Enabling the promise of STEM CELLS

The remarkable potential of stem cells to generate several hundred differentiated cell types is driving their use for regenerative medicine and for supporting the traditional drug discovery and development process (**Figure 1**). However, sustained progress in these areas is dependent on advances in the ability to: grow these cells; direct, and control their differentiation; and produce the quantities needed for clinical trials and therapies in a cGMP (current Good Manufacturing Practices) environment. This article will examine advances taking place on both the lab bench and the pharmaceutical production floor that are helping to bring the promise of stem cells closer to reality.

By Robert Shaw and Dr Jeff Till

Every cell type has its own unique needs when grown *in vitro* and stem cells are no exception. Stem cells, and in particular human embryonic stem (ES) cells, have earned a reputation for being labour intensive and difficult to grow and control in culture. A number of factors contribute to the labour-intensive upkeep of stem cell cultures: the cells must typically be fed daily; manual rather than enzyme-based passaging is often required; and differentiated colonies may require separation from undifferentiated colonies under a dissection microscope, which can be quite tedious. In addition, this excessive handling can increase the risk of contamination.

Further complicating matters, human ES cells are typically co-cultured with feeder layers of mouse fibroblast cells. Use of feeder layers requires two cell types to be maintained in parallel and introduces mouse proteins into the culture system.

Alternatively, stem cell cultures can be grown on extracellular matrix extracts and supplemented with conditioned medium from mouse fibroblast cultures. Although this approach eliminates the need for feeder layers, the system still contains undefined animal proteins, as well as possible contaminants. Inclusion of fetal bovine serum in stem cell media further contributes to an ill-defined culture system. The use of mouse feeder layers and animal serum are particularly problematic in the culturing of stem cells for possible therapeutic applications.

While significant progress has been made towards more defined culture conditions, development of animal-free culture systems has lagged. Use of serum replacements has removed some variability associated with fetal bovine serum and has led to human ES cell media that is 'serum-free'. However, serum replacements still may contain bovine serum albumin.

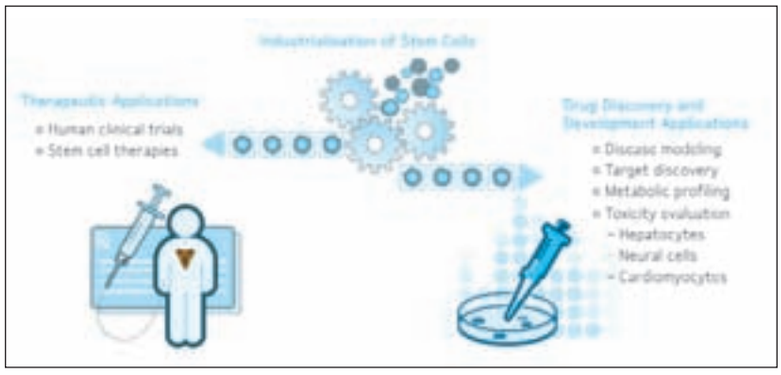

Figure 1: Application of stem cells for both therapeutic and drug discovery applications

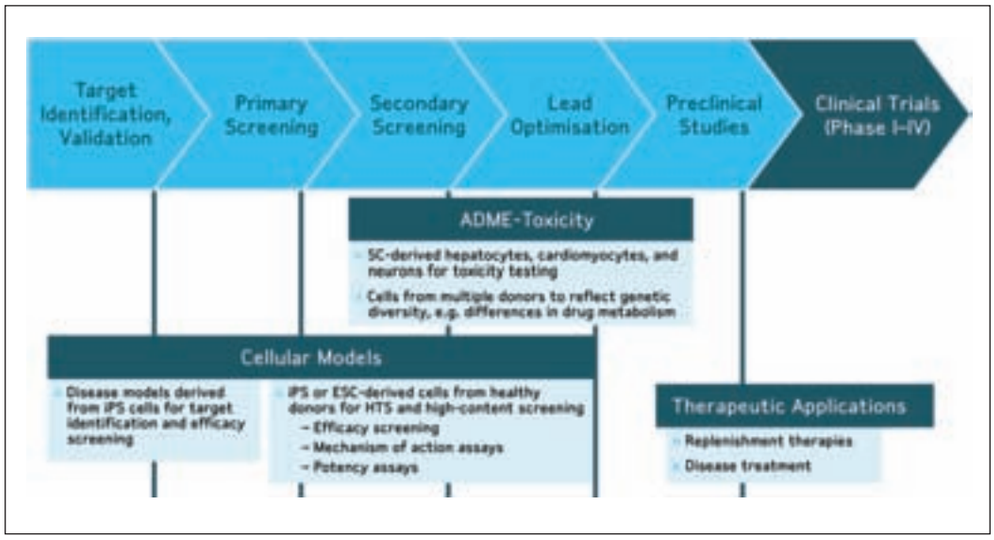

Figure 2
Potential uses for iPS cells for target identification, screening, ADME/toxicity testing and therapy

Several growth factors have been identified that promote growth of human ES cells in culture, most notably basic fibroblast growth factor (bFGF). However, the use of bFGF for human ES cell culture, particularly at the high levels used by some for 'feeder-free' culture, must be considered carefully, as bFGF may drive ES cells towards differentiation.

Xeno-free media that do not rely upon high levels of bFGF have been developed that contain only humanised or synthetic components and are engineered to support the undifferentiated growth and expansion of human ES cells. Use of human feeders derived under xeno-free conditions can maintain the xeno-free nature of the culture system and allow for the use of low levels of bFGF.

Use of progenitor cell lines can eliminate the need to culture challenging human ES cells. Progenitor cells are similar to embryonic stem cells in their capacity to differentiate into various cell types. However, progenitor cells can only differentiate into a limited number of cell types. Progenitor cells can be far easier to handle in culture than ES cells. For example, neural progenitor cells derived from a human ES cell line are easily propagated and require less handling than human ES cells. In addition, they do not require feeder layers and can differentiate into many neural lineages under the appropriate conditions[1].

The need to control differentiation of embryonic stem cells *in vitro* presents another set of challenges.

Identifying the right cocktail of media conditions, supplements and growth factors that successfully drive stem cells toward a desired lineage on a reproducible basis is a time-consuming, iterative exercise. A carefully choreographed series of signals must be recreated to guide cells down the chosen pathway. Fortunately, this labour-intensive work has already been done for a number of cell types. Kits and media containing an optimised set of factors necessary to differentiate stem cells to a chosen lineage are commercially available for generating neurons, oligodendrocytes, mesenchymal cells and osteocytes.

Cellular reprogramming

The discovery of a process whereby fully differentiated, adult somatic cells can be reprogrammed into induced pluripotent stem cells (iPS cells) has provided researchers entirely new approaches for target discovery, screening, metabolic profiling and toxicity evaluation[2].

iPS cells, which are similar to embryonic stem cells in their ability to differentiate into a wide variety of cell types, are now routinely generated from adult cells. Fibroblasts derived from a simple skin biopsy are a common starting point. For example, fibroblasts from a patient with Alzheimer's disease or amyotrophic lateral sclerosis (ALS) can be reprogrammed to yield iPS cells. The iPS cells can then be induced to differentiate into neurons and other cell types that might be affected in the disease.

Initial efforts to generate iPS cells required simultaneous co-infection of cells with four separate retroviral expression vectors. Each vector carried one transcription factor, which resulted in a high number of genomic integrations which may activate or inactivate critical host genes. Alternative approaches to iPS generation have included use of plasmids and non-integrating

adenovirus vectors to deliver the transcription factors. However, the reprogramming efficiency of these methods (ie, the rate at which cells convert to pluripotency) is currently lower than use of retroviral vectors[3].

Generation of human and mouse iPS cells have been accomplished using a single, excisable lentiviral vector that delivers all four 'Yamanaka' transcription factors[4]. Use of a single vector significantly reduces the number of viral integrations required – in some cases, iPS clones possessing only a single viral integrant can be isolated[5].

Tools for discovery and development

The ability to revert somatic cells to an embryonic state and subsequently differentiate them into a variety of cell types offers a wealth of opportunities for personalised regenerative medicine and disease research. Because iPS cells can be generated from individuals with different clinical phenotypes and genotypes, they can offer a strategic advantage over embryonic stem cells for use in patient-specific cell replacement therapies and drug discovery and development (**Figure 2**).

Disease modelling

For decades, researchers have relied on animal models, immortalised cell lines, or short-lived primary cultures to dissect the mechanisms and pathogenesis of diseases. Genetic manipulations including over-expression, knock down, knock out, and knock in strategies are often employed in animal models in an attempt to replicate genetic patterns linked to specific disease phenotypes.

Differentiated cells derived from iPS cells have the potential to overcome the inherent limitations of existing disease model systems. Cells derived from patient-specific iPS cells can potentially provide a more relevant model system as their properties more closely resemble those found in the patient's own system and they do not require genetic manipulation.

Diseases that arise from single base mutations or deletions are well-suited for modelling with iPS technology. iPS cells also offer tremendous opportunity modelling diseases that do not have robust animal models and more complex conditions that involve a number of different cell types such as obesity and metabolic disorders.

For example, while a researcher can easily obtain fat cells from a patient with a metabolic disorder, these cells cannot be cultured over the long term and may only allow for a one-time endpoint assay. By leveraging iPS cells to generate a sustained supply of patient-specific fat cells, the complexities of the metabolic disorder can be examined more effectively.

With iPS cells, the researcher can conduct dozens of assays to identify differences in fat cells from a person with a metabolic disorder such as type 2 diabetes versus an unaffected individual. The ability to take a single genotype and potentially make any of the tissues that might be involved in a metabolic disorder – such as pancreatic beta cells, hepatocytes, or hypothalamic cells – can lead to a powerful disease model.

Drug screening

Using iPS derivative cells, potential therapeutics can be screened against a large number of patient-specific cells prior to initiating clinical trials. Variation in the response to drugs by cells of patients with genetic differences can guide more targeted selection of patients for enrolment in clinical trials, resulting in trials that are smaller and more likely to be successful.

IPS cells can be particularly useful when primary cells are difficult or impossible to obtain. For example, drug screening applications for cystic fibrosis can be developed using lung cells derived from iPS cells[6]. Obtaining lung cells from a patient with cystic fibrosis is typically only possible if the patient is undergoing a lung transplant. However, these patients have dramatic lung infections so it is difficult to establish primary cell lines. And even if a cell line can be successfully established, the cells will have a limited ability to be passaged, which greatly limits the ability to generate enough cells for the screening process. Through use of iPS cells, large numbers of cells can be generated from a range of patients, and genomic patterns can be cross-referenced to drug screening results to predict the effectiveness of different drugs on different phenotypes.

Investigative toxicity

Drug-induced liver injury has been the most frequent single cause of safety-related withdrawals of marketed drugs over the past 50 years[7] and is the principal reason clinical trials are suspended. More efficient and predictive toxicity studies can be expected to reduce development costs associated with the late stage failure of drug candidates. Identification of drug candidates with toxicity issues earlier in the discovery process will most likely result in improved safety for clinical trial participants and patients.

Investigative *in vitro* liver toxicity studies are typically conducted using primary human hepatocytes or an immortalised (genetically transformed)

Figure 3
Requirements to support large-scale manufacturing of stem cells

Process Needs	Basic Research	Translational Research	Clinical Adoption
cGMP Media & Growth Factors	Defined media	Transition to cGMP media	Defined cGMP media
Bioreactors/ Automation	Some need at research scale	Needed by Phase II/III transition	Needed for clinical scale bioprocessing
Downstream Processing	Little need at research scale	Needed by Phase II/III transition	Needed for clinical scale bioprocessing
Cell Characterisation	Assays for cell identity and function	Transition to process monitoring and release	Process monitoring and release assays
Cells for Screening	Pharma toxicity testing and drug discovery	Not applicable	Not applicable

High Medium Low

liver-derived cell line such as HepG2. Despite routine use for investigative toxicity, both of these options present significant drawbacks:

- Primary human hepatocytes are derived from fresh liver tissue, typically sourced from cadavers or cancer resections. Supply of these cells can be limited and the tissue can vary widely among donors.
- Primary hepatocytes cannot be sustained for more than a few days in culture without losing function. Securing a consistent supply of cells requires repetitive sourcing, which further contributes to variability.
- Immortalised hepatocyte cell lines can be cultured indefinitely, which addresses the supply and variability issues associated with use of primary human hepatocytes. However, these cells display distinct differences from normal liver cells and may not exhibit normal cell behaviour or response. For example, most cytochrome P450 enzymes (responsible for drug metabolism) are expressed only weakly in HepG2 cells compared to normal human hepatocytes.

Although the pharmaceutical industry relies on animal models for preclinical metabolism and toxicity testing, these models also have limitations. Animal models may not be fully and reliably predictive of human toxicity. In addition, animal models are low throughput, expensive and raise ethical concerns for some.

Cost and throughput often relegate use of animal models to the later stages of preclinical development. By this time, a company has invested significant time and resources in a lead compound. This delayed evaluation of toxicity contributes to the high failure rate of compounds in late stage preclinical testing, which is extremely costly.

Earlier, more effective assessment of drug candidate toxicity has the potential to reduce the

attrition rate of drugs in later stages of development. Differentiation and expansion of human stem cells into hepatocytes for use in investigative toxicity studies could overcome the shortcomings of primary hepatocytes and immortalised cell lines. Use of stem cell-derived hepatocytes (and other cell types commonly used for toxicity studies) offers a number of important advantages to investigative toxicity studies including:

- Availability of a consistent source of cells that more closely match *in vivo* phenotype and physiology.
- Elimination of reliance on donor sources which can have sporadic availability.
- A more standardised, reproducible process for toxicity testing.
- Reduction in the use of animal models and animal tissue.
- Improvement in the predictive capabilities of early toxicity studies leading to reduction in late stage attrition of drugs.

More efficient and predictive toxicity studies enabled by iPS-derived cells can be expected to reduce development costs associated with the late stage failure of drug candidates. Identifying drug candidates with toxicity concerns earlier in the discovery process can improve the safety and, ultimately, the success of clinical trials.

Engineering large-scale stem cell production

As more stem cell-based therapeutics progress towards clinical testing[8], process-scale stem cell manufacturing systems must be engineered. Achieving 'industrialised' production of stem cells while meeting rigorous quality and regulatory standards will depend on further progress in the areas of cell culture and scale-up, characterisation, enrichment, purification and process control to deliver a consistent and reproducible supply of cells in a safe and cost-effective manner (**Figure 3**).

Although industrial-scale cell culture methods exist for the manufacture of protein therapeutics, these processes are always directly transferrable to the culture of stem cells where the cell itself is the 'product'.

Culture conditions

Industrial-scale stem cell cultures must adhere to Current Good Manufacturing Practice (cGMP) standards, incorporating well-defined, well-characterised media and supplements to support cell expansion and differentiation as desired. The FDA does not mandate the complete absence of animal products in stem cell cultures. However, there is interest in moving away from use of undefined serum, which is incompatible with the tightly controlled culture conditions necessary for producing human therapeutics.

Incorporation of cGMP-compliant supplements contributes to high quality, consistent, reproducible culture conditions. When manufactured under cGMP conditions, supplements enabling robust proliferation of stem cells in culture without the need for feeder layers are fully characterised and validated for activity, potency and purity. In addition, cGMP supplements are generally produced in larger lot sizes, which contribute to the reproducibility of culture conditions.

Although some cell culture supplements are currently available in cGMP form, many are not, including the widely used epidermal growth factor and fibroblast growth factor. In those labs where stem cells are being cultured for evaluation in the clinical setting, researchers often develop their own cGMP supplements. This process can be time-consuming and labour-intensive. It also doesn't allow for standardisation of culture conditions across different labs and organisations.

Process monitoring and characterisation

Once *in vitro* conditions are optimised, culture parameters and cell phenotypes must be continually monitored to ensure conditions do not drift outside established limits. Reproducibility and consistency of the stem cell 'product' is highly dependent on the ability to monitor culture conditions and cell phenotypes in real time (or as close to real time as possible). Media and external factors can be readily monitored and adjusted following standard practices that have been applied to the soluble components of protein production, but assessing phenotypic characteristics is more challenging.

Control strategies for biopharmaceutical production using transformed cell lines or microbes are generally based on stabilisation of cultures at a steady state condition to optimise recombinant protein yield. However, stem cell therapy production is a dynamic process. Cell populations evolve with time. Therefore, dynamic, rather than static, controls will likely be more effective. Efforts are currently under way to create more timely feedback loops that can assess the effects a process is having on the phenotypic characteristics of a culture while there is still time to modify process.

Integrated cell processing systems

Consistent and reproducible stem cell manufacturing requires scalable, optimised solutions that

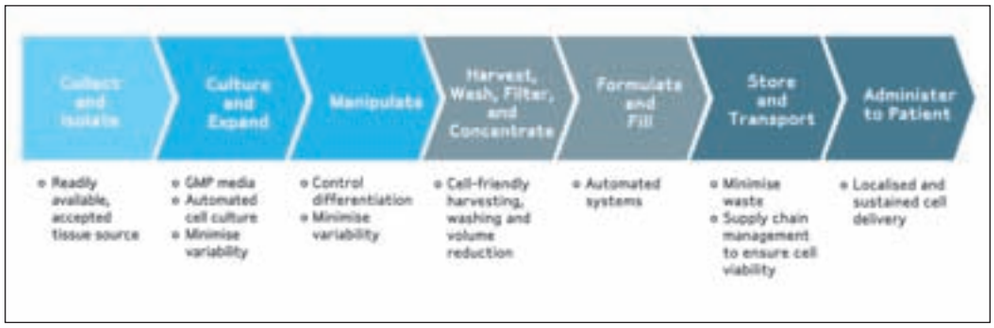

Figure 4
Large-scale stem cell production requires scalable, optimised solutions that impact every phase of the development pipeline

address each phase of the development pipeline (**Figure 4**). The physical system in which the cells are grown must also be able to:

- Minimise variability in the cell population.
- Enable harvesting and formulation without damaging cells.
- Incorporate processes to ensure cell viability during storage, transport and administration to the patient.

An important element in these integrated systems is the substrate upon which the stem cells grow. Anchorage-dependent stem cells must affix to a solid surface when grown *in vitro*. Currently, microcarriers are the only convenient way to scale up cultures of stem cells in a stirred bioreactor system. Unfortunately, the use of microcarriers presents a number of challenges. Small particulates or 'fines' are often generated during the microcarrier manufacturing process. These fines can end up in the culture system. Fines can also result from beads being crushed during the cell harvest process. Filtration of the stem cell cultures cannot remove these particulates, so they are co-purified with the cells. The presence of foreign particulate matter – such as microcarrier fines – is unacceptable for injectable products.

In addition, the ability of a stem, or derivative, cell, to grow on a microcarrier is influenced by how strongly the cells bind to the surface. If the surface chemistry causes the cells to adhere too tightly to the microcarriers, the cells' ability to grow and divide may be limited. The strength of adherence can also influence cellular phenotypes, as well as the success of the harvesting process. Cells must be recovered at the end of the scale-up process. The more tightly the cells are bound to the microcarrier surface, the more difficult it can be to remove the cells. Repeated trypsin digests can damage the cells, cause phenotypic changes and reduce viability.

It is likely that expansion of different stem cell lines will require a slightly different microcarrier surface to ensure optimal growth. A less than optimal surface may lead to an undesired cell population. This is especially true when differentiation is carried out on the microcarrier because the process is quite susceptible to both elasticity and surface chemistry.

A number of technological solutions are being developed including scaffolds made from a variety of biocompatible materials such as polyacrylate, hydroxyapitite and polylactic acid as well as natural materials including silk, chitin, collagen and polyglycolic acids such as hyaluronic acid. Scaffolds made from fibrous mats and hollow fibres, have been shown applicable with stem cells because they afford the ability to culture with perfusion, better simulating the natural environment.

Alternatives to microcarriers are in the early stages of development and are not yet feasible for large-scale cultures. Techniques are being developed to culture these cells in suspension culture without the need for a surface. In these systems, cells clump into small aggregates or are encapsulated in gel microdroplets. Cell densities can be increased limited by availability of nutrients. This approach has been demonstrated with murine stem cells, but has yet to be proven with human cells which are less robust.

The final step in the development pipeline – harvesting and packaging of live cells – is also a focus of intense development efforts.

Existing centrifugation and filtration technologies are not optimised for the harvest and recovery of live cells. Centrifugation is often used to collect cells for research applications. However, centrifugation is not always practical for the collection of large numbers of stem cells. Centrifugation is typically not a closed system. In addition, shear forces can damage cells. After cells are harvested, they must be rapidly concentrated, the media washed away with buffer solution, and packaged into containers for freezing

or administration to patients. New approaches must be developed to efficiently manage this fill/finish process for stem cells.

The road ahead

Stem cells represent seemingly limitless application in the clinical setting and can offer greater efficiency and physiological relevance to the drug discovery and development process. Yet along with this great promise come numerous challenges. While significant progress has been made, there is still much to do. Taking full advantage of the unique properties of stem cells will require a greater understanding of their inner workings as well as innovative technology to enable large-scale, consistent production. DDW

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