STEM CELL differentiation

Stem cells are extraordinary cells, capable of both self-renewal and differentiation to mature somatic cells *in vivo* and *in vitro*[1,2]. They have many features and advantages which could revolutionise drug development and healthcare applications.

By Dr Lilian Hook

Stem cells offer a limitless, consistent supply of physiologically relevant cells from validated pathogen-free sources for applications such as cell replacement therapies, drug discovery, disease modelling and toxicology studies. 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. iPS cells present the opportunity to generate patient and disease specific cells for cell therapy and disease modelling. However, a critical barrier to the use of stem cells in these therapeutic and drug development applications is the difficulty in routinely directing their differentiation to reproducibly and cost-effectively generate pure populations of specific cell types. In this article we will describe the current state-of-the-art in stem cell differentiation and some of the latest innovative technologies that are greatly accelerating improvements thus driving the adoption of stem cells in biopharmaceutical applications.

Stem cell applications

Stem cells have been utilised in cell replacement therapies for more than 40 years in the form of bone marrow transplantation[3]. Haematopoietic stem cells (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, for example ReNeuron's neural stem cells for treatment of Stroke[5] and Mesoblast's adult stem cell Revascor™ therapy for congestive heart failure. 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.

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 through to toxicology studies. Since they can be propagated for prolonged periods of time, cryopreserved and differentiated to physiologically relevant cell types they have significant advantages over currently used models such as recombinant cell lines and primary cells. Furthermore, iPS cells now offer the opportunity to generate disease-specific somatic cells[6] and to rapidly generate panels of stem cells with a range of genetic phenotypes, allowing genetic effects on drug performance to be studied. 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. There are few reports of true high throughput screening (HTS) campaigns using stem cells, however Pfizer has carried out one such screen providing proof of

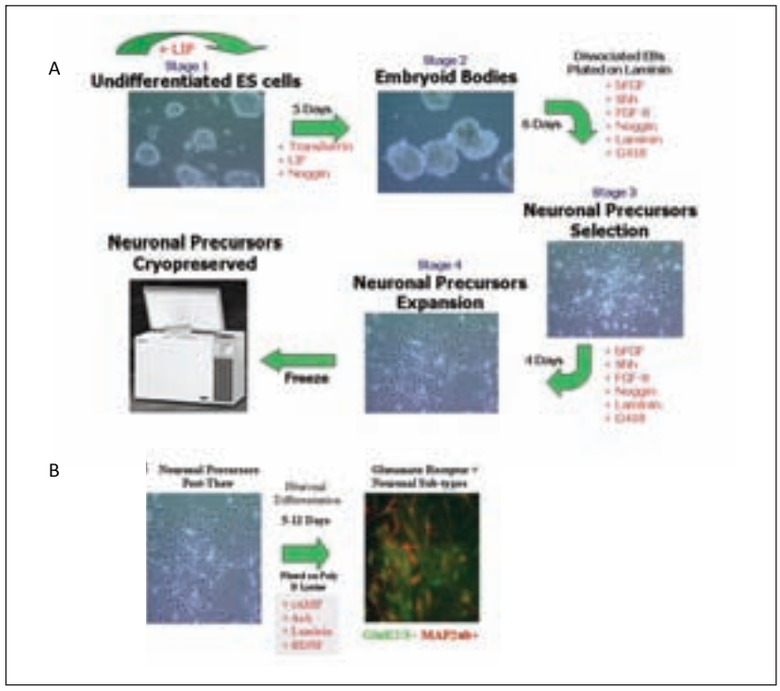

Figure 1

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.

From: McNeish, J et al. High-throughput screening in embryonic stem cell-derived neurons identifies potentiators of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate-type glutamate receptors. J Biol Chem, 2010. 285(22): p. 17209-17

concept for this application[7]. Mouse embryonic stem (mES) cells were differentiated into neuronal cells that express AMPA receptors and are pharmacologically responsive to standard AMPA potentiation agents. A library of 2.4 x 10^6 compounds was screened against these cells and novel chemical hits for AMPA potentiation were identified, followed by validation of leads in secondary assays using human embryonic stem (hES) cell-derived neurons. There is increasing evidence that pharmaceutical companies are realising the potential of stem cells for drug discovery applications. For example, Roche has invested $20 million in a deal with Harvard University to use cell lines and protocols to screen for drugs to treat cardiovascular and other diseases and are already using iPS-derived cardiomyocytes[8] (supplied by Cellular Dynamics International) in their drug discovery and toxicity processes.

Another application of stem cells is the development of disease models, either by generation of disease-specific somatic cells or *in vivo* animal models. iPS cells hold particular promise for this application since they can be generated from patients with a variety of diseases. iPS cells 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[6]. 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, eg Huntingdon's, ALS, SCID, juvenile diabetes and spinal muscular atrophy (SMA)[9].

For stem cells to be utilised to their full potential, two major challenges have to be overcome. The first is to be able to expand stem cell numbers *in vitro*, while maintaining a homogeneous culture of undifferentiated cells. The second is to be able to routinely direct stem cell differentiation *in vitro* to generate fully functional, specific cell types of choice. Production of stem cells and their differentiated progeny at large scale, in a robust and cost-effective manner, as required for cell therapy and drug discovery applications, is even more challenging. There are many different types of stem cells, of diverse origins and differentiation potential (**Figure 1**) and each requires unique culture conditions for growth and differentiation.

Stem cell types

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* (**Figure 1**). For example, of particular interest to the pharmaceutical industry, human pluripotent stem cells have been differentiated *in vitro* to haemopoietic, cardiac, multiple neuronal (eg dopaminergic, GABAergic, motor neuron), hepatic and pancreatic cells. There are two types of pluripotent stem cells. Embryonic stem (ES) cells[10], are derived from the inner cell mass of pre-implantation embryos. Induced pluripotent stem (iPS) cells are generated by reprogramming adult somatic cells to a pluripotent state through expression of a combination of genes or reprogramming factors[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[11]. In addition, it has been discovered that reprogramming of somatic cells can induce genomic alterations such as copy number variations and point mutations[12,13].

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 (**Figure 1**), eg neural stem cells under normal circumstances are only capable of

differentiating into the three neural lineages of neurons, astrocytes and oligodendrocytes[14] (**Figure 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[15]. Adult stem cells can be isolated from many adult and foetal tissues, eg haemopoietic, neural, mesenchymal and muscle[2]. Additionally, in some cases stable proliferating adult stem cells can be generated from pluripotent stem cells *in vitro*[15,16].

Many factors have to be considered when developing methods to culture and differentiate stem cells. The ability of stem cells to differentiate to multiple mature cells can be problematic in terms of obtaining high yield, pure populations of a particular cell type. Stem cell differentiation typically requires serial cell culture steps with sequential addition of particular combinations of growth and patterning factors, essentially mimicking processes that occur *in vivo* during development[17]. 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[18]. Testing a significant number of these variables is very labour intensive and time-consuming, limiting the development of optimised methods. Efficient identification of optimal stem cell differentiation protocols would greatly accelerate the widespread use of stem cells in industrial applications. Below we will describe some of the strategies for directing stem cell differentiation and the novel technologies which are driving increased understanding of stem cell biology and leading to improved methods for their differentiation.

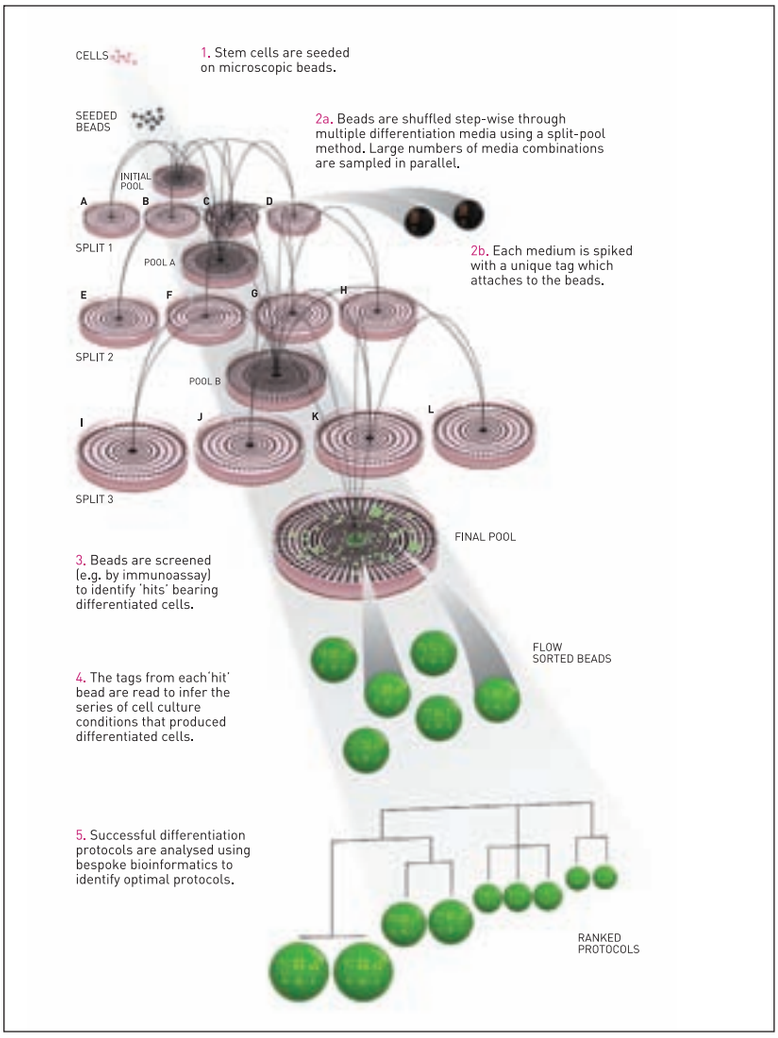

Figure 2: Combinatorial cell culture Combicult™ is a high throughput platform for the rapid identification of stem cell differentiation protocols. Stem cells on beads are exposed to multiple combinations of media, containing active agents such as growth factors or small molecules, using a split-pool technique. The optimal combinations for effective differentiation can be deduced rapidly and cost-effectively

Stem cell differentiation strategies

1. Soluble factors

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. Different combinations of factors are typically added in a sequential manner, particularly for the differentiation of pluripotent stem cells, reflecting progressive lineage commitment (**Figure 1**). For example, the differentiation of hES cells to pancreatic cells requires a series of four different culture medium, over 36 days, 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. Each medium contains a combination of growth factors and/or small molecules[19].To date, the development of such complicated protocols has been carried out empirically. However, recently high throughput approaches have been developed to accelerate protocol discovery.

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[20]. 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 (ie differentiation protocol) of beads bearing cells of the desired lineage to be deduced. Bespoke bioinformatics software, which uses criteria such as hierarchical clustering and probability analysis is used to analyse the positive protocols and select the optimal ones for further validation. The system has been successfully used to discover novel differentiation protocols for many different starting stem cell types and differentiated progeny, eg 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, eg 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 molecule libraries for their effect on self-renewal and stem cell differentiation[21-23]. The use of small molecules in place of standard growth factors and cytokines is preferable in terms of increasing reproducibility and cost-effectiveness. In one example, Studer's group performed an automated screen of more than 2,900 compounds for their effects on hES cell fate. Following compound treatment, cells were assessed by automated immunostaining and high content analysis. Four compounds were identified that support short-term self-renewal of hES cells in the absence of factors normally required, while 10 compounds were identified that resulted in early differentiation, inducing commitment to different lineages, ie trophectoderm, mesendoderm and neurectoderm[24]. In another example, more than 5,000 small molecules 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[23].

2. Lineage selection

Although the addition of optimal combinations of soluble factors can direct the differentiation of stem cells to a particular lineage, enriching for a chosen cell type, purity can vary extensively and never reaches 100%. For many applications there is therefore the need to purify populations of a specific cell type from the heterogeneous mix generated even during directed stem cell differentiation. Lineage marking and lineage selection strategies allow for the identification and selection of specified cell populations and the elimination of cells which are not of interest, generating highly purified populations of cells. The targeted introduction of a reporter gene such as green fluorescent protein (GFP) to one allele of a lineage restricted gene makes it possible to monitor appearance of that lineage during stem cell differentiation and to purify a specific population of GFP-expressing differentiated cells by fluorescence-activated cell sorting (FACS). Similarly, targeted insertion of a drug resistance gene (eg neomycin) enables the purification of a population of lineage restricted cells by positive selection with drug treatment. Such a strategy was used by Pfizer in the generation of neurons from mES cells for a high throughput drug screen (cf Stem Cell Applications)[7]. With recent advances in the efficiency of genetic manipulation in hES cells, in particular for homologous recombination, such strategies are increasingly being applied in hES cell differentiation. For example, hES cells have been generated that contain a neomycin resistance gene under the control of the lung alveolar type II (ATII) specific gene, SPC. Exposure of these cells to G418 during differentiation, resulted in generation of more than 99% pure populations of ATII cells which were morphologically and functionally normal. Without G418 selection only 12% of cells were of an ATII phenotype[25]. In another example, an eGFP gene was targeted to the Nkx2.5 allele in hES cells and eGFP expression used to track cardiac differentiation and to purify committed cardiac progenitors and cardiomyocytes[26]. Negative selection strategies can also be applied through the targeting of suicide genes such as thymidine kinase under the control of lineage specific promoters allowing unwanted cells to be eliminated from cultures[27]. This would be particularly valuable in the development of cell therapies from ES cells where transplant of con-

taminating undifferentiated ES cells could result in tumourigenesis.

3. Microenvironment/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. It is clear that factors other than those added to the cell culture medium can have a profound effect on stem cell self-renewal and differentiation[28]. Stem cell niches are 3D environments that subject cells to many different interactions which all combine to affect their fate. These include immobilised growth factors, extracellular matrix (ECM) components and neighbouring cell types, in addition to soluble growth factors. 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.

The ECM signals to cells principally via integrin receptors causing changes in cell shape, migration and protein expression[28]. In addition, the ECM provides a physical framework, affecting the mechanical forces cells are subjected to. There are several examples of how biochemical and mechanical forces affect stem cell differentiation and increasingly 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 on to microarrays in various combinations, allowing screens of tens to 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 1,200 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 results were consistent with what has previously been published, providing proof of concept for this approach.

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. Culture of MSCs on soft, intermediate or stiff gels resulted 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].

The above studies were all carried out in 2D culture systems, however the *in vivo* stem cell niche is a 3D structure and there is much interest in studying the effects of 3D culture on stem cell differentiation. Fernandes et al utilised a microarray spotter to deposit cells on to a modified glass surface to yield an array consisting of cells encapsulated in alginate spots[33]. 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. In another very elegant study, 3D hydrogel scaffolds with different growth factors immobilised at different positions were generated using 2-photon chemistry. Sonic hedgehog and ciliary neurotrophic factor were simultaneously immobilised in distinct patterns on a 3D gel and shown to differentially affect the differentiation of neural progenitor cells[34]. Such bioactive 3D patterned scaffolds are an important step forward in reconstructing the stem cell niche *in vitro*.

Conclusions

For the enormous potential of stem cells to be realised, methods for their differentiation need to be improved. This in turn requires improvements in how differentiation protocols are discovered – moving away from empirical experimentation to higher throughput techniques. Additionally, if optimal protocols are to be developed, focus needs to be on integrating all the signals that affect stem cell differentiation – ie soluble factors, cell-cell interactions, 3D configurations and the chemical and mechanical properties of cell substrates. As the techniques described above, and others, in particular new imaging technologies for tracking cells in complex 3D micro structures are further developed, methods for stem cell differentiation will greatly improve. This will lead to more reproducible, defined, cost-effective cell production protocols that will facilitate advancement of stem cells to the forefront of the pharmaceutical industry where their potential for transforming cell therapy and drug development can be realised. DDW

Dr Lilian Hook *is Research Director at Plasticell Limited. She has more than 15 years' experience in stem cell research, gained both in academia and industry. Her work has focused on the developmental biology of haemopoietic stem cells and the use of embryonic stem and adult stem cells in drug discovery applications, particularly in the neural field.*

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