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For the full text of this licence, please go to: http://creativecommons.org/licenses/by-nc-nd/2.5/ The larger scale expansion of pluripotent stem cells for regenerative medicine purposes

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Nomenclature

Abstract

Introduction

The last 15 years has seen the growth of a new and global healthcare industry based on human proteins produced in genetically engineered cells with an estimated current market value of ~£30 billion a year. There is now an opportunity to replicate this growth in the new industry of regenerative medicine. The next healthcare revolution will apply regenerative medicines, creating biological therapies or substitutes for the replacement or restoration of tissue function lost through failure or disease. However, whilst science has revealed the potential (ref), and early products have shown the power of such therapies (ref), there is now a need for the long term supply of human pluripotent stem cells (PSCs) in sufficient numbers to create reproducible and cost effective therapeutic products. Since human cells are known to develop both genetic and epigenetic instability over many passages, as well as loss of functionality⁷, there is a defined need for technologies that will allow the maximal expansion of each cell line for cryobanking at low passage numbers that will enable larger scale healthcare applications. The scale-up techniques to be developed for PSC's are analogous to those already developed for biopharmaceutical production using mammalian cells at large scales but there are a number of unique challenges that need to be addressed not least of which is that it is the cell that is now the product rather than the proteins that they express.

Human PSCs are adherent, dependent on attachment to either a feeder layer of cells or an extracellular matrix substitute and thus, until recently, have been cultured manually in planar, culture systems (T flasks). Although successful automation of these manual hESC culture processes, using for example the CompacT SelecT (The Automation Partnership) or the Cell^{host} (Hamilton Robotics), has been demonstrated {{379 Thomas,R.J. 2009;86 Terstegge,S. 2007;}} and may be suitable for the cGMP production of smaller quantities of cells for autologous therapies, they are limited ultimately by the necessity of a large footprint within a facility as well as the need for highly trained operators. Additionally, such automated systems often use wasteful quantities of expensive growth media and are only linearly scalable i.e. by adding more vessels or culture platforms, rather than increasing vessel size geometrically. Now that reports have started to emerge of the successful larger scale culture of PSC's in conventional stirred tank systems using either microcarriers as surfaces for cell attachment (see Table 1) or by growing them as suspension aggregates (see Table 2) the aim here is to critically review the progress that has been made in the area of suspension culture of PSC's in the last few years. Further we present a detailed analysis of the viability of these methods for realising the end-goal of larger-scale production of therapeutic materials from human pluripotent cells.

Conventional T flask Culture

The ability of PSCs to self-renew and differentiate into specific cell types are key characteristics of the final cell as a product, that must be retained regardless of the method of culture or passaging. In addition, genetic or epigenetic instability, which is known to be associated with long-term culture of hPSC's {{105 Allegrucci,C. 2007;}} and can lead to tumourogenicity must also be avoided. When mouse and human ESC's were first derived and propagated it was found that they were not only adherent, but in order to retain pluripotency they had to be grown on feeder layers {{117 Thomson,J.A. 1998;583 Evans,M.J. 1981;}} of, for instance, inactivated

mouse embryonic fibroblasts cells (mESC's). Such feeder layers introduce a highly variable, and xenogenic, component into hESC culture, which needs extra processing steps to ensure removal before analysis or further processing. Although it was rapidly discovered that by adding leukaemia inhibitory factor (LIF), mESC's could be propagated without the need for a feeder layer {{586 Ying,Q.L. 2003;}}, the equivalent single factor that can maintain pluripotency of hPSC's in extended culture has not yet been found, although basic fibroblast growth factor (FGF) and transforming growth factor- β have been shown to be important for self-renewal and maintaining the undifferentiated state {{587 Azarin,S.M. 2010;}}. Importantly, the use of xenogenic materials for the production of human therapies is not likely to gain regulatory approval so to overcome some of these issues, a number of human cell types have been used as feeders, including foetal muscle {{588 Richards, M. 2003;}}, neonatal foreskin {{589 Hovatta,O. 2003;}} and hESC-derived fibroblasts {{590 Stojkovic, P. 2005; }}. However, separation technologies don't yet exist, at least not at the larger scales, that will prevent contamination of these feeder cells of the final product.

Other work has concentrated on the use of feeder-conditioned medium in conjunction with an extracellular matrix substitute, such as Matrigel, in order to culture hPSCs {{591 Xu,C. 2001;592 Totonchi,M. 2010;}}. Matrigel is the extracellular matrix secreted by an Engelbroth Holm Swarm mouse tumour cell-line and is a mixture of extracellular matrixes, proteoglycans and growth factors {{491 Kleinman,H.K. 2005;593 Hughes,C.S. 2010;}}. Additionally, several defined media, which represent a departure from the use of foetal bovine serum, have also been developed such as StemPro (Invitrogen) and TeSR ({{138 Ludwig,T.E. 2006;}}; Stem cell Technologies). The vast array of feeder- and serum-free conditions that have been developed for particular cell lines have been extensively reviewed elsewhere {{292 McDevitt,T.C. 2008;594 International Stem Cell Initiative Consortium 2010;}} and whilst these are a step in the right direction, from a manufacturing point of view, many still require cells to be cultured on Matrigel which, like the mouse embryonic fibroblasts is xenogenic, unlikely to gain regulatory approval and introduces lot-to-lot variation. These media are also very expensive and, consequently, are not compatible with cost-effective, large-scale culture. Some success has been reported growing hPSC's on individual extracellular matrix components such as fibronectin, vitronectin or laminin {{131 Amit,M. 2004;596 Rodin,S. 2010;597 Braam,S.R. 2008;}} which might result in a more defined and xeno-free system however, recently, research into synthetic alternatives has shown more promise {{598 Kolhar,P. 2010;137 Li,Y.J. 2006;601 Mahlstedt,M.M. 2010;602 Melkoumian,Z. 2010;}}

Human PSC's are typically grown in colonies because cell-cell interactions are important for the retention of self-renewal and pluripotency but large colonies often show high levels of spontaneous differentiation {{587 Azarin,S.M. 2010;}} and thus these must be passaged regularly. However, if colonies are dissociated to single cells, they are prone to anoiksis {{136 Watanabe,K. 2007;}} and karyotypic abnormalities can occur{{595 Brimble,S.N. 2004;}} therefore they are often manually forcibly microdissected into small clumps for reseeding which is highly labour intensive and unsuitable for large scale processing. Although long-term enzymatic dissociation has been linked with genetic abnormalities {{105 Allegrucci,C. 2007;}} and can lead to variable colony sizes, it is frequently used to rapidly passage colonies of hPSC's into small clumps for reseeding. It has also been found that supplementing media with a

Rho-associated kinase (ROCK) inhibitor, Y-27632, before and after plating, or introducing this inhibitor into the Matrigel, can promote survival of hPSC's that have been dissociated to the single cell level {{603 Pakzad,M. 2010;136 Watanabe,K. 2007;}}, creating more uniform colonies and allowing more reproducible seeding densities. Enzymatic dissociation has also been used to move away from colony culture altogether and the successful monolayer culture of a range of hESC lines has been reported {{379 Thomas,R.J. 2009;596 Rodin,S. 2010;601 Mahlstedt,M.M. 2010;604 Priddle,H. 2010;605 Chin,A.C. 2010;}}. Such continuous monolayer cultures are more suited to automated processing {{379 Thomas,R.J. 2009;}} and provide a more homogenous, controlled environment for differentiation protocols as soluble factors will be equally available to all cells {{596 Rodin,S. 2010;}}.

Stem cell culture in larger scalable bioreactors.

The culture of stem cells in stirred tank bioreactors offers marked advantages over the standard T flask methods; simplicity of scale-up, availability of continuous, online, process monitoring and enhanced medium utilisation. The necessity for large quantities of pluripotent stem cells {{139 Mallon,B.S. 2006;}}), and the associated developments in culture methods, closely mirrors the same progression in mammalian cell culture from flask to full production-scale. There are a number of technologies available which are capable of growing cells in a format compatible with the extant culture method, such as roller bottles and hollow fibre bioreactors, although these methods are limited in their scalability.

One of the most significant obstacles to cultivating pluripotent stem cells in a stirred tank bioreactor environment is their requirement for attachment to a surface {{117

Thomson, J.A. 1998; }}. This difficulty was addressed with respect to mammalian cells (1976, {{574 van Wezel, A.L. 1976;}}), where the cells were attached to a "microcarrier" – a (usually) spherical particle manufactured of a material conducive to cell attachment and proliferation, with a diameter approximately an order of magnitude greater than the size of the cell. These microcarriers whilst themselves in free suspension provide a 'static' surface on which the cells can attach and grow. Most of the early work utilising this technology was focussed on viral vaccine production processes {{576 Kallel, H. 2003;575 Wu, S.C. 2004;}}, and then following the development of hybridoma culture, microcarrier-based methods were also applied to mammalian cell cultures producing biopharmaceuticals. However, quite early on in the development of such systems, free suspension culture in stirred tanks became available and quickly dominated because it is easier to perform (607 Schutt,C. 1997; Nienow, 2006 606 Kumar, N. 2008;). The development of culture methods for mouse PSC's has preceded those for human cells considerably since their prior discovery and isolation in 1981 {{583 Evans, M.J. 1981;}}. Some work has been carried out demonstrating culture of mouse PSC's on microcarriers, however, methods are now available to grow these cells as aggregates {{577 Niebruegge, S. 2008; 87 zur Nieden, N.I. 2007; } }. Work towards a suspension culture solution for hPSC's has been slower for a number of reasons, the primary one being the lack of discovery of a single factor directing cells to maintain pluripotency in suspension culture; as LIF seems to for mPSC's {{579 Daheron,L. 2004;578 Humphrey,R.K. 2004;}}.

A number of reviews in the area of scale-up technologies for hPSC production have highlighted the desire to move away from animal-derived components during manufacture {{587 Azarin,S.M. 2010;}}. Matrigel is one of the major materials of

this type used for routine culture, but, despite the work mentioned earlier {{131 Amit, M. 2004;596 Rodin, S. 2010;597 Braam, S.R. 2008;598 Kolhar, P. 2010;137 Li,Y.J. 2006;601 Mahlstedt,M.M. 2010;}} striving to replace it, it is still the default substrate for most hPSC culture. The only three microcarriers which have been shown to support hPSC proliferation without the prior coating of matrigel are Cytodex 3 {{467 Fernandes, A.M. 2009;}}, Hillex II {{262 Phillips, B.W. 2008;}} and Cultispher S {{610 Storm, M.P. 2010;}}. It should be noted that each of these microcarriers were trialled in the initial stages of the work by Oh et al {{468 Oh,S.K. 2009;}}, with limited success, indicating that the differences between cell-lines is significant enough to increase the challenge of developing a single, platform, process for native microcarrier hPSC cultivation. Cultispher S and Cytodex 3 also contain animal-derived materials (Gelatin and Collagen, respectively), therefore representing a smaller departure from the Matrigel paradigm than first appears. Phillips et al {{262 Phillips, B.W. 2008; } managed to cultivate two different hESC lines on a synthetic surface, although after a number of passages the ability of the cells to grow on the microcarriers was reduced to almost zero. Interestingly, the expression of Tra-1-81 (a cell surface marker of pluripotency) remained high throughout the microcarrier culture, however Oct3/4 (also markers of pluripotency) showed a 13 % drop, compared with equivalent cells cultured in T flasks on a feeder layer.

The work of Phillips *et al* {{262 Phillips,B.W. 2008;}} is notable for the very high FGF concentration relative to the other studies presented in Table 1. FGF is still a very expensive component of the culture medium, therefore, for larger scales its use should be minimised as far as possible. Significantly, all of the studies in Table 1. have taken place over short timescales with limited passages (typically five or fewer).

Thus, the ability to cultivate cells over a period which would be able to create a clinically-relevant number of cells is, as yet, unproven, with this system. The work performed by Serra *et al* {{609 Serra,M. 2010;}} has taken the first step in this process, being the first report of successful cultivation of hESC within a fully-instrumented stirred-tank reactor. This has enabled tight control over the dissolved oxygen within the bioreactor, and subsequent adjustment to investigate the effect of hypoxic conditions.

There is currently a great deal of conflicting data regarding the effect of hypoxia on the growth and maintenance of pluripotency in hPSC's. Restriction of oxygen (1.5-8 %) in standard T flask cultures has been shown to reduce the level of spontaneous differentiation of hPSC cultures {{580 Ezashi,T. 2005;581 Prasad,S.M. 2009;}}. In contrast, Chen et al {{611 Chen,H.F. 2009;}} found no significant difference between growth or maintenance of pluripotency under normoxic and hypoxic conditions except where the cells were allowed a larger than normal inter passage interval. One major difference, which is unsurprising, is the large reduction in growth potential as a result of hypoxic conditions. All of the studies which have investigated hypoxia as a method of maintaining pluripotency have reported a 75% reduction in cell density when compared to that for normoxic cultures.

Due to the lack of a cell wall mammalian cells are vulnerable to changes in osmolarity and have long been perceived to be 'shear' sensitive i.e., they are physically damaged by the rotating impeller used in conventional bioreactors. However, it is now recognised that this early concern for 'shear' sensitivity was excessive and the majority of industrial processes for protein production use mammalian cells in free suspension stirred tank bioreactors especially on the large scale (Nienow). Indeed work has shown that cells are much more likely to be damaged by the action of bursting bubbles (for aeration) at the liquid free surface than by the action of fluid shear rates or stresses generated by impellers (Oh et al., 1992). This damage via the action of bursting bubbles can be easily eliminated by the addition of antifoaming agents such as Pluronic F68 to the culture medium (Nienow, 2006). The same may apply to the culture of stem cells however, since it is now the cell that is the product rather than the proteins that they produce the situation remains unclear. Indeed it is known (Wang and Thampatty 2008) that mechanical forces, including tension, gravity, hydrostatic pressure, compression, and so called 'shear stress', play an important role in human development. In particular they influence extracellular matrix (ECM) gene expression, ECM protein production, and expression of inflammatory mediators of many mechanically sensitive adult cell types e.g. fibroblasts, chondrocytes and endothelial cells. Therefore careful studies will need to be conducted that can accurately assess the effect of the fluid mechanical stresses that exist in conventional stirred tank bioreactors before any detrimental effects on the successful bioprocessing of stem cells can be fully discounted.

Recently, it has also become viable to grow hPSC's as clumps or aggregates in suspension, without microcarriers (Table 2.). This method has the considerable attraction of obviating the need for removal of cells from microcarriers which has received very little attention to date as there in no comparable need and therefore no established method for taking mammalian cells off such particles. Aggregate culture has the potential downside of necessarily exposing cells within the aggregate to a different environment from those in the centre, however, periodic "passaging" by

enzymatic disruption of aggregates, prevents them from becoming large and necrotic in the centre. Prior to 2008, several studies, Cameron et al {{89 Cameron,C.M. 2006;}}, Gerecht-Nir et al {{203 Gerecht-Nir,S. 2004;}}, Schulz et al {{446 Schulz,T.C. 2004;}} and Yirme et al {{582 Yirme,G. 2008;}} all successfully expanded hESCs as aggregates, however, their primary focus was on differentiation. Table 2. highlights the results of more recent publications which have focused on expansion as the end-goal.

Some comment on length of culture, number of population doublings in comparison to CHO and suspension?

All of the studies where hPSC have successfully been grown in aggregate suspension culture have used the ROCK inhibitor Y-27632. The protective effect of this compound on dissociated cells has been well established {{136 Watanabe,K. 2007;}} and it suggested that this is mediated by inhibiting apoptosis. Krawetz et al {{534 Krawetz, R.J. 2009; }} have, however, demonstrated that this ROCK inhibitor does not decrease susceptibility of cells to apoptosis. Here, based on a TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay, it is argued that Y-27632 is sufficient to inhibit specific stages of apoptosis but is not able to arrest the progression of apoptosis once it has been initiated. They posit that cells, by treatment with Y-27632, increase their affinity for attachment to each other. This increase in the level of cell-cell attachment is suggested to mitigate the lack of attachment to the surface substrate, preventing subsequent activation of apoptosis pathways. It should also be noted in passing that the TUNEL assay, measures the presence of DNA "nickends" which are not solely caused by apoptotic cell death {{617 Higuchi, Y. 2003;}}. Therefore in order to fully understand the role of Y-27632 further studies will be needed perhaps using a number of apoptosis detection assays in parallel.

Although the same concentration of Y-27632 (10 μ M) was used in each study, exposure times vary considerably; with one reports continuous exposure {{612 Olmer,R. 2010;}}, whilst others report that periodic feeding of the medium with Y-27632 to be detrimental to growth, hence suggesting that shorter exposure times; ranging from 1 h pre-passage – 30 mins post-passage – 48 h post-passage would be beneficial.

All of the work that is described here is performed using colony culture prior to the suspension phase. These colonies are manually or enzymatically removed from their substrate (usually with collagenase) and subsequently dissociated into single-cells using a combination of enzyme treatment and physical agitation. There has not been any description, thus far, of transition of monolayer-cultured hPSC to aggregate suspension. The suspended aggregates are a *de facto* colony, which may influence the ease of transition between planar colony culture and aggregate suspension compared with monolayer systems. Indeed it has been shown {{608 Kehoe, D.E. 2010;}} that the addition of 10 % Matrigel to the culture medium is necessary, implying that, cellcell attraction alone was not sufficient for aggregates to form in suspension culture. The requirement for Matrigel in the process suggests that the aggregates in this type of culture are not self-forming, but are likely to be nucleating around Matrigel particles comprising a "nano-carrier" of sorts. Similar effects may also be apparent in {{616 Steiner, D. 2010;}}, where the culture medium was supplemented with gelatin, laminin and fibronectin. All of the suspension aggregate cultures required periodic disruption, by a combination of enzymatic and physical methods. Without this treatment, the aggregates often grew quickly in size, and developed differentiated or necrotic segments within the aggregate. The latter presumably due to mass transfer limitations within the developing cluster. It has also previously been observed that aggregate size can have a significant impact on differentiation trajectories (Bauwens), indicating that the propriety of this methodology may depend on the therapeutic cell type required.

The majority of the work presented here achieved levels of expansion lower than that expected from static, planar, culture where a 10-fold expansion in 5 days is standard (Mahlstedt). However, the most successful work, achieved a 1000 fold expansion over 35 days in culture, using aggregate rather than microcarrier culture (Steiner). This larger expansion could be attributable to the presence of neurotrophic factors, such as BDNF or other growth stimulating components, such as b-D-xylopyranose which has been demonstrated to improve growth potential in mammalian cell cultures (US Patent 5063157). The higher concentration of FGF contained in the Neurobasal medium could also be responsible for this high level of growth, however where a high FGF concentrations was used in microcarrier studies {{262 Phillips,B.W. 2008;}} a similar effect was not observed suggesting that FGF concentration alone is not the controlling factor. It is also a concern that the presence of neurotrophic factors in the medium(Steiner) may predispose cells towards differentiation towards neural cell lineages especially if this protocol were to be used as a scale-up technique for generic hPSC production.

Since the hPSC is the final product the cells once removed from the microcarrier or freed from the aggregate (typically *via* enzymatic treatment) must have retained their pluripotency and typical human karyotype with no chromosomal abnormalities More here. This is important since the presence of any differentiated cells or karyotype

abnormalities in cells for delivery to a patient can lead to tumour formation and even patient death so it is not an insignificant problem. Currently most research is focussed on preventing the heterogeneity during the growth phase (ref), however, if these heterogeneities cannot be completely eradicated (and history tells us that will be almost impossible to achieve over long term and large scale culture (ref), then novel downstream processes will have to be conceived and developed that will separate out all non-hESC elements from the process stream. Such technologies do exist that really on the appropriate immunolabelling, namely magnetic activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) are available however they are costly and not scalable perhaps only useful if small amounts of cells are needed for autologous therapies. Clearly the ideal purification technique should combine high cell purity, yield and function coupled with fast processing and affordability. Larger scale techniques are under development that combine immunochemistry with monolith or membrane technology but little progress has been made and there is much work to be done in this area.

Need something on differentiation??

All of the work discussed so far demonstrates the potential for larger scale hPSC culture whether on microcarriers or in aggregate suspension however just how suitable or viable are these methods for the larger scale expansion of pluripotent stem cells for regenerative medicine purposes?

Feasibility Analysis of Scalable Culture Methods

The ability to manufacture therapeutic quantities of cells is dependent on the capacity of the productive means. There are a number of limitations intrinsic to a viable culture process, which must be considered; the productive capacity of the manufacturing equipment (cells/process unit), efficiency of production process, efficiency of differentiation from pluripotent to target cell, number of cells per patient, number of patients per year. Separation efficiencies? Obviously, the number of cells per patient and patients per year are a function of a particular disease state to be treated, and so these are fixed as far as the process engineer is concerned. This number of cells per year would, however, indicate the viability, or otherwise, of a route of manufacture for a particular illness.

Based on laboratory experience cultivating pluripotent cells, and literature estimations of production capacity and demand, it is possible to define the conditions that are necessary for production through automated platforms, or stirred-tank methods. In adherent cultures, the output of manufacturing methods is contingent on the total surface area available within a single reactor (whether automated system, or microcarrier-based stirred-tank).

The principle of hPSC growth within an automated T flask based system has already been demonstrated using the Compact Select (The Automation Partnership TAP) (Thomas et al). As such, the model which has been applied here is the larger capacity instrument, from the same company, the Select. The total surface area available for cell growth within a Select is 31580 cm² (based on 182 T175 flasks), as such, this is assumed to be the capacity of the system. Based on the manufacturer's instructions (GE Healthcare, ??) for Cytodex microcarriers, the surface area available for cell growth is limited to 88,000 cm² per litre. Assuming a liquid volume within the stirred-tank of 10,000 L allows a capacity of 8.8 x 10^8 cm².

Whilst the aggregate culture paradigm does not have a basis in surface area, attempts have been made (Amit et al) to enumerate the contents in such clusters, with typical estimates in the range of $10^5 - 10^7$ cells.mL⁻¹. A value of the order of 10^6 cells.mL⁻¹ is also consistent with the concentrations achieved during stirred-tank reactor culture of adherent mammalian cells (Wlaschin; Meuwly). Therefore given the same reactor volume (10,000 L) this gives a potential output of 10^{10} cells.m⁻³ (switching between m³ and litres here).

From laboratory experiments in standard T flasks, hPSC's can routinely achieve densities of 1.6×10^5 cells.cm⁻² without reduction in quality, defining the benchmark for this value for both microcarrier and automated planar culture. Throughout this analysis, the primary disease model that has been used is cardiomyocyte replacement for acute cardiovascular disease. Mummery et al suggest that an infarcted heart may require up to 10^9 functional cardiomyocytes, in order to replace tissue which has died during an ischaemic episode. Furthermore, European Cardiovascular Disease Statistics (2008 edition) indicate that there are 2,000,000 deaths per year as a result of cardiovascular disease within the EU member states alone. As a result it has been assumed here that 250,000 is a reasonable number (why?) of patients which might be treated by this type of cell replacement therapy. Similarly it has been established

(Keymuelen et al) that 2-5 x 10^8 would be necessary for islet replacement for treatment of diabetes.

Similarly, the 5 billion cardiomyocytes required to repair an infarcted heart in a single patient¹⁸ would require 100 billion starting pluripotent cells. Manually a single operator can culture no more than 10 x T175 flasks (300 million) of pluripotent cells per week, falling far short of the manufacturing scale required to produce a feasible product, especially considering the 150 lines estimated to be needed to cover the immune haplotypes of most Caucasians¹⁹ Do we need to consider haplotype here? this here? **18)** Mummery (2005) *Nature*. 10;433(7026):585-7. **19)** Taylor et al., (2005). *Lancet*. 10;366(9502):2019-25?

The two remaining variables to be considered are; differentiation efficiency and production efficiency. In any other process, these would be considered together to comprise an overall production efficiency, however, in this case, it is important to highlight what is expected of the differentiation step of the process in order to realise the potential of these cells. Production efficiency has been assumed how? to be 90% (this is far higher than from a pharmaceutical process especially when you consider separation efficiencies) throughout, which, although lower than King et al, is a satisfactory estimate based on the complexity of the process involved.

Figure 1. demonstrates that for a production process to be viable (based on what criteria?) at a large-scale, differentiation efficiencies must be 6-8-fold higher than they are presently. What are they presently? It is apparent that for widespread diseases, requiring high cell-doses, the TAP Select will be unlikely to satisfy demand,

rendering suspension culture the most appropriate route to market. Even when a shortened process is applied, by reducing the number of purification units, as in Figure 2., enormous production capacities are still required (> 200 m³ bioreactor or nearly 1,000,000 Full TAP Selects). However, if differentiation efficiency can be improved to even 40 %, suspension bioreactor culture becomes a very real possibility.

A comparison of the production techniques over a range of possible patient numbers is shown in Figure 3. At this stage, the TAP select is beginning to be a viable alternative to more conventional scale-up methods. This effect would be even more pronounced for conditions requiring lower cell-doses per patient, for example implantation of 3×10^5 retinal epithelial cells in Parkinson's patients has been shown to improve motor symptoms (allogeneic or autologous?) (ref Mason and dunnill 2009; Regen Med, 4(2): 153-157). Use of the Corning Hyperflask triple flasks? would further reduce the number of Selects required by almost 10-fold, with the caveat that no-one has yet demonstrated that hPSC can be cultivated routinely in such a system. The relatively large volume within this type of flask would likely lead to heat and mass transfer deficiencies, resulting in intra- and inter-flask heterogeneity. The Hyperflask also exchanges the problem of insufficient surface area for excessive medium use, because the 10-fold higher surface area requires a similar increase in medium volume.

Conclusions and Future Perspectives

Something in hear about timing throw it over the wall etc...Something about defining the problem....

By the generation of new therapies, regenerative medicine promises both to revolutionise clinical practise and to promote significant economic growth. Realisation of this promise requires robust and scalable manufacturing techniques for the larger scale production, recovery and differentiation of fully functional human cells for specific purposes. Whilst both aggregate suspension and microcarrier culture of hPSCs can expand cell numbers, a standard larger scale culture process has not yet been established and there is much work still to be done. The work described here utilise a variety of media and additive formulations as well as widely differing passaging techniques. Currently, aggregate suspension appears to produce the highest fold expansion, however, the feasibility study we have performed suggest that microcarrier culture has a theoretical advantage in achieving the requisite numbers of cells for therapy. In order to realise this theoretical potential, more research is needed to further understand the effects of microcarrier culture on cells and optimise the culture conditions.

This feasibility study demonstrates that current low differentiation efficiencies are a major hurdle to the exploitation of hPSCs as a source of cells for human therapy. Research is ongoing in this area and it is expected that these will increase. In addition, more effort needs to be put into developing potential purification technologies specific to these cell therapies in order that manufacturing can be as efficient as possible. Streamlined purification processes are particularly important given that purification accounts for 80 % of the production cost of a biopharmaceutical. Throughout the development of these new processes for expansion and purification of hPSCs, the quality of the cell is paramount and must be

maintained in contrast to traditional mammalian cell culture for recombinant biologics.

Publication	Cell-line(s)	Microcarrier	Medium ([FGF])	Fold Expansion (days)
Phillips et al {{262 Phillips,B.W. 2008;}}	ESI017 HUES9	Hillex II (HyClone)	HFF- conditioned (150 ng.mL ⁻¹)	2.5 (5)
Nie et al {{471 Nie,Y. 2009;}}	H1 H9	Cytodex 3 (GE Healthcare)	MEF- conditioned (8 ng.mL ⁻¹)	3.25 (2.5)
Lock et al {{473 Lock,L.T. 2009;}}	H1 H9	Collagen (HyClone)	MEF- conditioned (8 ng.mL ⁻¹)	34 (8) 45 (12)
Oh et al {{468 Oh,S.K. 2009;}}	HES-2 HES-3	DE53 (Whatman)	mTeSR StemPro	5.8 (7)
Fernandes et al {{467 Fernandes,A.M. 2009;}}	Н9	Cytodex 3	MEF- conditioned (8 ng.mL ⁻¹)	6.8 (14)
Kehoe et al {{608 Kehoe,D.E. 2010;}}	B12-3 (iPS)	Collagen (HyClone)	MEF- conditioned (8 ng.mL ⁻¹)	7 (7)
Leung et al	HES2 HES3	DE53 (Whatman)	MEF- conditioned (10 ng.mL ⁻¹)	<mark>10 (7)</mark>
Serra et al {{609 Serra,M. 2010;}}	SCED461	Cytodex 3	MEF- conditioned (10 ng.mL ⁻¹)	15 (14)
Storm et al {{610 Storm,M.P. 2010;}}	SHEF3	Cultispher S (HyClone)	MEF- conditioned (4 ng.mL ⁻¹)	10 (7)

Table 1.

			Agitated	Static
Study Author	Cell-line(s)	Medium	Fold expansion	Fold expansion
		([FGF])	(days)	(days)
Olmer et al	hIPS-2	mTeSR1	4 (4)	4 (4)
{{612	hES-3	(10 ng.mL^{-1})		6.5 (4)
Olmer,R.				
2010;}}				
Krawetz et al	H1	mTeSR1	25 (6)	N/A
{{613		(10 ng.mL^{-1})		
Krawetz,R.				
2010;}}				
Singh et al	HES2	mTeSR1	2.25 (5)	0.25 (5)
{{614 Singh,H.	HES3	(10 ng.mL^{-1})		2 (5)
2010;}}	ESI049			0.25 (5)
Kehoe et al	H1	MEF-conditioned	6 (7)	N/A
{{608		(not stated)		
Kehoe, D.E.				
2010;}}				
Amit et al	I3	MEF-conditioned	25 (10)	N/A
{{615 Amit,M.	I4	(4 ng.mL^{-1})		
2010;}}	I6			
	H9.2			
Steiner et al	HES1	Neuroblast	N/A	1000 (35)
{{616	HES2	(20 ng.mL^{-1})		
Steiner,D.	H7			
2010;}}				

Table 2



Figure 1.



Figure 2.



Figure 3.

Table 1. Summary of the papers published concerning expansion of hPSC on microcarriers. All except the highlighted study used matrigel or MEF-coated microcarriers.

 Table 2. Summary of papers on expansion potential of hPSCs in aggregate suspension

 culture.

Figure 1. Necessary capacity at varying differentiation efficiency; assuming a 6-step manufacturing process, with each stage having a 90 % yield. The hatched area indicates the approximate current differentiation efficiencies that are achievable for ?? (Burridge).

Figure 2. Necessary capacity at varying differentiation efficiency; assuming a 3-step manufacturing process, with each stage having a 90 % yield. The hatched area indicates the approximate current differentiation efficiencies that are achievable for?? (Burridge).

Figure 3. Production requirements for varying patient numbers treatment for what?. Assuming differentiation efficiency of 25 % and 6-step production process with each stage having a 90 % yield