

# Bioprocess Development for the Expansion of Embryonic Stem Cells

Megan M. Hunt<sup>1,3</sup>, Roz Alfred<sup>1,3</sup>, Derrick E. Rancourt<sup>2</sup>,  
Ian D. Gates<sup>3</sup> and Michael S. Kallos<sup>1,3</sup>

<sup>1</sup>*Pharmaceutical Production Research Facility (PPRF), Schulich School of Engineering,  
University of Calgary, Alberta*

<sup>2</sup>*Department of Biochemistry and Molecular Biology, Faculty of Medicine,  
University of Calgary, Alberta*

<sup>3</sup>*Department of Chemical and Petroleum Engineering, Schulich School of Engineering,  
University of Calgary, Alberta,  
Canada*

## 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
<b>Pluripotency markers</b>		
SSEA-1	+	-
SSEA-3	-	+
SSEA-4	-	+
TRA-1-60	-	+
TRA-1-81	-	+
Oct-4	+	+
<b>Factors that affect self renewal</b>	LIF + Serum – activates JAK/STAT3 pathway <sup>1</sup>  LIF+BMP (serum free) – activates SMAD and/or MAPK pathways <sup>1</sup>	bFGF+MEF+SR or high bFGF+SR (no MEFs)- suppresses BMP signalling and/or upregulates expression of TGF $\beta$ ligands, activation of ERK &PI3K <sup>1</sup>  TGF $\beta$ / Activin/Nodal <sup>1</sup>
<b>Teratoma formation in vivo</b>	+	+
<b>Colony morphology</b>	Tight rounded multilayered	Lose rounded monolayers
<b>Passaging</b>	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;<sup>1</sup> Yu & Thomson, 2008. bFGF= basic fibroblast growth factor, BMP= bone morphogenetic protein, LIF= leukemia inhibitory factor, MEF= mouse embryonic fibroblasts, SR= serum replacement, TGF $\beta$ = 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
<b>Kehoe 2008</b>	E14Tg2a	0.1% Gelatin	DMEM+10%FBS	TrypLE
<b>Veraitch 2008</b>	E14Tg2a	0.1% Gelatin	GMEM+10%FBS	Trypsin
<b>Abranches 2009</b>	E14Tg2a 46C S25	0.1% Gelatin	GMEM+10%FBS	Not specified for maintenance conditions
<b>Fernandes, TG 2009</b>	46C	0.1% Gelatin	DMEM+10%FBS ESGRO complete KO-DMEM+15%KSR	Trypsin, Accutase
<b>Marinho 2009</b>	USP-1	Gelatin MEFs	DMEM/F12+15%KSR+CHO-CM (for LIF)- changed every 2 days	TrypLE
<b>Alfred 2010</b>	D3	Gelatin	DMEM+15%FBS	Not specified for static
<b>Jing 2010</b>	E14Tg2a	0.1% Gelatin	DMEM+10%FBS	TrypLE
<b>Taiani 2010</b>	D3	MEFs	DMEM+15%FBS	Not specified for static
<b>Ito 2010</b>	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 (Invitrogen), 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  $2.1 \times 10^{10}$  cells would be required per treatment. If one T-75 culture flask supports the production of  $2 \times 10^6$  cells in 2-3 days, production of  $2.1 \times 10^{10}$  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  $1-2 \times 10^6$  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 <sup>4</sup>	DMEM+15%FBS	Trypsin	Bellco (50 mL)	60,100
Cormier 2006	R1	3.75-10x10 <sup>4</sup>	DMEM+15%FBS	Trypsin	Corning (100 mL)	60-120
Zur Neiden 2007	R1	3.75x10 <sup>4</sup>	DMEM+15%FBS	Trypsin	NDS (100 mL)	100
Hwang 2008	E14Tg2a	3.0x10 <sup>5</sup>	DMEM+10%FBS	N/A	Synthecon (50 mL)	25
Kehoe 2008	E14Tg2a	1-7.5x10 <sup>4</sup>	DMEM+10%FBS DMEM+10-20% KSR	TrypLE	Corning (100 mL)	60-120
Tsuji 2008	D3, E14, EB5	(1-500)x10 <sup>4</sup>	DMEM+15%FCS	Trypsin	96 well plates	N/A
Alfred 2010	D3	3.75x10 <sup>4</sup>	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 <sup>4</sup>	MEF-CM+ Matrigel+ROCK	Accutase+ ROCK	Not specified	60
Krawetz 2010	H9	1.8x10 <sup>4</sup>	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 <sup>6</sup>	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 <sup>5</sup>	mTeSR+/-ROCK KO-DMEM+ KSR+bFGF+/-ROCK KO-DMEM+FBS +/- ROCK	Collagenase	6well plate	NA
Singh 2010	HES2, HES3, ES1049	2.5x10 <sup>5</sup> 1x10 <sup>6</sup> 1x10 <sup>6</sup>	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 <sup>6</sup>	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 <sup>4</sup>	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:

$$\text{Input} + \text{Generation} = \text{Output} + \text{Accumulation} \quad (1)$$

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:

$$\text{OTR} - \text{OUR} = 0 + dC_{O_2}/dt \quad (2)$$

where OTR and OUR are as follows:

$$\text{OTR} = k_L a \Delta C_{O_2} \quad (3)$$

$$\text{OUR} = q_{O_2} X \quad (4)$$

where  $k_{L,a}$  is the volumetric mass transfer coefficient,  $\Delta C_{O_2}$  is the difference in oxygen concentration between oxygen saturation concentration in the bulk medium ( $C_{O_2}^*$  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 ( $C_{O_2}$ ). The term  $q_{O_2}$  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:

$$dC_{O_2}/dt = k_{L,a} \Delta C_{O_2} - q_{O_2}X \quad (5)$$

As can be seen from the preceding equations, the  $k_{L,a}$  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  $k_{L,a}$ . 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  $k_{L,a}$  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  $k_{L,a}$  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  $k_{L,a}$ . 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  $k_{L,a}$  values experimentally. There have been thorough reviews elsewhere which have described several approaches to experimentally determine  $k_{L,a}$  (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,  $C_{O_2}$  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:

$$k_{L,a} = q_{O_2} X / C_{O_2}^* \quad (6)$$

The specific oxygen uptake ( $q_{O_2}$ ) 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:

$$dC_{O_2}/dt = q_{O_2}X \quad (7)$$

Once the specific uptake rate for a cell type is known, the limiting  $k_L a$  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:

$$k_L a = 1.08 Re^{0.78} [D_{O_2} a / D_T] \quad (8)$$

Where  $Re$  and  $D_{O_2}$  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  $k_L a$  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 <sup>3</sup> )	Bead Diameter (μm)	Surface Area (cm <sup>2</sup> /g)
<b>CultiSpher S</b>	PerCELL Biolytica	Crosslinked pharmaceutical grade gelatin	Porcine gelatin	1.04	130 – 380	<b>7500</b>
<b>Collagen</b>	SoloHill Engineering	Crosslinked Polystyrene, modified with gelatin	Type1 porcine gelatin	1.02	90-150	<b>480</b>
<b>Fact III</b>	SoloHill Engineering	Crosslinked Polystyrene, modified with cationic gelatin	Cationic, type1 porcine gelatin	1.02	90-150	<b>480</b>
<b>Glass</b>	SoloHill Engineering	Crosslinked Polystyrene, modified with high silica glass	High silica glass	1.02	125-212	<b>360</b>
<b>Pronectin F</b>	SoloHill Engineering	Crosslinked Polystyrene, modified with recombinant fibronectin	Recombinant fibronectin	1.02	125-212	<b>360</b>
<b>Hillex II</b>	SoloHill Engineering	Modified Polystyrene, modified with cationic trimethyl-ammonium	Cationic, trimethyl ammonium	1.11	160 - 180	<b>515</b>
<b>Cytodex 3</b>	Amersham Biosciences	Crosslinked dextran, Denatured Collagen on the surface	Porous Porcine gelatin	1.04	141 - 211	<b>2700</b>
<b>Cytodex 1</b>	Amersham Biosciences	Crosslinked Dextran with N,N-diethylaminoethyl groups	Porous Porcine gelatin Cationic	1.03	147-248	<b>4400</b>

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 <sup>4</sup>	Cytodex 3, Glass coated styrene	60
Abranches 2007	S25	GMEM+10%FBS	Trypsin	1x10 <sup>4</sup> , 5x10 <sup>4</sup> or 1x10 <sup>5</sup>	Cytodex 3	60
Fernandes 2007	46C	DMEM+10%FBS ESGRO Complete	Trypsin	5x10 <sup>4</sup>	Cytodex 3, Cultispher S	40
Phillips 2008b	R1	MEF-CM	Trypsin	1x10 <sup>5</sup>	Hillex II	40
Marinho 2009	USP-1	MEF-CM + DMEM/F12 + 15%KSR + CHO- CM	TrypLE	2x10 <sup>5</sup>	Cytodex 3	70
Storm 2010	E13tg2a	KO-DMEM+ 20%KSR GMEM+10%Serum	Trypsin	6x10 <sup>4</sup>	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 <sup>4</sup>	Hillex II	Not specified
Fernandes 2009	H9	MEF-CM	TrypLE	2-2.5x10 <sup>5</sup>	Cytodex 3	60
Lock 2009	H1, H9	DMEM/F12+ 20%KSR+bFGF	TrypLE	5-20x10 <sup>4</sup>	Collagen+ Matrigel coated	45-80
Nie 2009	H1, H9	MEF-CM + bFGF	Trypsin	3-7x10 <sup>4</sup> cells/cm <sup>2</sup>	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 <sup>5</sup> (6well) 6x10 <sup>5</sup> (spinner)	Matrigel coated cellulose	100 (6well) 25(spinner)
Chen 2010	HES2, HES3	MEF-CM mTeSR StemPRO	Mechanical	2x10 <sup>5</sup>	DE-53+ matrigel	120(6well)
Storm 2010	SHEF-3	MEF-CM+bFGF KO-DMEM+ 20%KSR	Trypsin+ ROCK	6x10 <sup>4</sup>	Cultispher S	45
Serra 2010	SCED- 461	MEF- CM+ROCK	TrypLE	1.5,3,4.5x10 <sup>5</sup>  4.5x10 <sup>5</sup>	Cytodex 3	100mL Wheaton 24rpm 300mL BIOSTAT 50- 65rpm
Leung 2011	HES2, HES3	MEF-CM	Mechanical	0.8-6x10 <sup>5</sup> (6well) 3.2-6x10 <sup>5</sup> (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.

## 6. References

- Abranches, E., Bekman, E., Henrique, D., & Cabral, JM. (2007). Expansion of mouse embryonic stem cells on microcarriers. *Biotechnology & Bioengineering*. Vol. 96, No. 6, pp.1211-1221. eISSN 1097-0290.

- Abranches, E., Silva, M., Pradier, L., Schulz, H., Hummel, O., Henrique, D., & Bekman, E. (2009). Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo. *PLoS One*. Vol. 4, No. 7, e6286. eISSN 1932-6203.
- Ahsan, T., & Nerem, RM. (2010). Fluid shear stress promotes an endothelial-like phenotype during the early differentiation of embryonic stem cells. *Tissue Engineering Part A*. Vol. 16, No. 11, pp. 3547-53. eISSN 1937-335X.
- Alfred, R., Radford, J., Fan, J., Boon, K., Krawetz, R., Rancourt, D., & Kallos, MS. (2011). Efficient suspension bioreactor expansion of murine embryonic stem cells on microcarriers in serum-free medium. *Biotechnology Progress*. [ePub ahead of print, 27 April 2011]. eISSN 1520-6033.
- Alfred, R., Gareau, T., Krawetz, R., Rancourt, D., & Kallos, MS. (2010). Serum-free scaled up expansion and differentiation of murine embryonic stem cells to osteoblasts in suspension bioreactors. *Biotechnology & Bioengineering*. Vol. 106, No. 5, pp. 829-840. eISSN 1097-0290.
- Amit, M., Chebath, J., Margulets, V., Laevsky, I., Miropolsky, Y., Shariki, K., Peri, M., Blais, I., Slutsky, G., Revel, M., & Itskovitz-Eldor, J. (2010). Suspension culture of undifferentiated human embryonic and induced pluripotent stem cells. *Stem Cell Reviews and Reports*. Vol. 6, No. 2, pp. 248-59. eISSN 1558-6804.
- Audet, J. (2010). Adventures in time and space: Nonlinearity and complexity of cytokine effects on stem cell fate decisions. *Biotechnology & Bioengineering*. Vol. 106, No. 2, pp. 173-82. eISSN 1097-0290.
- Aunins, JG., Woodson, BA Jr., Hale, TK., & Wang, DI. (1989). Effects of paddle impeller geometry on power input and mass transfer in small-scale animal cell culture vessels. *Biotechnology & Bioengineering*. Vol. 34, No. 9, pp. 1127-32. eISSN 1097-0290.
- Bauwens, CL., Peerani, R., Niebruegge, S., Woodhouse, KA., Kumacheva, E., Husain, M., & Zandstra, PW. (2008). Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells*. Vol. 26, No. 9, pp. 2300-10. Epub 2008 Jun 26. eISSN 1549-4918.
- Baghbaderani, BA., Behie, LA., Sen, A., Mukhida, K., Hong, M., & Mendez, I. (2008). Expansion of human neural precursor cells in large-scale bioreactors for the treatment of neurodegenerative disorders. *Biotechnology Progress*. Vol. 24, No. 4, pp. 859-70. eISSN 1520-6033.
- Baghbaderani, BA., Mukhida, K., Sen, A., Kallos, MS., Hong, M., Mendez, I., & Behie, LA. (2010). Bioreactor expansion of human neural precursor cells in serum-free media retains neurogenic potential. *Biotechnology & Bioengineering*. Vol. 105, No. 4, pp.823-833. eISSN 1097-0290.
- Bedell, MA., Jenkins, NA., & Copeland NG. (1997). Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. *Genes & Development*. Vol. 11, No. 1, pp. 1-10. eISSN 1549-5477.
- Bedell, MA., Largaespada, DA., Jenkins, NA., Copeland, & NG. (1997). Mouse models of human disease. Part II: recent progress and future directions. *Genes & Development*. Vol. 11, No. 1, pp. 11-43. eISSN 1549-5477.
- Bendall, SC., Hughes, C., Campbell, JL., Stewart, MH., Pittock, P., Liu, S., Bonneil, E., Thibault, P., Bhatia, M., & Lajoie, GA. (2009). An enhanced mass spectrometry approach reveals human embryonic stem cell growth factors in culture. *Molecular & Cellular Proteomics*. Vol. 8, No. 3, pp. 421-32. Epub 2008 Oct 20. eISSN 1535-9484.

- Bongso, A., Fong, CY., Ng, SC., & Ratnam, S. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Human Reproduction*. Vol. 9, No. 11, pp. 2110-7. eISSN 1460-2350.
- Borys, MC., & Papoutsakis, ET. (1992). Formation of bridges and large cellular clumps in CHO-cell microcarrier cultures: effects of agitation, dimethyl sulfoxide and calf serum. *Cytotechnology*. Vol. 8, No. 3, pp. 237-48. eISSN 1573-0778.
- Chawla, M., Bodnar, CA., Sen, A., Kallos, MS., & Behie, LA. (2006). Production of islet-like structures from neonatal porcine pancreatic tissue in suspension bioreactors. *Biotechnology Progress*. Vol. 22, No. 2, pp. 561-567. eISSN 1520-6033.
- Chen, X., Chen, A., Woo, TL., Choo, AB., Reuveny, S., & Oh, SK. (2010). Investigations into the metabolism of two-dimensional colony and suspended microcarrier cultures of human embryonic stem cells in serum-free media. *Stem Cells and Development*. Vol. 19, No. 11, pp. 1781-92. Epub 2010 Sep 10. eISSN 1557-8534.
- Cherry, RS., & Kwon, KY. (1990). Transient shear stresses on a suspension cell in turbulence. *Biotechnology & Bioengineering*. Vol. 36, No. 6, pp. 563-71. eISSN 1097-0290.
- Chin, AC., Fong, WJ., Goh, LT., Philp, R., Oh, SK., & Choo, AB. (2007). Identification of proteins from feeder conditioned medium that support human embryonic stem cells. *Journal of Biotechnology*. Vol. 130, No. 3, pp. 320-328. eISSN 1573-0972.
- Chu, L., & Robinson, DK. (2001). Industrial choices for protein production by large-scale cell culture. *Current Opinion in Biotechnology*. Vol. 12, No. 2, pp. 180-187. ISSN 0958-1669.
- Chung, CA., Yang, CW., & Chen, CW. (2006). Analysis of cell growth and diffusion in a scaffold for cartilage tissue engineering. *Biotechnology & Bioengineering*. Vol. 94, No. 6, pp. 1138-1146. eISSN 1097-0290.
- Cormier, JT., zur Nieden, NI., Rancourt, DE., & Kallos, MS. (2006). Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors. *Tissue Engineering*. Vol. 12, No. 11, pp. 3233-3245. ISSN 2152-4955.
- Evans, MJ., & Kaufman, MH. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature*. Vol. 292, No. 5819, pp. 154-156. ISSN 0028-0836.
- Fernandes, AM., Fernandes, TG., Diogo, MM., da Silva, CL., Henrique, D., & Cabral, JM. (2007). Mouse embryonic stem cell expansion in a microcarrier-based stirred culture system. *Journal of Biotechnology*. Vol. 132, No. 2, pp. 227-36. Epub 2007 Jun 7. eISSN 1573-0972.
- Fernandes, AM., Marinho, PA., Sartore, RC., Paulsen, BS., Mariante, RM., Castilho, LR., & Rehen, SK. (2009). Successful scale-up of human embryonic stem cell production in a stirred microcarrier culture system. *Brazilian Journal of Medical and Biological Research*. Vol. 42, No. 6, pp. 515-22. eISSN 1678-4510.
- Fernandes, TG., Fernandes-Platzgummer, AM., da Silva, CL., Diogo, MM., & Cabral, JM. (2009). Kinetic and metabolic analysis of mouse embryonic stem cell expansion under serum-free conditions. *Biotechnology Letters*. Vol. 32, No. 1, pp. 171-9. Epub 2009 Aug 25. eISSN 1573-6776.
- Fok, EY., & Zandstra, PW. (2005). Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation. *Stem Cells*. Vol. 23, No. 9, pp. 1333-1342. eISSN 1549-4918.
- Freshney, RI. (2000). Transformation, In: *Culture of animal cells: a manual of basic technique (4<sup>th</sup> Edition)*, pp. 269-84, John Wiley & Sons, ISBN 9780-471-34889-9, New York.

- Galban, CJ., & Locke, BR. (1999) Analysis of cell growth kinetics and substrate diffusion in a polymer scaffold. *Biotechnology & Bioengineering*. Vol. 65, No. 2, pp. 121-132. eISSN 1097-0290.
- Garcia-Cardena, G., & Adamo, L. (2011). Directed Stem Cell Differentiation by Fluid Mechanical Forces. *Antioxidants & Redox Signaling*. 2011 Feb 5. [Epub ahead of print]. eISSN 1557-7716.
- Garcia-Ochoa, F., & Gomez, E. (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnology Advances*. Vol. 27, No. 2, pp. 153-76. Epub 2008 Nov 12. ISSN 0734-9750.
- GE Healthcare. (2005). *Microcarrier Cell Culture: Principles and Methods*. Ed. Piscataway. Retrieved from:  
<[http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/180CFF1511F005C9C1257628001CBE22/\\$file/18114062AB.pdf](http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/180CFF1511F005C9C1257628001CBE22/$file/18114062AB.pdf)>
- Gibson, JD., Jakuba, CM., Boucher, N., Holbrook, KA., Carter, MG., & Nelson, CE. (2009). Single-cell transcript analysis of human embryonic stem cells. *Integrative Biology (Camb)*. Vol. 1, No. 8-9, pp. 540-51. Epub 2009 Jul 30. eISSN 1757-9708.
- Gilbertson, JA., Sen, A., Behie, LA., & Kallos, MS. (2006). Scaled-up production of mammalian neural precursor cell aggregates in computer-controlled suspension bioreactors. *Biotechnology & Bioengineering*. Vol. 94, No. 4, pp. 783-792. eISSN 1097-0290.
- Hentze, H., Soong, PL., Wang, ST., Phillips, BW., Putti, TC., & Dunn, NR. (2009). Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. *Stem Cell Research*. Vol. 2, No. 3, pp. 198-210. Epub 2009 Feb 12. ISSN 1873-5061.
- Ito, S., D'Alessio, AC., Taranova, OV., Hong, K., Sowers, LC., & Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*. Vol. 466, No. 7310, pp. 1129-33. ISSN 0028-0836.
- Jing, D., Parikh, & A., Tzanakakis, ES. (2010). Cardiac cell generation from encapsulated embryonic stem cells in static and scalable culture systems. *Cell Transplantation*. Vol. 19, No. 11, pp. 1397-412. Epub 2010 Jun 29. ISSN 0963-6897.
- Kallos, MS., Sen, A., & Behie, LA. (2003). Large-scale expansion of mammalian neural stem cells: a review. *Medical & Biological Engineering and Computing*. Vol. 41, No. 3, pp.271-282. eISSN 1741-0444.
- Kallos, MS., & Behie, LA. (1999). Inoculation and growth conditions for high-cell-density expansion of mammalian neural stem cells in suspension bioreactors. *Biotechnology & Bioengineering*. Vol. 63, No. 4, pp. 473-483. eISSN 1097-0290.
- Kehoe, DE., Lock, LT., Parikh, A., & Tzanakakis, ES. (2008). Propagation of embryonic stem cells in stirred suspension without serum. *Biotechnology Progress*. Vol. 24, No. 6, pp. 1342-1352. eISSN 1520-6033.
- Kehoe, DE., Jing, D., Lock, LT., & Tzanakakis, ES. (2010). Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. *Tissue Engineering Part A*. Vol. 16, No. 2, pp. 405-21. eISSN 1937-335X.
- King, JA., & Miller, WM. (2007) Bioreactor development for stem cell expansion and controlled differentiation. *Current Opinion in Chemical Biology*. Vol. 11, pp. 394-398. ISSN 1367-5931.
- Kirouac, DC., & Zandstra, PW. (2008). The systematic production of cells for cell therapies. *Cell Stem Cell*. Vol. 3, No. 4, pp. 369-81. ISSN 1934-5909

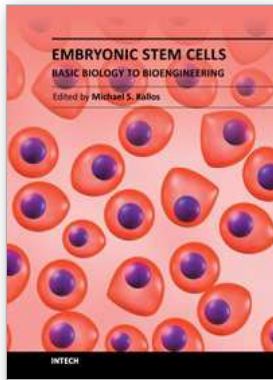
- Krawetz, R., Taiani, JT., Liu, S., Meng, G., Li, X., Kallos, MS., & Rancourt, D. (2009). Large-scale expansion of pluripotent human embryonic stem cells in stirred suspension bioreactors. *Tissue Engineering Part C: Methods*. [Epub ahead of print]. DOI: 10.1089/ten.tec.2009.0228. eISSN 1937-3392.
- Larijani, MR., Seifinejad, A., Pournasr, B., Hajihoseini, V., Hassani, SN., Totonchi, M., Yousefi, M., Shamsi, F., Salekdeh, GH., & Baharvand, H. (2011). Long-Term Maintenance of Undifferentiated Human Embryonic and Induced Pluripotent Stem Cells in Suspension. *Stem Cells and Development*. 2011 Feb 24. [Epub ahead of print]. eISSN 1557-8534.
- Lee, LH., Peerani, R., Ungrin, M., Joshi, C., Kumacheva, E., & Zandstra, P. (2009). Micropatterning of human embryonic stem cells dissects the mesoderm and endoderm lineages. *Stem Cell Research*. Vol. 2, No. 2, pp. 155-62. Epub 2008 Dec 3. ISSN 1873-5061.
- Lemon, G., Waters, L., Rose, FRAJ., & King, JR. (2007) Mathematical modeling of human mesenchymal stem cell proliferation and differentiation inside artificial porous scaffolds. *Journal of Theoretical Biology*. Vol. 249, No. 3, pp. 543-553. ISSN 0022-5193.
- Leung, HW., Chen, A., Choo, AB., Reuveny, S., & Oh, SK. (2011). Agitation can induce differentiation of human pluripotent stem cells in microcarrier cultures. *Tissue Engineering Part C: Methods*. Vol. 17, No. 2, pp. 165-72. Epub 2010 Nov 4. eISSN 1937-3392.
- Levenstein, ME., Ludwig, TE., Xu, RH., Llanas, RA., VanDenHeuvel-Kramer, K., Manning, D., & Thomson, JA. (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells*. Vol. 24, No. 3, pp. 568-74. Epub 2005 Nov 10. eISSN 1549-4918.
- Lock, LT., & Tzanakakis, ES. (2009). Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture. *Tissue Engineering Part A*. Vol. 15, No. 8, pp. 2051-63. eISSN 1937-335X.
- Ludwig, TE., Levenstein, ME., Jones, JM., Berggren, WT., Mitchen, ER., Frane, JL., Crandall, LJ., Daigh, CA., Conard, KR., Piekarczyk, MS., Llanas, RA., & Thomson, JA. (2006). Derivation of human embryonic stem cells in defined conditions. *Nature Biotechnology*. Vol. 24, No. 2, pp. 185-7. Epub 2006 Jan 1. eISSN 1546-4696.
- Mantzaris, NV., Daoutidis, P., & Srienc, F. (2001) Numerical solution of multi-variable cell population balance models: I. Finite difference methods. *Computers & Chemical Engineering*. Vol. 25, No. 11-12, pp. 1411-1440. ISSN 0098-1354.
- Marinho, PA., Fernandes, AM., Cruz, JC., Rehen, SK., & Castilho, LR. (2010). Maintenance of pluripotency in mouse embryonic stem cells cultivated in stirred microcarrier cultures. *Biotechnology Progress*. Vol. 26, No. 2, pp. 548-55. . eISSN 1520-6033.
- Martin, GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. Vol. 78, No. 12, pp. 7634-7638. ISSN 0027-8424.
- Martin, Y., Eldardiri, M., Lawrence-Watt, DJ., & Sharpe, JR. (2011). Microcarriers and their potential in tissue regeneration. *Tissue Engineering Part B: Reviews*. Vol. 17, No. 1, pp. 71-80. Epub 2010 Dec 29. eISSN 1937-3376.
- Montes, R., Ligerio, G., Sanchez, L., Catalina, P., de la Cueva, T., Nieto, A., Melen, GJ., Rubio, R., García-Castro, J., Bueno, C., & Menendez, P. (2009). Feeder-free maintenance of

- hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-beta and IGF-II. *Cell Research*. Vol. 19, No. 6, pp. 698-709. eISSN 1748-7838.
- Mouse Genome Sequencing Consortium, et al. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*. Vol. 420, No. 6915, pp. 520-62. ISSN 0028-0836.
- National Institutes of Health, U.S. Department of Health and Human Services. (2009). Appendix B: Mouse Embryonic Stem Cell Cultures . In Stem Cell Information, Accessed Wednesday, April 20, 2011. Available from: <<http://stemcells.nih.gov/info/scireport/appendixb>>
- Nelson, TJ., Behfar, A., Yamada, S., Martinez-Fernandez, A., & Terzic, A. (2009). Stem cell platforms for regenerative medicine. *Clinical and Translational Science*. Vol. 2, No. 3, pp. 222-227. eISSN 1752-8062.
- Nie, Y., Bergendahl, V., Hei, DJ., Jones, JM., & Palecek, SP. (2009). Scalable culture and cryopreservation of human embryonic stem cells on microcarriers. *Biotechnology Progress*. Vol. 25, No. 1, pp. 20-31. eISSN 1520-6033.
- Niebruegge, S., Bauwens, CL., Peerani, R., Thavandiran, N., Masse, S., Sevaptisidis, E., Nanthakumar, K., Woodhouse, K., Husain, M., Kumacheva, E., & Zandstra, PW. (2009). Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. *Biotechnology & Bioengineering*. Vol. 102, No. 2, pp. 493-507. eISSN 1097-0290.
- Noble, WS. (2006). What is a support vector machine? *Nature Biotechnology*. Vol. 24, No. 12, pp. 1565-7. eISSN 1546-4696.
- Oh, SK., Fong, WJ., Teo, Y., Tan, HL., Padmanabhan, J., Chin, AC., & Choo, AB. (2005). High density cultures of embryonic stem cells. *Biotechnology Bioengineering*. Vol. 91, No. 5, pp. 523-533. eISSN 1097-0290.
- Oh, SK., Chen, AK., Mok, Y., Chen, X., Lim, UM., Chin, A., Choo, AB., & Reuveny, S. (2009). Long-term microcarrier suspension cultures of human embryonic stem cells. *Stem Cell Research*. 2(3):219-30. Epub 2009 Mar 4. ISSN 1873-5061.
- Olmer, R., Haase, A., Merkert, S., Cui, W., Palecek, J., Ran, C., Kirschning, A., Scheper, T., Glage, S., Miller, K., Curnow, EC., Hayes, ES., & Martin, U. (2010). Long term expansion of undifferentiated human iPS and ES cells in suspension culture using a defined medium. *Stem Cell Research*. Vol. 5, No. 1, pp. 51-64. Epub 2010 Mar 30. ISSN 1873-5061.
- Ouyang, A., & Yang, ST. (2008). A two-stage perfusion fibrous bed bioreactor system for mass production of embryonic stem cells. *Expert Opinion on Biological Therapy*. Vol. 8, No. 7, pp. 895-909. eISSN 1744-7682.
- Papoutsakis, ET. (1991). Fluid-mechanical damage of animal cells in bioreactors. *Trends in Biotechnology*. Vol. 9, No. 12, pp. 427-37. ISSN 0167-7799.
- Peerani, R., Rao, BM., Bauwens, C., Yin, T., Wood, GA., Nagy, A., Kumacheva, E., & Zandstra, PW. (2007). Niche-mediated control of human embryonic stem cell self-renewal and differentiation. *European Molecular Biology Organisation Journal*. Vol. 26, No. 22, pp. 4744-55. Epub 2007 Oct 18. ISSN 0261-4189.
- Phillips, BW., Lim, RY., Tan, TT., Rust, WL., & Crook, JM. (2008a). Efficient expansion of clinical-grade human fibroblasts on microcarriers: cells suitable for ex vivo expansion of clinical-grade hESCs. *Journal of Biotechnology*. Vol. 134, No. 1-2, pp. 79-87. Epub 2008 Jan 8. eISSN 1573-0972.

- Phillips, BW., Horne, R., Lay, TS., Rust, WL., Teck, TT., & Crook, JM. (2008b). Attachment and growth of human embryonic stem cells on microcarriers. *Journal of Biotechnology*. Vol. 138, No. 1-2, pp. 24-32. Epub 2008 Aug 14. eISSN 1573-0972.
- Prudhomme, WA., Duggar, KH., & Lauffenburger, DA. (2004) Cell population dynamics model for deconvolution of murine embryonic stem cell self renewal and differentiation responses to cytokines and extracellular matrix. *Biotechnology & Bioengineering*. Vol. 88, No. 3, pp. 264-72. eISSN 1097-0290.
- Reubinoff, BE., Pera, MF., Fong, CY., Trounson, A., & Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nature Biotechnology*. Vol. 18, No. 4, pp. 399-404. eISSN 1546-4696.
- Schmidt, FR. (2005) Optimization and scale up of industrial fermentation processes. *Applied Microbiology and Biotechnology*. Vol. 68, No. 4, pp. 425-435. eISSN 1432-0614.
- Sen, A., Kallos, MS., & Behie, LA. 2002. Expansion of mammalian neural stem cells in bioreactors: effect of power input and medium viscosity. *Brain Research: Developmental Brain Research*. Vol. 134, No. 1-2, pp. 103-113. ISSN 0165-3806.
- Sen, A., Kallos, MS., & Behie, LA. (2010). Bioprocess Engineering of Neural Stem Cells. In: *Wiley Encyclopedia of Industrial Biotechnology*, Ed. MC Flickinger, Publ. John Wiley & Sons, ISBN 978-0-471-79930-6.
- Serra, M., Brito, C., Sousa, MF., Jensen, J., Tostões, R., Clemente, J., Strehl, R., Hyllner, J., Carrondo, MJ., & Alves, PM. (2010). Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control. *Journal of Biotechnology*. Vol. 148, No. 4, pp. 208-15. Epub 2010 Jun 17. eISSN 1573-0972.
- Singh, H., Mok, P., Balakrishnan, T., Rahmat, SN., & Zweigerdt, R. (2010). Up-scaling single cell-inoculated suspension culture of human embryonic stem cells. *Stem Cell Research*. Vol. 4, No. 3, pp. 165-79. Epub 2010 Mar 12. ISSN 1873-5061.
- Steiner, D., Khaner, H., Cohen, M., Even-Ram, S., Gil, Y., Itsykson, P., Turetsky, T., Idelson, M., Aizenman, E., Ram, R., Berman-Zaken, Y., & Reubinoff, B. (2010). Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension. *Nature Biotechnology*. Vol. 28, No. 4, pp. 361-4. Epub 2010 Mar 28. eISSN 1546-4696.
- Storm, MP., Orchard, CB., Bone, HK., Chaudhuri, JB., & Welham, MJ. (2010). Three-dimensional culture systems for the expansion of pluripotent embryonic stem cells. *Biotechnology & Bioengineering*. Vol. 107, No. 4, pp. 683-95. eISSN 1097-0290.
- Taiani, J., Krawetz, RJ., Nieden, NZ., Wu, YE., Kallos, MS., Matyas, JR., & Rancourt, DE. (2009). Reduced differentiation efficiency of murine embryonic stem cells in stirred suspension bioreactors. *Stem Cells and Development*. Vol. 19, No. 7, pp. 989-98. eISSN 1557-8534.
- Terstegge, S., Laufenberg, I., Pochert, J., Schenk, S., Itskovitz-Eldor, J., Endl, E., & Brüstle, O. (2007). Automated maintenance of embryonic stem cell cultures. *Biotechnology & Bioengineering*. Vol. 96, No. 1, pp. 195-201. eISSN 1097-0290.
- Terstegge, S., Rath, BH., Laufenberg, I., Limbach, N., Buchstaller, A., Schütze, K., & Brüstle, O. (2009). Laser-assisted selection and passaging of human pluripotent stem cell colonies. *Journal of Biotechnology*. Vol. 143, No. 3, pp. 224-30. Epub 2009 Jul 15. eISSN 1573-0972.
- Thomas, RJ., Anderson, D., Chandra, A., Smith, NM., Young, LE., Williams, D., & Denning, C. (2009). Automated, scalable culture of human embryonic stem cells in feeder-

- free conditions. *Biotechnology & Bioengineering*. Vol. 102, No. 6, pp. 1636-44. eISSN 1097-0290.
- Thomson, JA., Kalishman, J., Golos, TG., Durning, M., Harris, CP., Becker, RA., & Hearn, JP. (1995). Isolation of a primate embryonic stem cell line. *Proceedings of the National Academy of Sciences of the United States of America*. Vol. 92, No. 17, pp. 7844-8. ISSN 0027-8424.
- Thomson, JA., Itskovitz-Eldor, J., Shapiro, SS., Waknitz, MA., Swiergiel, JJ., Marshall, VS., & Jones, JM. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*. Vol. 282, No. 5391, pp. 1145-7. eISSN 1095-9203.
- Toh, YC., & Voldman, J. (2011). Fluid shear stress primes mouse embryonic stem cells for differentiation in a self-renewing environment via heparan sulfate proteoglycans transduction. *Journal of the Federation of American Societies for Experimental Biology*. Vol. 25, No. 4, pp. 1208-17. Epub 2010 Dec 23. eISSN 1530-6860.
- van't Reit, & K., Temper, J. (1991) *Basic bioreactor design*. (7<sup>th</sup> Edition). Dekker, ISBN 978-0-8247-8446-1, New York.
- Veraitch, FS., Scott, R., Wong, JW., Lye, GJ., & Mason, C. (2008). The impact of manual processing on the expansion and directed differentiation of embryonic stem cells. *Biotechnology & Bioengineering*. Vol. 99, No. 5, pp. 1216-29. eISSN 1097-0290.
- Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, JB., Nishikawa, S., Nishikawa, S., Muguruma, K., & Sasai, Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology*. Vol. 25, No. 6, pp. 681-6. Epub 2007 May 27. eISSN 1546-4696.
- Williams, JA. (2002). Keys to bioreactor selections. *Chemical Engineering Progress Magazine*, March:24-41.
- Xu, RH., Peck, RM., Li, DS., Feng, X., Ludwig, T., & Thomson, JA. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nature Methods*. Vol. 2, No. 3, pp. 185-90. Epub 2005 Feb 17. eISSN 1548-7105.
- Yener, B., Acar, E., Aguis, P., Bennett, K., Vandenberg, SL., & Plopper, GE. (2008). Multiway modeling and analysis in stem cell systems biology. *BMC Systems Biology*. Vol. 2, No. 63. ISSN 1752-0509
- Yu, J., & Thomson, JA. (2008). Pluripotent stem cell lines. *Genes & Development*. Vol. 22, No. 15, pp. 1987-97. eISSN 1549-5477.
- Zandstra, PW., Eaves, CJ., & Piret, JM. (1994). Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells. *Biotechnology (N Y)*. Vol. 12, No. 9, pp. 909-14. ISSN 0733-222X.
- zur Nieden, NI., Cormier, JT., Rancourt, DE., & Kallos, MS. (2007). Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors. *Journal of Biotechnology*. Vol. 129, No. 3, pp. 421-432. eISSN 1573-0972.





## **Embryonic Stem Cells - Basic Biology to Bioengineering**

Edited by Prof. Michael Kallos

ISBN 978-953-307-278-4

Hard cover, 478 pages

**Publisher** InTech

**Published online** 15, September, 2011

**Published in print edition** September, 2011

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.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Megan M. Hunt, Roz Alfred, Derrick E. Rancourt, Ian D. Gates and Michael S. Kallos (2011). Bioprocess Development for the Expansion of Embryonic Stem Cells, *Embryonic Stem Cells - Basic Biology to Bioengineering*, Prof. Michael Kallos (Ed.), ISBN: 978-953-307-278-4, InTech, Available from: <http://www.intechopen.com/books/embryonic-stem-cells-basic-biology-to-bioengineering/bioprocess-development-for-the-expansion-of-embryonic-stem-cells>

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No.65, Yan An Road (West), Shanghai, 200040, China  
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Phone: +86-21-62489820  
Fax: +86-21-62489821

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