

# Large-scale expansion of stem cells for therapy and screening

Recently, pre-clinical and clinical studies of stem cells in regenerative medicine have demonstrated great promise as treatments for various diseases. Their ability to self-renew and differentiate makes stem cells valuable for both cell therapy and tissue engineering fields, both of which drive the demand for large-scale cell expansion. In addition, having adequate and reproducible numbers of both stem cells and differentiated cells for compound screening provides a powerful research tool in drug discovery. Here, we outline recent advancements of single use cell scale-up tools and vessels that can improve the ability to generate large quantities of stem cells for therapeutic purposes and basic research.

Stem cells are undifferentiated cells with the ability to self-renew and differentiate into specialised cell types during early development and growth. When a stem cell divides, each of the daughter cells may remain as a stem cell or undergo differentiation into a cell lineage with specialised function. There are two main classes of stem cells: embryonic (pluripotent), isolated from the inner cell mass of developing blastocysts and adult (multipotent), located in various tissues throughout the body. Adult stem cells, in their natural state, act as a repair system, dividing without limit to replace and replenish obsolete damaged tissue.

Stem cells are unspecialised cells that do not possess any tissue-specific structures to perform specific functions. However, these unspecialised cells can give rise to specialised and differentiated cells with unique structures and functions through several growth stages. Given their unique ability to regenerate, stem cells offer new and exciting potential for treating diseases as cell-based therapies or

regenerative medicine. Recent clinical trials in both areas are exploring the use of stem cells to differentiate into specific cell types to replace or repair damaged cells or tissues. This review covers the implications of stem cells in cell therapy and regenerative medicine, as well as addressing various methods needed to achieve large numbers of cells for therapeutic purposes and basic research.

Cell therapy, as defined by the FDA, is ‘The prevention, treatment, cure or mitigation of disease or injuries in humans by the administration of autologous (derived from same cells), allogeneic or xenogeneic cells that have been manipulated or altered *ex vivo*’<sup>1</sup>. In autologous stem cell administration, cells are derived from the donor and applied to the donor, while allogeneic stem cell administration involves the transfer of cells from healthy donors to afflicted recipients.

Like regenerative medicine, cell therapy relies on healthy tissues and cells to repair and heal cellular damage, as well as replace failing organs. More

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Product	Growth Area (cm <sup>2</sup> )	Units required	Spatial footprint (ft <sup>2</sup> )
T175-flask	18,200	104	4.5
CellFactory-10	18,960	3	1.4
CellSTACK-10	19,080	3	1.4
HYPERStack-36	18,000	1	0.7
Microcarriers in 1- 1L spinner	18,000	1 Spinner flask, 4 – 50g*	0.2

**Table 1:** Projected unit required for a typical 2 billion cell production using different vessel and platforms. Cell production is calculated using 18,000cm<sup>2</sup> of growth surface. Typical harvest density at 100,000 cells/cm<sup>2</sup>.

\* Amount of microcarriers required dependent on product used

recently, cell therapy has also been proposed to counter the effects of ageing, improvement of general health and reversal of degenerative diseases.

Currently, donated organs and tissues are commonly used to replace failing or destroyed tissue, but the need for transplantable tissue far outweighs the available supply. Stem cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat multiple diseases. In the last decade, the usage of stem cells to treat degenerative and debilitating diseases, autoimmune conditions, musculoskeletal injuries, as well as cosmetic treatments have all been proven feasible. Spinal cord injuries, once thought incurable, represents an area of research where stem cells may provide better treatments and even cure<sup>2</sup>. Neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's and Parkinson's diseases, have all exhibited the benefits of using stem cells to better understand the fundamental biology underlying disease pathogenesis<sup>3-6</sup>. Similarly, cardiovascular diseases such as myocardial infarction have relied on stem cells to repair damaged heart tissue<sup>7,9</sup>.

Human stem cells can also be used to evaluate the efficacy and safety of novel investigational drugs, thereby providing a physiologically relevant approach for high throughput screening of potential drug candidates. Recently, studies have been performed evaluating human stem cells, programmed to become tissue specific cells, and their response to novel drugs for oncology and neurodegenerative research<sup>10-12</sup>. Such experiments are similar to high throughput screens currently performed using engineered cell lines. These latter cells may give rise to false positives and negatives, and as a result primary cells derived from stem

cells are gaining acceptance. Consequently, many new drugs could be screened and profiled for their potential efficacy to a specific disease while simultaneously identifying potential side-effect toxicity and metabolic activities.

A more challenging aspect of cell therapy is the isolation of stem cells. Isolation of embryonic stem cells relies on the extraction of the inner cell mass from developing blastocysts. For autologous adult stem cells, bone marrow, adipose tissue and blood are three accessible sources. Although adult stem cells are more limited in their ability to differentiate into different lineages, emerging evidence has shown that they have the ability to generate unrelated cell types via genetic reprogramming<sup>13-15</sup>.

Many studies have shown that stem cells rapidly expand after *in vivo* transplantation. Yet a major challenge remains with the *in vitro* expansion and culture of self-renewable stem cells and the subsequent differentiation of these cells. Recently, several protocols have been reported that alter culturing conditions and other factors (eg medium and design of cell culture vessel) that support reliable amplification of immature and differentiated stem cells. However, challenges still exist in optimising the wide variety of platforms capable of supporting cell therapy needs.

The ability to generate and manufacture lot sizes of cells in the 100 billion to 1 trillion ranges is a major challenge in cell therapy. The number of cells (or lot size) needed require different approaches to those used in classical cell culture techniques. Traditional cell culture flasks provide, on average, a growth area less than 200cm<sup>2</sup>, generating cells in the low million unit range. Protocols to support the expansion of stem cells on these flasks are very labour intensive and are limited in their scale-up potential. Recently multilayer planar vessels (626-36,000cm<sup>2</sup>) have been used to expand stem cells. These multilayer planar vessels, (eg Corning® CellSTACK®, HYPERStack® and Nunc® Cell Factory) provide added benefits including (i) decreased risk of contamination; (ii) increased total surface area within each vessel; (iii) reduced number of vessels required per lot size and (iv) reduced labour and operating costs. Scaling up from traditional cell culture flasks into these vessels tends to be an easier and direct translation leading to a decrease in the process development and optimisation time compared to other technologies. Additionally, it is feasible to achieve the required lot size by either increasing the vessel size or the layer number in a multilayer vessel. Recent data has shown that 50-70 10-layer vessels can be used to produce desired lot sizes using roughly

400,000cm<sup>2</sup> of growth area using robotic EPA-TEC manipulators. Many of these vessels have the ability to be accessorised with venting filters and single-use connectors enabling a closed system approach to reduce the risk of contamination.

Although multilayer planar vessels address many of the scale-up needs, the demand for ready-to-use cell scale-up products have pushed manufacturers to further develop technology that increases surface area while requiring no sterilisation or labour-intensive cleaning steps, thereby minimising the risk of cross-contamination and providing adequate growth environments. These novel technologies must provide efficient mixing and delivery of gasses to accommodate high density cell growths and, with the diverse lot size demands, they should incorporate aseptic connectors, allowing multiple units to be connected with each other. This, in turn, will lead to a more precise and accurate control over the vessels required to meet specific lot size challenges and demands.

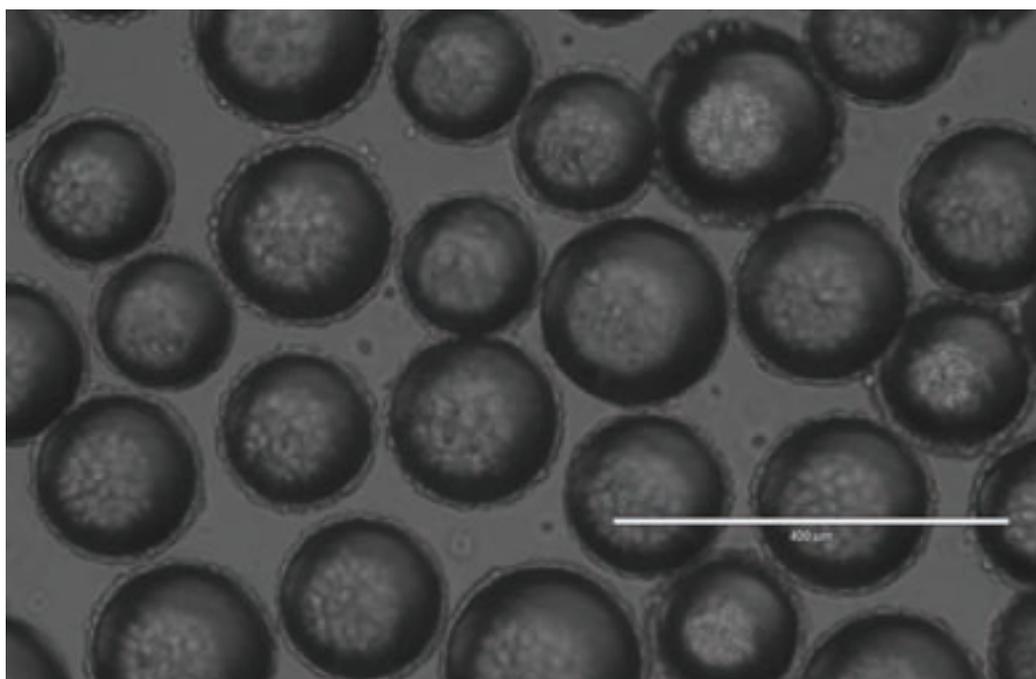
To fill the cell expansion demands in industry, multiple types of bioreactors have entered the market. Some of these technologies that could be used for stem cell expansion are perfusion type parallel plate (eg Corning Cell Cube and ATMI Integrity Xpansion) and fixed bed bioreactors (eg New Brunswick Fibra-Cel Disk System and FiberCell System, ATMI iCELLis and Beckman Coulter's Quantum). The parallel plate bioreactors are simi-

lar to multilayer vessels with the added benefits of controlling and monitoring multiple variables such as pH, dissolved oxygen (dO), and dissolved CO<sub>2</sub> with the use of computerised controllers. Like most multilayer vessels, these parallel plate bioreactors can be further connected in a sterile environment in series or parallel with other units thus providing larger platform lots. Additionally, since the technology used for a multilayer vessel and a parallel plate bioreactor are similar, the time spent optimising cell growth and harvest conditions may be minimised. A fixed bed bioreactor, however, offers the advantage of decreased mL/cm<sup>2</sup> without an increase in shear stress. Though, cell harvest and proper maintenance of a homogenous axial and radial nutrient distribution from fixed bed bioreactors are often more difficult and therefore a large amount of time may be required for optimisation.

In addition to parallel plate and fixed bed bioreactors, researchers may also obtain the surface area for cell growth using microcarrier beads. Microcarriers offer several advantages over other forms of technology. High scale expansion of cells using microcarriers is obtained in bioreactors (stainless steel or single-use) under a controlled environment. Bioreactors allow the researcher to control and monitor multiple parameters such as pH, dO and temperature. The constant mixing provides researchers with the ability to reduce the amount of medium from 0.2-0.5mL/cm<sup>2</sup> (found in

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Vero cells expanded on Corning® collagen coated microcarriers in a 1L glass spinner flask. Images were captured using the AMG EVOS® FI microscope. Scale bar represents 400µm

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Corning® CellBIND®  
HYPERStack® 12 and 36 layer  
Cell Culture Vessel

traditional flask technology) to  $>0.1\text{mL}/\text{cm}^2$ ), thereby decreasing medium costs and increasing overall efficiency. Additionally, researchers can purchase large bioreactors (eg 2,000L) to generate massive quantities of cells in one run to reduce variability and risk of contamination. Studies have also demonstrated that to scale up stem cells (eg hMSC) one can add more microcarriers for further expansion without the use of dissociation reagents<sup>16</sup>. hMSC expansion, for example, has been demonstrated on various microcarriers (eg collagen, Matrigel®, laminin or vitronectin-coated) in spinner flasks and stirred-tank bioreactors<sup>16</sup>.

Nonetheless, when expanding stem cells (eg hMSC) using microcarriers there are multiple obstacles that need to be addressed. For instance, the agitation rate, sparger choice (eg micro versus macrosparger), sparging rate and impeller design can significantly impact cell attachment and thus expansion on microcarrier beads<sup>16</sup>. Secondly, the type of microcarrier selected can greatly impact cell yields (eg positive charged versus coated microcarriers). Therefore, careful consideration should be taken when selecting the type of bioreactor, as well as the type of microcarrier for stem cell scale-up. Lastly, cell harvest may be more challenging on microcarriers when compared to cell culture ves-

sels, and multiple studies are often performed to select the optimal dissociation reagent for the selected microcarriers. A comprehensive review of the application of stem cell microcarrier cultures in cellular therapy can be found in reference 16.

As mentioned above, high scale expansion of stem cells may be achieved in either stainless steel or single-use bioreactors. Single use, disposable systems have found widespread adoption in areas of advanced cell culture, as well as in industrial scale up activities. In the former, there is growing use of single-use culture vessels, microplates and roller bottles, while in the latter there is now widespread adoption of disposable biomanufacturing equipment, including storage bags, fermenters (with volumes up to 2,000L), filters and other components. The present market share of single-use manufacturing systems of about 10% is changing rapidly, as more biomanufacturing activities are outsourced and both BioPharma and Contract Manufacturing Organizations (CMOs) require reduced capital investment, decreased cross contamination and maximal flexibility. These trends are particularly evident in the area of 'upstream' bioprocessing. To facilitate the broader use of single use systems, there are increasing efforts under way in terms of standardisation on connectors, fitting and valves, as well as testing for extractables and leachables. Extractables are chemical entities released from the plastic under conditions of elevated temperature in the presence of certain solvents. Leachables are released from the plastic under conditions of actual use. Standardisation efforts will undoubtedly continue in this area as the adoption of single use technologies grow and mature.

The high interest in cell-based therapies, and stem cells in particular, are also driving adoption of new technologies, particularly those of a single use disposable nature. A key problem in this area lies in the generation of large numbers of cells, accompanied by high reproducibility, for use in clinical trial evaluation and ultimately as therapeutics. To some extent, a range of systems are available for stem cell production in the 'upstream' bioprocessing stages, but new technologies are clearly needed for scale up 'downstream' activities. Several technologies in this area are showing promise, including the use of perfusion bioreactors, parallel plate bioreactors, hollow fibre bioreactors and fixed or fluidised bioreactors. It is likely that scale up of stem cell bioprocessing will require an 'in-series and in-parallel' modular use of bioreactors and consequently the use of single use disposable systems will proportionally increase.

Multiple parameters can influence cell yield

either on microcarriers, perfusion type parallel plate, or fixed bed bioreactors. Some of these conditions include the medium, bioreactor and microcarrier employed. Typically, stem cells are cultured in the presence of serum (5-10%); however, in clinical applications serum-containing medium is a risk for the cell therapy industry, due to potential viral and prion transmission, immunological reactions, as well as variability between serum lots. In addition, the presence of animal-derived components in the serum increases the downstream processing steps necessary to clarify/purify the end product. Studies have demonstrated that by switching to chemically-defined media there is a reduction in downstream processing, leading to time and cost savings. Consequently, ideal medium conditions for stem cell expansion in clinical applications would be under animal-free, xeno-free, chemically-defined conditions.

While optimisation in upstream processing is a key challenge in stem cell therapy, downstream processing is another major consideration. For protein and vaccine therapies, downstream processing is a more defined and optimised process, but for stem cell therapies the process is more difficult since the cells are the product, and viability must be maintained. During this process the cells are concentrated, washed (through processes such as tangential flow filtration and continuous centrifugation) and then packaged and cryopreserved for future use. Large scale cryopreservation of cells is a critical, final step in downstream processing and therefore this process needs to be well controlled (eg frozen at a controlled rate and quickly thawed) to ensure viable cells. Like upstream processing, downstream processing would ideally include the use of disposable or single-use technologies, as well as automation steps to reduce the risk of contamination and maintain consistency between lots.

After upstream and downstream processing steps it is critical to confirm that the stem cells remain multipotent. A common method to evaluate multipotency is to measure expression of cell surface antigens (eg CD105 – positive marker for hMSC) via flow cytometry. Stem cell functionality can also be assessed using quantitative trilineage assays. Lonza, for example, offers an Adipogenesis Assay Reagent, AdipoRed™, to quantitate differentiated adipocytes from hMSC. Lastly, additional characterisation assays (eg migration assays, induction of endothelial cell proliferation, etc), which are dependent on the desired application, should be performed prior to release of cells for clinical applications.

### Stem cells as therapeutics: a promising frontier

Stem cells are truly master cells with unique properties and abilities. They can proliferate virtually without limit to produce large quantities of autologous unspecialised cellular copies. Furthermore; they can differentiate to produce other cell types that can be used to treat damaged tissues. As a consequence, they are a powerful tool to both understand fundamental disease pathology and act as therapeutics.

In the last decade, many research laboratories have developed protocols to isolate, grow and apply stem cells for use as revolutionary treatment options for many major debilitating diseases. During this same period of time, great strides have been taken to further expand the growth of stem cells from classical cell culture flasks to novel multi-layer vessels, microcarriers and/or single use bioreactors. In the next few years, further development will undoubtedly involve optimising conditions that will focus on animal-free, xeno-free technologies, thus alleviating the risk associated with the use of serum and animal-derived coatings. **DDW**

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