4

Bioprocess Development for the Expansion of Embryonic Stem Cells

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1. Introduction

As the North American population demographic shifts towards an older profile, the demand for regenerative therapies will rise as more people face age-related degenerative diseases ranging from osteoarthritis to neurological disorders such as Parkinson's disease. Current treatment options focus on palliative measures to alleviate symptoms rather than addressing the underlying cause. Tissue engineering has emerged as an interdisciplinary field of work that aims to restore function to the tissues identified as the underlying cause of illness. The most important building blocks used in tissue engineering are living cells. These cells are generally obtained from one of two sources — adult tissues or embryos. Adult tissues have very sparse and difficult-to-isolate stem cell populations. Further, their use for large scale treatment is not realistic due to their limited proliferation capabilities (Thomson & Odorico, 2000). Embryonic stem cells (ESCs), in contrast, are rapidly emerging as a promising cell source due to their unique characteristics — namely their ability to readily proliferate in culture as well as their potential to differentiate into all cell types of the adult body.

A major roadblock that has been anticipated on the path to clinical implementation of ESC derived cellular therapies is the labour intensive and highly variable nature of small scale cultures. It has been estimated that as many as 26 billion human ESCs may be required as a starting point for treating one patient taking into account losses during culture, inefficient differentiation protocols, downstream processing and purification of mature cell types (Ouyang & Yang 2008). Based on current small scale culture techniques, thousands of static tissue culture flasks and several weeks to months of culture time would be required to generate this number of cells (Ouyang & Yang 2008). There are notable disadvantages in producing this quantity of cells in static tissue culture including heterogeneity between flasks, lack of environmental controls, large quantities of materials, large amounts of incubator space and the many hours of labour required to maintain the cultures. Several

automated bioprocessing systems exist which aim to reduce the amount of labour required to maintain static cultures, however these systems are still limited by surface area which is a key control on mass transfer of nutrients (Terstegge et al., 2007; Terstegge et al., 2009; Thomas et al., 2008). More effective methods are required to generate the large numbers of pluripotent ESCs needed for subsequent differentiation into functional tissue types. Suspension bioreactors offer a means to scale-up production of ESCs in a controlled culture environment not bound by the same surface area limitations as static culture techniques.

The development of protocols for bioreactor expansion of stem cells has progressed rapidly in the past decade. Groups have successfully cultured hematopoietic stem cells, neural stem cells, human pancreatic progenitor cells and more recently both mouse and human embryonic stem cells in suspension (Zandstra et al., 1994; Kallos et al., 1999; Chawla et al., 2006; Cormier et al., 2006; Krawetz et al., 2009; Kehoe et al., 2010). Despite the advances in bioreactor design, there is still a need for optimization and standardization of suspension culture protocols to ensure reproducible and predictable cell populations for use in clinical applications. In this chapter our aim is to review recent progress in the large scale culture of both murine and human ESCs as well as discuss fundamental bioprocess issues to be considered when using stirred suspension bioreactor culture systems.

2. Mouse embryonic stem cells as a model

The use of animal models in medical research goes back at least a century (Mouse Genome Sequencing Consortium, 2002). New therapies and drugs must be assessed for safety and efficacy; however, testing them on humans is not an ethical or realistic option. While single cells have proven to be invaluable resources for many studies, intact living animals have full organ systems which can undergo complex disease progression dynamics. Different animal models serve different purposes — larger animals models such as pigs and dogs allow for studies complex systems (i.e. joint loading) whereas smaller ones such as mice are useful for studies of genetics and progression of degenerative diseases. Mice have been shown to have similar genetics and physiological structures to humans. They naturally develop several diseases (cancer and diabetes for example) typically attributed to humans and can also be induced to present symptoms of neurological disorders such as Alzheimer's (see Bedell et al., 1997 for a review of mice models for many human diseases). Animal models such as mice provide an excellent means for evaluating cell therapies: it is cost effective and provides fast results (the murine gestational period is a matter of weeks which allows for quick assessment of germline transmission which is paramount in murine ESC derivation evaluation).

Knowledge gained from isolation of mouse ESCs has laid a solid foundation for isolation of ESCs from other animals including non-human primates and ultimately humans (Bongso et al., 1994; Thomson et al., 1995; Thomson et al., 1998; Reubinoff et al., 2000). Within our own laboratory, work with mouse cell lines has established foundation protocols for human studies although the methods have not been directly transferable. Specifically, successful expansion of murine neural stem cells as aggregates in suspension bioreactors (Kallos & Behie 1999; Gilbertson et al., 2006) enabled us to develop protocols for human neural stem cells (Baghbaderani et al., 2010). Similarly, expanding murine ESCs as aggregates (Cormier et al., 2006; zur Nieden et al., 2007) allowed us to successfully expand pluripotent human ESCs as aggregates in suspension bioreactors (Krawetz et al., 2010). The fact that protocols are not directly transferrable is expected since cells from different species have been

observed to present different growth morphologies, growth kinetics (doubling times, for example), as well as different markers for cell pluripotency and differences in associated signaling pathways (illustrated in Table 1). However, from a bioengineering point of view, there is still tremendous value in conducting murine experiments first.

	Murine ESCs	Human ESCs
Pluripotency markers		
SSEA-1	+	-
SSEA-3	-	+
SSEA-4	-	+
TRA-1-60	-	+
TRA-1-81	-	+
Oct-4	+	+
Factors that affect self renewal	LIF + Serum—activates JAK/STAT3 pathway [1] LIF+BMP (serum free) – activates SMAD and/or MAPK pathways [1]	bFGF+MEF+SR or high bFGF+SR (no MEFs)- suppresses BMP signalling and/or upregulates expression of TGFβ ligands, activation of ERK &PI3K[1] TGFβ/Activin/Nodal[1]
Teratoma formation in vivo	+	+
Colony morphology	Tight rounded multilayered	Lose rounded monolayers
Passaging	Single cells	Clumps

Table 1. Summary of culture similarities and differences between mouse and human embryonic stem cells. Adapted from National Institutes of Health, 2009; [1] Yu & Thomson, 2008. bFGF= basic fibroblast growth factor, BMP= bone morphogenetic protein, LIF= leukemia inhibitory factor, MEF= mouse embryonic fibroblasts, SR= serum replacement, TGFβ= transforming growth factor beta.

3. Bioprocess development for suspension culture of ESCs

Many types of cell culture systems can produce cells and tissues in culture but they can generally be grouped into two categories: 1. adherent culture (tissue culture flasks) and 2. non-adherent culture. Both cultures can further be classified as stationary (or static), where the culture media is not moving, or suspension, where the medium is agitated or perfused (Sen et al., 2010). Conventional culture methods for propagation of ESCs use static tissue culture flasks (stationary adherent culture). As this is the current standard method for ESC culture, it is necessary to understand the effect of static culture environment on ESCs before successful development of suspension culture protocols. Static culture also provides a baseline for comparison of cell production from suspension culture.

3.1 Adherent culture

In 1981, Evans and Kaufman reported that isolation and culture of pluripotent cells were dependent on: 1) the stage at which pluripotent cells exist in the embryo (in other words, day at which cells are harvested from the blastocyst), 2) explantation of sufficiently large

numbers of these cells from the embryo and 3) tissue culture conditions conducive to propagation (multiplication) of the cells rather than differentiation. These considerations have been the basis for isolation of mammalian ESC lines. Currently, however, the focus of many groups is on the third point: trying to determine the optimum combination of factors to support long term expansion of these cells while maintaining functionality (pluripotency and self-renewal). Based on their knowledge of embryonic carcinoma cells, which are pluripotent cells isolated from germ cell tumors, Evans and Kaufman isolated the inner cell mass of a mouse blastocyst and cultured the cells on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in 20% serum containing medium. The resulting cells exhibited a normal karyotype in contrast to embryonic carcinoma cells, which did not, and thus the first line of mouse ESCs was derived. This reliance on a feeder layer initially restricted ESC research to static adherent culture conditions. Some years later it was found that conditioned medium was able to support the growth of murine ESCs in the absence of a feeder layer (Yu & Thomson, 2008). Subsequent fractionation of conditioned medium led to identification of leukemia inhibitory factor (LIF) as one of the key ingredients that supported viability (Williams et al., 1988; Smith et al., 1988 as cited in Yu & Thomson 2008). Standard lab scale cultures today use gelatin as a substrate for mouse ESC adherence together with DMEM-based medium supplemented with LIF. This combination has repeatedly demonstrated long term propagation of stable pluripotent cells. Table 2 summarizes static culture conditions described in recent publications.

Based on previous methods of ESC derivation, Bongso et al. (1994) isolated the inner cell mass of a human blastocyst on a feeder layer of human oviductal epithelial cells in a medium containing human LIF and 10% human serum. While initial colonies had promising morphologies, the cells either differentiated or died after only two passages. This is an example of difficulties encountered in transferring protocols directly between species. Shortly after, another group successfully derived a non-human primate ESC line (Thomson et al., 1995). The cells isolated from a rhesus monkey embryo were cultured on irradiated mouse embryonic fibroblasts in DMEM based medium supplemented with human LIF and 20% fetal bovine serum (FBS). Although human LIF was used in the initial derivation of these cells, it was subsequently removed and the cells were successfully cultured for over a year while maintaining a stable karyotype and the ability to differentiate into all three germ layers. Conversely, when human LIF was used and MEFs removed, the cells differentiated (Thomson et al., 1995). Based on this success, Thomson then used a similar protocol to derive the first human ESC lines using irradiated MEFs and DMEM medium with 10% FBS (Thomson et al., 1998). Subsequent work has led to the refinement of medium and identification of optimum substrates with many groups now reporting the use of DMEM with serum replacement medium or the commercially available defined medium, mTeSR™ with matrigel™ (an ECM extract of mouse sarcoma) as the substrate. These culture protocols have proven to give rise to stable pluripotent cell populations (Xu et al., 2005; Ludwig et al., 2006; Levenstein et al., 2006). Table 3 provides a brief summary of static culture conditions reported in recent publications of human ESCs. It is apparent that these small scale adherent culture conditions are well suited to most laboratory research. However, if we look to the future of stem cell based therapies in clinical settings, these small scale culture techniques are simply not feasible on a large scale.

Ouyang & Yang (2008) provided a useful summary of cell numbers estimated to be required for different clinical applications. For treatment of an adult with leukemia, they calculated

Reference	Cell Lines	Substrate	Medium	Passaging
Kehoe 2008	E14Tg2a	0.1% Gelatin	DMEM+10%FBS	TrypLE
Veraitch 2008	E14Tg2a	0.1% Gelatin	GMEM+10%FBS	Trypsin
Abranches 2009	E14Tg2a 46C S25	0.1% Gelatin	GMEM+10%FBS	Not specified for maintenance conditions
Fernandes, TG 2009	46C	0.1% Gelatin	DMEM+10%FBS ESGRO complete KO-DMEM+15%KSR	Trypsin, Accutase
Marinho 2009	USP-1	Gelatin MEFs	DMEM/F12+15%KSR+CHO-CM (for LIF)- changed every 2 days	TrypLE
Alfred 2010	D3	Gelatin	DMEM+15%FBS	Not specified for static
Jing 2010	E14Tg2a	0.1% Gelatin	DMEM+10%FBS	TrypLE
Taiani 2010	D3	MEFs	DMEM+15%FBS	Not specified for static
Ito 2010	E14Tg2a J1	Gelatin MEFs	GMEM+15%FBS	Not specified

Table 2. Summary of recent publications with static maintenance of murine ESCs. DMEM= Dulbecco's Modified Eagle's Medium, GMEM= Glasgow's Minimal Essential Medium, KO-DMEM= KnockOut™ DMEM (Inivitrogen), KSR= KnockOut™ Serum Replacement (Invitrogen), ESGRO Complete= defined serum-free medium (Chemicon/Millipore)-contains LIF, CHO-CM= Chinese Hamster Ovary Conditioned Medium. Unless otherwise stated, culture medium also included LIF, 2-mercaptoethanol and non-essential amino acids.

Reference	Cell Lines	Substrate	Medium	Passaging
Peerani 2008	H9, CA1, I6	MEFs Matrigel	KO-DMEM+20%KSR+bFGF XVIVO10+bFGF+TGFb1	Collagenase
Bauwens 2008	H9, H2B	MEFs	KO-DMEM+20%KSR+bFGF	Collagenase
Niebruegge 2008	H9, HES2	Matrigel MEFs	XVIVO10+bFGF+TGFb1 KO-DMEM+20%KSR+bFGF	Collagenase
Phillips 2008a	ESI-017	HFF+ Fibronectin Fibronectin	KO-DMEM+20%KSR+bFGF HFF-CM	Collagenase NB6
Bendal 2009	H1, H9	Matrigel	MEF-CM with and without bFGF	Collagenase
Fernandes 2009	H9	MEFs	DMEM/F12+20%KSR+bFGF	TrypLE
Gibson 2009	H9	MEFs	DMEM/F12+20%KSR+bFGF	Collagenase
Hentze 2009	HES2, HES3, HES4, ESI-014, 017, 035, 049, 051, 053	HFFs	KO-DMEM+20%KSR+bFGF	Collagenase, TrypLE
Lee 2009	H9, I6, HES2	MEFs	KO-DMEM+20%KSR+bFGF	Not specified
Lock 2009	H1, H9	MEFs Matrigel	DMEM/F12+20%KSR+bFGF MEF-CM+bFGF	Collagenase
Montes 2009	HSI81, SHEF1	Matrigel	MSC-CM + bFGF HFF-CM +bFGF	Collagenase
Nie 2009	H1, H9	MEFs Matrigel	DMEM/F12+20%KSR+bFGF MEF-CM + bFGF	Collagenase
Oh 2009	HES2, HES3	Matrigel	MEF-CM + bFGF	Collagenase
Amit 2010	I3, I4, I6, H9.2	MEF	DMEM/F12+15%KSR+bFGF	Collagenase
Chen 2010	HES2, HES3	Matrigel	mTeSR, MEF-CM, StemPRO	TrypLE
Krawetz 2010	H9	HFF Matrigel	mTeSR	Collagenase+ TrypLE

Reference	Cell Lines	Substrate	Medium	Passaging
Singh 2010	HES2, HES3, ES1049	HFF	KO-DMEM+20%KSR+bFGF	TrypLE
Larijani 2011	Royan H5, H6, hiPSC1, hiPSC4	Matrigel	DMEM/F12+20%KSR+bFGF	Not specified for adherent cultures
Leung 2011	HES2, HES3	Matrigel	MEF-CM+bFGF	Enzymatic

Table 3. Summary of recent publications with static maintenance of human ESCs. bFGF = basic fibroblast growth factor, TGFb1= transforming growth factor beta 1, XVIVO10= serum free medium (Lonza), HFF-CM= human foreskin fibroblast conditioned medium, MEF-CM= mouse embryonic fibroblast conditioned medium, mTeSR= serum-free defined medium (STEMCELL Technologies), StemPRO= serum-free defined medium (Invitrogen).

that $2.1x10^{10}$ cells would be required per treatment. If one T-75 culture flask supports the production of $2x10^6$ cells in 2-3 days, production of $2.1x10^{10}$ cells would require over 10,000 tissue culture flasks and up to 5 weeks of culture time (Ouyang & Yang, 2008). They also determined flask count and time estimates for Parkinson`s disease and diabetes. In both cases similarly large numbers of tissue culture flasks were calculated. The handling of this number of tissue culture flasks would be extremely labor-intensive. Additionally, this type of culture lacks continuous monitoring and environmental controls that may result in spontaneous stem cell differentiation. Alternatively, suspension bioreactors provide a controlled environment to produce the same number of cells. Studies from our lab have produced densities of mouse ESCs in suspension of approximately $1\text{-}2x10^6$ cells/mL (Alfred et al., 2010; Cormier et al., 2006). As such, production of clinical numbers would require suspension volumes of the order of liters.

3.2 Suspension bioreactor culture

There have been arguments that not enough is known about ESCs to take them out of adherent cultures and culture them in suspension conditions as reduced adhesion in anchorage dependent cells has been associated with disorganized growth patterns and changes in cell-to-cell contact (Freshney, 2000). However, the final test of success for any new culture format is the functionality of the cells. Refinement of suspension culture protocols to ensure stable karyotypes, continued expression of pluripotency markers as well as demonstration of pluripotency through embryoid body and/or teratoma formation is the goal of embryonic stem cell bioprocess development.

It is clear upon review of recent publications that culture conditions vary a great deal between mouse and human ESCs as illustrated in Tables 4 and 5, respectively. Mouse ESCs were able to readily form aggregates in suspension whereas human ESCs required the addition of ROCK inhibitor to survive non-adherent conditions and form aggregates. As for differences within each cell type, it is clear that there is greater variation between different human ESC protocols than between different mouse ESC protocols. As murine ESCs were established years before human ESCs, culture medium and passaging techniques are far more standardized and suspension protocols appear to be fairly similar between publications.

While these studies illustrate that it is possible to culture human ESCs in suspension (i.e. proof of concept), work must be done with respect to optimization and standardization of protocols. An understanding of key culture variables is necessary to achieve this.

Reference	Cell Lines	Inoculation Density (cells/mL)	Medium	Passaging	Spinner Type (Volume)	Agitation Rate (rpm)
Fok 2005	R1, CCE	$5x10^4$	DMEM+15%FBS	Trypsin	Bellco (50 mL)	60,100
Cormier 2006	R1	$3.75\text{-}10x10^4$	DMEM+15%FBS	Trypsin	Corning (100 mL)	60-120
Zur Neiden 2007	R1	$3.75x10^4$	DMEM+15%FBS	Trypsin	NDS (100 mL)	100
Hwang 2008	E14Tg2a	$3.0x10^5$	DMEM+10%FBS	N/A	Synthecon (50 mL)	25
Kehoe 2008	E14Tg2a	$1\text{-}7.5x10^4$	DMEM+10%FBS DMEM+10-20% KSR	TrypLE	Corning (100 mL)	60-120
Tsuji 2008	D3, E14, EB5	$(1\text{-}500)x10^4$	DMEM+15%FCS	Trypsin	96 well plates	N/A
Alfred 2010	D3	$3.75x10^4$	DMEM+15%KSR	Trypsin	NDS (100 mL)	100

Table 4. Summary of suspension culture conditions for undifferentiated murine ESCs as aggregates. Unless otherwise stated, culture medium also included LIF, 2-mercaptoethanol and non-essential amino acids.

Reference	Cell Lines	Inoculation Density (cells/mL)	Medium	Passaging	Bioreactor Type	Agitation Rate (rpm)
Kehoe 2010	H1	$6.0x10^4$	MEF-CM+ Matrigel+ROCK	Accutase+ ROCK	Not specified	60
Krawetz 2010	H9	$1.8x10^4$	mTeSR+ROCK+ rapamycin	Accutase+ ROCK	NDS (100mL)	100

Reference	Cell Lines	Inoculation Density (cells/mL)	Medium	Passaging	Bioreactor Type	Agitation Rate (rpm)
Amit 2010	I3, I4, I6, H9.2	1-5x10^6	DMEM/F12+KSR+ bFGF DMEM/F12+KSR+ bFGF+IL6RIL6 DMEM/F12+KSR+ bFGF+LIF DMEM/F12+KSR+ bFGF+IL6	Collagenase Trypsin+ ROCK	Erlenmeyer (25mL)	90
Olmer 2010	HES3	0.3x10^5	mTeSR+/-ROCK KO-DMEM+ KSR+bFGF+/-ROCK KO-DMEM+FBS +/- ROCK	Collagenase	6well plate	NA
Singh 2010	HES2, HES3, ES1049	2.5x10^5 1x10^6 1x10^6	KO-DMEM+20%KSR +bFGF mTeSR	Collagenase +TrypLE+ ROCK	Low att. plate Stirred dish (10mL) CELLSPN (100mL)	NA 35 40
Steiner 2010	HES1, HES2, H7	0.37-1.2x10^6	KO-DMEM+14%KSR +bFGF +Activin A+ fibronectin+ laminin+ gelatin+BDNF+NT3 +NT4+Nutridoma-CS	Collagenase Trituration+ ROCK	12well plate	NA
Larijani 2011	Royan H5, H6, hiPSC1, hiPSC4	15x10^4	DMEM/F12+20%KSR + bFGF MEF-CM+bFGF MEF-CM+bFGF+ N2+B27 (all with ROCK)	Trypsin+ ROCK	6well plate	NA

Table 5. Summary of suspension culture conditions for human ESCs as aggregates. ROCK = p160-Rho-associated coiled kinase inhibitor (Y-27632) (Watanabe et al., 2007).

3.2.1 Suspension bioreactor design considerations

Traditional reactor types that may be used in biological processes include batch reactors, continuous stirred tank reactors, and plug flow reactors. Bioreactors support and control living biological entities and therefore require process control and stringent steps to

eliminate contamination (Williams, 2002). Mammalian cells are far more sensitive to culture conditions and far less stable with respect to maintenance of cell functionality than many other cell types such as yeast, bacteria and fungi. Additionally, shear is normally not a concern for these cell types whereas ESCs are far more sensitive to their environment. Human ESCs for example will spontaneously transform, differentiate or undergo apoptosis as a reaction to small changes in culture conditions (Chu & Robinson, 2001). This becomes a challenge as mixing is an integral consideration in reactor design for nutrient and oxygen mass transfer and the requirement of achieving target shear places an additional constraint on agitation speed (Schmidt, 2005). The aim of bioreactor design is to minimize the cost of production while retaining the desired quality of the product all within biological, fluid mechanical, and mass transfer constraints (van't Riet & Temper, 1991). With stem cells, the objective is to robustly produce large quantities of viable undifferentiated cells for further differentiation and purification steps. Downstream processing will be the cost determining step and therefore the goal is maximization of product concentration obtained from the bioreactors (van't Riet & Temper, 1991). To accomplish this, some key design areas must be considered including: materials (bioreactor walls and agitators), medium (chemical composition, pH, temperature), rheological conditions (fluid dynamics and mass transfer systems), and residence time of cells within the bioreactor. Issues such as temperature, pH, and medium formulation are fairly well defined for human ESC culture (King & Millar, 2007). Areas that are less understood with respect to suspension culture are those of the physical and geometric properties of the bioreactor itself. Parameters falling within this area are broad ranging from rheology and hydrodynamics, to mixing and agitator design, to heat and mass transfer, to issues of scale up and process control. Throughout the work within our lab group we have observed that culture parameters with the largest impact on cell populations are agitation rate, inoculation density and oxygen transfer.

3.2.2 Agitation speed

The rate of agitation within a stirred suspension bioreactor is an important consideration as it affects not only the mixing of oxygen and nutrients within the medium, but it also maintains cell aggregate sizes and keeps cells in suspension. However, development of effective bioprocesses for suspension culture requires an understanding of the nature of the biomass within the reactor. Embryonic stem cells along with many other mammalian cell types have been observed as being extremely sensitive to shear (Garcia-Cardena & Adamo, 2011; Toh & Voldman, 2011). While the medium must be agitated at a speed sufficient to ensure nutrient mixing, this speed must also be kept within limits so as not to exert undue shear on the sensitive biomass. We therefore need to understand the forces caused by agitation, how these forces act on cells, how we can quantify these forces, and the relationship between the forces and cell viability. There have been a very few reports on the effect of shear in stirred bioreactors on murine ESC growth. Cormier et al., (2006) found that aggregate diameter correlated with agitation rate but did not quantify the correlation. To date there have not been any comprehensive investigations into shear effects on growth kinetics in human ESC suspension cultures.

3.2.3 Inoculation density

Inoculation density is an important parameter that tends to be taken for granted in many studies. In our lab, we have seen that when inoculating suspension spinners, too few cells

will fail to initiate agglomeration and therefore aggregates do not form and cells die due to lack of cell-to-cell interaction. Too many cells may form massive aggregates that create issues with oxygen transfer to cells at the aggregate centre (necrosis). Additionally, rapid depletion of nutrients in the medium and subsequent rapid build-up of waste materials can adversely affect growth and expansion of the cell population (Sen et al., 2010).
In general, inoculating at the lowest possible cell density allows for maximum cell number amplification, however many have observed that this also causes a longer lag phase which results in an extended culture period required to reach maximum cell density. Extremely low and high initial densities have been observed to affect the exponential growth phase with a reduction in specific growth rates (Cormier et al., 2006; Fernandes, TG et al., 2009).
A review of literature fails to turn up a great deal of further information on inoculation densities with respect to ESC culture. With human ESCs the passaging techniques used (passaged as clumps until recently) prevented quantification of cells.

3.2.4 Nutrient and oxygen uptake

Oxygen is essential for cell proliferation and viability. In smaller vessels, surface aeration as the main mode of oxygen supply is typically sufficient and commonly used due to its simplicity. However, as culture volumes increase and cell densities increase, the surface area to volume ratios decrease and surface aeration may no longer be sufficient to ensure oxygen transport to the cells (Gilbertson et al., 2006; Baghbaderani et al., 2008). Options to increase oxygen transport include increasing agitation rate of the impeller, sparging, or medium additives such as Perfluorocarbons (PFCs) which increase oxygen solubility within the liquid medium. To determine which method is best, the oxygen requirements of the system must be determined.
Starting with the basic mass conservation equation for a specific component within a control volume (in this case the oxygen within the liquid cell culture medium), we have:

$$\text{Input} + \text{Generation} = \text{Output} + \text{Accumulation} \tag{1}$$

The input of oxygen into the medium, also known as the oxygen transfer rate (OTR), by using surface aeration only (no sparging) is controlled by the concentration difference between the headspace of the vessel and bulk medium oxygen concentration. Since no oxygen is released from the system, the output term is zero. The accumulation term refers to the rate of change in oxygen concentration within the medium while the generation term actually refers to the oxygen uptake rate (OUR) of the cells which is negative to indicate the cells are consuming oxygen rather than producing it. With this in mind, the material balance becomes:

$$OTR - OUR = 0 + dC_{O2}/dt \tag{2}$$

where OTR and OUR are as follows:

$$OTR = k_La\ \Delta C_{O2} \tag{3}$$

$$OUR = q_{O2}X \tag{4}$$

where k_La is the volumetric mass transfer coefficient, ΔC_{O2} is the difference in oxygen concentration between oxygen saturation concentration in the bulk medium (C_{O2}* which may be determined via Henry's law for an assumed partial pressure of 20% O_2 in normal incubator air with 5% CO_2) and the measured bulk medium concentration (C_{O2}). The term q_{O2} is the specific oxygen uptake rate of the cells and X is the cell density within the medium (Garcia-Ochoa & Gomez., 2009).

Rearranging Equation 2 yields an expression for the rate of change in oxygen concentration within the bulk medium:

$$dC_{O2}/dt = k_La\ \Delta C_{O2} - q_{O2}X \quad (5)$$

As can be seen from the preceding equations, the k_La value is the unknown when determining the rate of oxygen transfer. There have been numerous methods described for the determination of the mass transfer coefficient depending on the nature of the system: factors such as aeration, vessel design, medium composition and the effect of the presence of a microorganism must all be taken into account.

Throughout the literature there have been many attempts to develop empirical relationships for the determination of k_La. These relationships are sometimes based on both dimensional and dimensionless values. Garcia-Ochoa & Gomez (2009) have presented a very comprehensive summary of a number of published correlations for the determination of k_La in stirred suspension bioreactors. Upon review, it is apparent there is considerable variation between the correlations presented. For example, in 1979, Van't Riet & Temper proposed a correlation based on the power input per unit volume (P/V) but stated there was no influence of the impeller geometry and placement within the bioreactor. Subsequent studies contradicted this statement by showing that changing the impeller geometry alone caused an increase in mass transfer rate and a change in impeller placement also had an effect (See Garcia-Ochoa & Gomez, 2009 for a full review). To date there still does not appear to be a firm consensus as to which correlation is best suited for the determination of k_La in a stirred vessel.

The wide range of empirical relationships developed may in part be due to the similarly wide range of protocols for the experimental determination of k_La. For culturing stem cells, the presence of the cells themselves is assumed to have a large impact on mass transfer in the system. As such, the biomass present within the system is the main consideration when determining k_La values experimentally. There have been thorough reviews elsewhere which have described several approaches to experimentally determine k_La (Garcia-Ochoa & Gomez., 2009). One example that we have used, described by Baghbaderani et al. (2008), is as follows. Briefly, the assumption is made that at some point in time the reactor reaches steady state during which any oxygen entering the medium would be immediately consumed by the cells (that is, C_{O2} equals zero in the medium). At this point the maximum cell density is attained and the limiting rate of oxygen transfer is reached. In this instance oxygen transfer into the medium equals the oxygen uptake rate of the cells and there would be no change in the oxygen concentration in the bulk liquid. In other words the rate of change in oxygen concentration is zero (dC/dt=0). Equation 5 then simplifies to:

$$k_La = q_{O2}\ X\ /\ C_{O2}^* \quad (6)$$

The specific oxygen uptake (q_{O2}) of the cells in the system can be determined by measuring the oxygen concentration in medium without cells and then placing a known number of cells in the medium and sealing off the vessel with no headspace to replenish the oxygen. Measurements of the oxygen concentration in the medium are taken over time and the resulting rate of oxygen decrease is directly attributed to the uptake by the cells in the medium. That is:

$$dC_{O2}/dt = q_{O2}X \quad (7)$$

Once the specific uptake rate for a cell type is known, the limiting k_La value for a desired cell concentration can be determined. This method, however, obviously does not take into account any dimensional effects of the system. As such, Baghbaderani et al. (2008) have recommended a correlation put forth by Aunins et al. (1989) for a 500mL Corning spinner which takes the following form:

$$k_La = 1.08\ Re^{0.78}\ [D_{O2}\ a\ /\ D_T] \quad (8)$$

Where Re and D_{O2} are the Reynolds' number (dimensionless) and diffusion coefficient of oxygen ($m^2\ s^{-1}$) in the bioreactor medium, a, is the specific mass transfer interfacial area (taken as surface area/volume, m^{-1}) and D_T is the tank diameter (m). Determining this value based on system parameters and then comparing to the k_La determined experimentally for the cell type allows for an indication of whether or not oxygen transfer limitations exist within the system.

3.3 Suspension bioreactor culture of ESCs on microcarriers

Microcarriers are small, usually spherical or nearly spherical beads on which adherent cell types may grow. These beads are available in a multitude of materials including gels, polymers, and collagen. Based on their surface topography, they are generally described as macroporous (allowing cells to expand within the microcarrier), microporous (cell attachment occurs on the surface of the microcarrier, however, cells are exposed to medium on the attached surface as well), or non-porous microcarriers (cells are exposed to medium only on surfaces not attached to the microcarrier). When added to a suspension bioreactor, microcarriers provide high surface area to volume ratio which enables higher cell densities compared to that obtained in static culture. This area can be adjusted by varying the number of microcarriers in the culture (see Table 6 for a summary of microcarrier types and specifications). Microcarriers in cell culture offer several advantages including: better control of culture macro-environment within bioreactors compared to static tissue cultures and roller bottles, a reduction in labor costs, ease of downstream clinical applications as cells can be transplanted while on microcarriers, significant reduction in the space required for a given-sized operation and hence higher cell densities per unit volume. In addition, by allowing cells to grow on a surface, microcarrier cultures harness all the advantages of a static tissue culture system, as well as the controlled environment of a bioreactor system. This provides the cells direct exposure to the medium and reduces mass transfer limitations of oxygen and nutrients. Comprehensive reviews on microcarrier materials and specifications as well as their role in tissue engineering can be found elsewhere (GE Healthcare, 2005; Martin et al., 2011).

Though it has been over four decades since microcarriers were developed (Van Wezel, 1967), only recently have microcarriers been investigated as suitable scaffold materials for

Type	Company	Core Material	Surface Coating	Density (g/cm^3)	Bead Diameter (μm)	Surface Area (cm^2/g)
CultiSpher S	Percell Biolytica	Crosslinked pharmaceutical grade gelatin	Porcine gelatin	1.04	130 – 380	**7500**
Collagen	SoloHill Engineering	Crosslinked Polystyrene, modified with gelatin	Type1 porcine gelatin	1.02	90-150	**480**
Fact III	SoloHill Engineering	Crosslinked Polystyrene, modified with cationic gelatin	Cationic, type1 porcine gelatin	1.02	90-150	**480**
Glass	SoloHill Engineering	Crosslinked Polystyrene, modified with high silica glass	High silica glass	1.02	125-212	**360**
Pronectin F	SoloHill Engineering	Crosslinked Polystyrene, modified with recombinant fibronectin	Recombinant fibronectin	1.02	125-212	**360**
Hillex II	SoloHill Engineering	Modified Polystyrene, modified with cationic trimethyl-ammonium	Cationic, trimethyl ammonium	1.11	160 - 180	**515**
Cytodex 3	Amersham Biosciences	Crosslinked dextran, Denatured Collagen on the surface	Porous Porcine gelatin	1.04	141 - 211	**2700**
Cytodex 1	Amersham Biosciences	Crosslinked Dextran with N,N-diethylaminoethyl groups	Porous Porcine gelatin Cationic	1.03	147-248	**4400**

Table 6. Summary of some of the commercially available microcarriers for cell culture (adapted from Alfred et al., 2011).

cultivation of ESCs (as shown in Tables 7 and 8). However, several issues need to be resolved before ESCs produced on microcarriers and their progeny can be used in drug discovery and regenerative medicine applications, respectively. These issues include excessive agglomeration, which has been shown to be detrimental to cell expansion as a result of necrotic centers (Borys and Papoutsakis 1992), mediocre to low cell yields (especially for human ESC cultures), as well as the elimination of serum and MEFs used in microcarrier cell cultures. Finally, lack of attachment of cells to the microcarriers and the formation of cystic structures in suspension (Abranches et al., 2007; Nie et al., 2009; Storm et al., 2010) must also be addressed to eliminate heterogeneities in cultures.

4. Future directions for large scale production of human ESCs

The publications summarised in Tables 6 and 7 coupled with our own initial successes culturing human ESCs in suspension are a promising step towards development of strategies for implementation of stem cell therapies on a large scale. However, it is also quite apparent that major discrepancies exist in protocols between lab groups. Significant cell-manufacturing and regulatory challenges must be overcome before clinical application of stem cell therapies will be viable. It must be noted that standards and methodologies are only *just* being developed for efficacy evaluation, product characterization and process validation and control. As such, human ESC culture must be approached as a multidimensional optimization problem with the goal to increase target cell output while decreasing cost and occurrences of adverse events (Kirouac & Zandstra, 2008). In 2008, Kirouac & Zandstra suggested that process design and optimization should incorporate: i) assessment of relevant cell properties, ii) measurement and control of key parameters, iii) robust predictive strategies for evaluating the parameters that may impact culture output, iv) approaches to test these many different parameters in a high throughput and scale relevant manner.

Development of predictive strategies includes computer modeling methods to assist in prediction of culture outcomes based on various input parameters with their inherent uncertainty. To date, many groups have used mathematical modeling techniques to describe cell proliferation in a variety of systems (Mantzaris et al., 2001; Galban & Locke., 1999;Lemon et al., 2007) as well as differentiation (Lemon et al., 2007; Yener et al., 2008; Prudhomme et al., 2004). This modeling approach is limited as only a small number of input variables can be considered to maintain the practicality of derived relationships and ease of computation. Empirical or correlation models on the other hand, do not rely on mathematically describable relationships between input and output variables. Some researchers have used factorial design for process optimization by using response surface maps that approximate relationships between variables and outcomes (Chung et al., 2006; Audet, 2010).

Both of these modeling approaches are reasonable and practical when the input variables are limited and generated data sets are of a manageable size. When these data sets become increasingly large, for example, using tools such as microarrays, other methods of analysis are beginning to surface. Methods such as neural networks and statistical learning methods may prove to be very useful. These algorithms learn by example, or are trained by a data set, to assign labels to objects and recognize patterns within very large amounts of data. The use of these methods has yet to be fully defined within biological systems but it is apparent that the process is underway (Nobel, 2006).

Reference	Cell Lines	Medium	Passaging/ Harvesting	Inoculation Density (cells/mL)	Microcarrier Type	Agitation Rate (rpm)
Fok 2005	CCE, R1, M8, 9J	DMEM+15%FBS	Trypsin	$5x10^4$	Cytodex 3, Glass coated styrene	60
Abranches 2007	S25	GMEM+10%FBS	Trypsin	$1x10^4$, $5x10^4$ or $1x10^5$	Cytodex 3	60
Fernandes 2007	46C	DMEM+10%FBS ESGRO Complete	Trypsin	$5x10^4$	Cytodex 3, Cultispher S	40
Phillips 2008b	R1	MEF-CM	Trypsin	$1x10^5$	Hillex II	40
Marinho 2009	USP-1	MEF-CM + DMEM/F12 + 15%KSR + CHO-CM	TrypLE	$2x10^5$	Cytodex 3	70
Storm 2010	E13tg2a	KO-DMEM+ 20%KSR GMEM+10%Serum	Trypsin	$6x10^4$	Collagen, FACT, Cultispher S	45

Table 7. Summary of suspension culture conditions for mouse ESCs on microcarriers. Medium components listed are only the base. See references for full medium components.

Reference	Cell Lines	Medium	Passaging/ Harvesting	Inoculation Density (cells/mL)	Microcarrier Type	Agitation Rate (rpm)
Phillips 2008b	ESI-017	HFF-CM	TrypLE	$6.25x10^4$	Hillex II	Not specified
Fernandes 2009	H9	MEF-CM	TrypLE	$2\text{-}2.5x10^5$	Cytodex 3	60
Lock 2009	H1, H9	DMEM/F12+ 20%KSR+bFGF	TrypLE	$5\text{-}20x10^4$	Collagen+ Matrigel coated	45-80
Nie 2009	H1, H9	MEF-CM + bFGF	Trypsin	$3\text{-}7x10^4$ cells/cm^2	Cytodex 3+ MEFs or matrigel	6well plate on rocker

Reference	Cell Lines	Medium	Passaging/ Harvesting	Inoculation Density (cells/mL)	Microcarrier Type	Agitation Rate (rpm)
Oh 2009	HES2, HES3	MEF-CM, mTeSR1, StemPRO	TrypLE Collagenase Mechanical	$2x10^5$(6well) $6x10^5$(spinner)	Matrigel coated cellulose	100 (6well) 25(spinner)
Chen 2010	HES2, HES3	MEF-CM mTeSR StemPRO	Mechanical	$2x10^5$	DE-53+ matrigel	120(6well)
Storm 2010	SHEF-3	MEF-CM+bFGF KO-DMEM+ 20%KSR	Trypsin+ ROCK	$6x10^4$	Cultispher S	45
Serra 2010	SCED-461	MEF-CM+ROCK	TrypLE	$1.5,3,4.5x10^5$ $4.5x10^5$	Cytodex 3	100mL Wheaton 24rpm 300mL BIOSTAT 50-65rpm
Leung 2011	HES2, HES3	MEF-CM	Mechanical	$0.8\text{-}6x10^5$ (6well) $3.2\text{-}6x10^5$ (spinner)	DE-53	100-120(6well) 25(spinner)

Table 8. Summary of suspension culture conditions for human ESCs on microcarriers. Medium components listed are only the base. See references for full medium components.

As computational methods become more refined along with our understanding of biological processes, it is clear that regenerative medicine will benefit. The use of modeling and scale up techniques has the potential to bring stem cell based therapies into mainstream application and greatly benefit those suffering from debilitating degenerative diseases.

5. Conclusions

Overall, though considerable progress has been made in the development of bioprocesses for the production of ESCs and their progeny, it is obvious that more basic research is needed prior to downstream application of these cells. While protocols developed so far have focused on scaling up production of ESCs and their derivatives, other fundamental issues including elimination of animal derived products in ESC cultures, immune rejection and tumor formation upon transplantation of ESC derivatives must also be addressed. Utilization of modeling techniques to address possible outcomes of interaction effects of various variables involved will be a cost effective method to address some of these issues. Finally, it will be necessary to develop optimized robust, controllable systems in production facilities designed to meet the manufacturing requirements established by various governing bodies.

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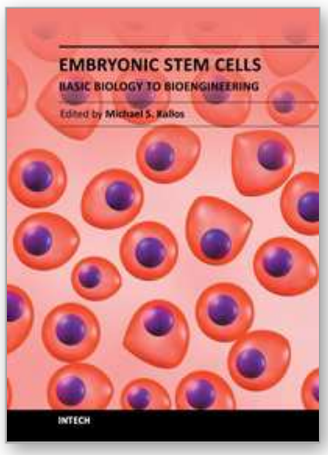

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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