatin remodelling enzymes in turn affecting stem cell fate. A study investigating different polyacrylamide gels showed that gel stiffness had a dramatic effect on the differentiation fate of MSCs, with culture on soft, intermediate or stiff gels resulting in differentiation to neurons, muscle and bone respectively[31]. High-throughput methods have also been developed to assess the effect of substrate stiffness on cell function. For example, libraries of micropost arrays of different heights, resulting in different stiffnesses, have been generated. These micropost arrays can also be microprinted with ECM components on their surface to investigate ECM binding and substrate rigidity together[32].

Conclusions

The unique properties of stem cells offer enormous potential to many biopharmaceutical applications. In the area of drug development discussed here, they are already being used to some degree, particularly in disease modelling and toxicity studies. 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, and in particular, direct their differentiation to specific lineages. Discovery and optimisation of stem cell differentiation protocols is technically challenging due to the large number of variables to consider, and adoption of higher-throughput techniques for protocol discovery would be advantageous. Focus needs to be on integrating all the signals that affect stem cell differentiation – i.e. soluble factors, cell-cell interactions, 3D configurations and the chemical and mechanical properties of cell substrates, to generate stem cell derived 'mini tissues' that are more physiologically relevant than current systems. As the techniques described above, and others - in particular new imaging technologies for tracking cells in complex 3D micro structures - are further developed, the use of stem cells will advance to the forefront of the pharmaceutical industry where their potential for transforming cell therapy and drug development can be realised.

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